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ANCIENT DNA STUDIES IN PHYSICAL ANTHROPOLOGY

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■ **Abstract** Nucleic acids are preserved in prehistoric samples under a wide range of depositional environments. The development of new molecular methods, especially the polymerase chain reaction, has made possible the recovery and manipulation of these molecules, and the subsequent molecular genetic characterization of the ancient samples. The analysis of ancient (a)DNA is complicated by the degraded nature of ancient nucleic acids, as well as the presence of enzymatic inhibitors in aDNA extracts. We review aspects of ancient DNA preservation, a variety of methods for the extraction and amplification of informative DNA segments from ancient samples, and the difficulties encountered in documenting the authenticity of ancient DNA template. Studies using aDNA to address questions in human population history or human evolution are reviewed and discussed. Future prospects for the field and potential directions for future aDNA research efforts in physical anthropology are identified.

INTRODUCTION

That DNA in ancient specimens could be extracted and characterized was first demonstrated in nonhuman material in 1984 by Higuchi and colleagues, who identified nucleic acids from a museum specimen of the extinct quagga and showed its phylogenetic affinity to modern zebra (Higuchi et al 1984). A year later, Pääbo (1985a,b, 1986) obtained DNA sequence data from a 2400-year-old Egyptian mummy. This result was surprising not only for its demonstration of the remarkable antiquity for which molecular genetic analysis was apparently possible, but also for the large DNA fragment sequenced (>3 kb). Both of these early efforts relied on extracting ancient (a)DNA fragments, cloning fragments into a vector, and subsequent sequencing of the cloned fragments. Following the nearly simultaneous development of the polymerase chain reaction [PCR (a molecular technique that uses the complementary nature of DNA bases and an enzyme involved in DNA replication to produce millions of copies of a single, specific DNA target

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sequence)] (Mullis & Faloona 1987, Saiki et al 1988), a number of researchers began extracting and characterizing aDNA from geographically dispersed human samples (for reviews, see Rogan & Salvo 1990, Herrmann & Hummel 1994, O'Rourke et al 1996, Audic & Béraud-Colomb 1997). The application of aDNA analyses to questions in physical anthropology had begun.

Following the early enthusiasm for aDNA research came fundamental observations on the nature of aDNA preservation, high failure rate of amplification of many samples, and concerns regarding the authenticity of aDNA samples. Nucleic acids of any antiquity are degraded and modified in various ways (Lindahl 1993, Höss et al 1996). Because of the degraded nature of aDNA extracts, mitochondrial (mt)DNA has proven to be the molecule of preference for genetically characterizing prehistoric samples. This is because mtDNA is present in several hundreds of copies per cell, in contrast to the single-copy nuclear genome. Thus, target sequences of mtDNA are more likely to be present in any single extract, and accessible for amplification, than are nuclear sequences. However, in some well-preserved samples, nuclear markers have been screened (e.g. Filon et al 1995, Zierdt et al 1996), and several methods for molecular determination of sex have been developed (e.g. Hummel & Herrmann 1991, Lassen et al 1996, Stone et al 1996, Palmirotta et al 1997, Ovchinnikov et al 1998, Faerman et al 1998, Cipollaro et al 1998). Additionally, genomic DNA has been used to confirm the presence of disease organisms in prehistoric samples (see below).

Despite difficulties associated with recovery and analysis of aDNA, numerous methods have been developed to optimize recovery, study, and authenticity of aDNA.

BIOCHEMISTRY OF aDNA

There are two principal obstacles to the recovery of aDNA. Molecular degradation limits the amount of amplifiable DNA available, and organic PCR inhibitors often coextract with the DNA. Nucleic acids gradually degrade over time through processes such as hydrolysis and oxidation (reviewed in Lindahl 1993). Hydrolysis is the breakdown of the N-glycosyl bond between the sugar and the base in the presence of water. Guanine and adenine are 20-fold more susceptible to removal (depurination) than are cytosine or thymine (depyrimidination), although the rate is temperature and pH dependent. Conversely, hydrolytic deamination of the bases affects the pyrimidines (30,000 years half-life in vivo) at a rate 40-fold higher than the purines. Oxidation is the process by which water-derived hydroxyl or superoxide radicals modify bases or distort the helix. Because mitochondria are the center of O_2 metabolism, oxidation primarily affects the mitochondrial rather than the nuclear genome. Hydantoins (oxidized pyrimidines) are suspected to do the most damage to DNA. Their presence is negatively correlated with success in extraction and amplification of aDNA, most likely due to the fact that they block extension during PCR (Höss et al 1996). These degradation processes occur continuously

in vivo (100–500 times $cell^{-1}$ day^{-1}), but in the nucleus are under stringent control by DNA repair mechanisms. Postmortem, these and other degradative events continue to accumulate. Such alterations to aDNA molecules have been detected by high-pressure liquid chromatography and electron microscopy (Pääbo 1989). For these reasons, recovery and amplification of aDNA, when possible, is usually limited to fragments <300–500 bp in length, and only for samples in the range of tens of thousands, or fewer, years old.

A 20°C decrease in temperature reduces base degradation 10- to 25-fold (Höss et al 1996). It is not surprising, then, that Pääbo and colleagues (Höss et al 1996, Pääbo 1989, Poinar et al 1996) observe an inverse correlation (nonsignificant) between long-term environmental temperature and DNA extraction and amplification success. This inverse correlation is also observed in our laboratory when comparing extraction and amplification success rates of prehistoric populations under study from the North American Arctic, the Great Basin, and the US southwest (DH O'Rourke, MG Hayes, SW Carlyle, unpublished data). Tuross (1994) reports an inverse correlation between sample age and total DNA yield. However, because this was assessed electrophoretically by comparative ethidium bromide staining, it may also reflect coextracting bacterial and/or fungal DNA. Tuross further suggests that at least for bone samples, the breakdown of DNA primarily occurs immediately postmortem, most likely because of the stabilization of DNA binding to hydroxyapatite (Tuross 1993, 1994), which slows the hydrolytic depurination rate twofold (Lindahl 1993). Environmental factors such as temperature (4°–37°C), humidity (20%–98%), pH (3.0–10.0), exposure to seawater, and burial in garden soil or sand does not significantly affect DNA yields from forensic dental samples (Schwartz et al 1991).

Because aDNA recovery is a destructive process, defining suitable candidates for aDNA extraction and amplification attempts is of considerable import. Nucleic acid degradation can be monitored several ways. Amino acid racemization, the transformation of L- into D-enantiomers (two optical isomers of amino acids), like depurination of DNA, is also affected by temperature and the presence of water. The rate of aspartic acid (Asp) racemization is approximately equal to that of DNA depurination and, therefore, is a good predictor of DNA preservation. Poinar et al (1996) report they could not retrieve and amplify DNA from samples in which the Asp D-form exceeded the Asp L-form by $\geq$9%. Another method, gas chromatography/mass spectrometry, can be used to measure the relative amounts of modified DNA bases.

The majority of extracted PCR inhibitors are tannins, humic acids, and fulvic acids, all common soil-derived degradation products (Hummel et al 1992, Tuross 1994). Because they are highly phenolic, they generally should be removed by phenol-chloroform extraction. Another class of inhibitors are Maillard products, by-products of sugar reduction, which cross-link macromolecules, including nucleic acids (Pääbo 1989). Humic acids, fulvic acids, and Maillard products often result in brown coloring of DNA extracts. These compounds often fluoresce blue in agarose gel under ultraviolet (UV) light (Tuross 1994, Hänni et al 1995, Kolman & Tuross 2000).

EXTRACTION METHODS

Prior to extraction, potential surface contamination of samples should be removed. The surface layer can be removed by scalpel or dremel/drill abrasion, briefly UV-irradiated, or soaked in 5% sodium hypochlorite (bleach). Regardless of tissue, reduction of the sample material should be completed prior to addition of an extraction buffer in order to provide as much contact surface area as possible for extraction enzymes and reagents. This is accomplished by reducing the sample chemically or mechanically. Mechanical reduction may be achieved by grinding the sample in a coffee grinder, mill, ball-bearing shaker, or mortar and pestle. Freezing the sample in liquid nitrogen may help this process, but the sample must be placed in a sealed bag prior to submersion since liquid nitrogen can be a source of DNA contamination (Fountain et al 1997). A potential concern with powdering skeletal samples is the extra manipulation of the samples, and the creation of increased sample surface area for the binding of contaminating DNA molecules. Despite such concerns, there have been few reports of increased contamination with modern DNA by those using this method. Bone samples also can be chemically reduced by decalcification in EDTA with agitation or rotation for 72 hours, changing the solution every 24 hours. Following this method, small bone fragments can be completely digested overnight in a proteinase K (PK) extraction buffer without the need for mechanical reduction (O'Rourke et al 1996, 1999).

aDNA extraction methods borrow heavily from forensic protocols (see review in Parsons & Weedn 1996), and use one of two approaches: proteinase K/phenol-chloroform or silica based extraction protocols. Proteinase K chemically reduces proteins in a sample with a protease (Blin & Stafford 1976) and unlike other proteases its activity is not inhibited by EDTA remaining from decalcification protocols. Extraction buffers also include various detergents or surfactants to emulsify the lipids and/or aid in the digestion of proteins (see Sambrook et al 1989). The addition of 1.0–2.0 ml extraction buffer to a small amount of sample (0.5–1.0 g) is sufficient to digest the protein in several hours at moderate temperatures (50–60°C) with rotation or agitation. Equal volumes of equilibrated phenol and DNA-containing post-PK digestion solution are combined, vortexed, and centrifuged to separate the aqueous and organic phases (phenolic), and the supernatant is removed to a fresh tube. Since DNA is an acid, it remains dissolved in the aqueous phase, whereas most other compounds commonly found in the tissue source and surrounding contextual matrix remain in the organic phase. This process is repeated once with 25:24:1 phenol:chloroform:isoamyl alcohol, and again with 24:1 chloroform:isoamyl alcohol. Carryover of organic solvents to subsequent extraction and amplification stages must be prevented since they either inhibit PCR or cause sample loss (by destroying micro-concentrator filters). The final aqueous phase is concentrated on a micro-concentrator to remove macromolecules less than 30,000 MW in size. Unfortunately, this also concentrates co-extracted PCR inhibitors along with the DNA. The concentrate is subsequently ethanol (or isopropanol) precipitated and the pellet redissolved in mild TE buffer. Hänni et al (1995) suggest substituting isopropanol for ethanol, because it has a greater selectivity for

DNA (Sambrook et al 1989). They also report that isopropanol is more efficient at removing the brown-colored PCR inhibitor suspected to be Maillard products (Poinar et al 1998). The addition of sodium chloride, sodium acetate, ammonium acetate, potassium acetate, or lithium chloride to the ethanol extraction may increase DNA yields (Miller et al 1988, Sambrook et al 1989), as does extending precipitation to 24 hours at −20°C (Vachot & Monnerot 1996).

The silica method (Höss & Pääbo 1993, Boom et al 1990) extracts DNA in a high concentration of guanidinium thiocyanate (GuSCN). GuSCN, like PK, has the ability to lyse proteins, and acts as a chaotropic agent facilitating the binding of DNA to silica particles. After incubation at moderate temperatures (~60°C) for several hours, the solution is centrifuged to pellet any remaining cellular debris. An aliquot of the supernatant is added to an equal volume of a silica suspension-containing GuSCN extraction buffer and briefly re-incubated. After centrifugation the silica pellet is washed once in a modified GuSCN extraction buffer, twice with ethanol, and once with acetone. The pellet is then re-dissolved in mild TE buffer. The advantage of this protocol is that it removes the necessity of a fume hood for handling the highly toxic phenol and chloroform vapors. It is also less likely to co-extract PCR inhibitors; but silica itself is a strong PCR inhibitor, so care must be taken to remove all silica during the washes. Also, due to its extreme affinity for DNA, the GuSCN can easily become contaminated with modern nucleic acids. Several silica-gel-based DNA extraction kits have been used to extract ancient DNA (Cano & Poinar 1993, Tuross 1994, Zierdt et al 1996, Yang et al 1998). Comparison of various combinations of phenol-chloroform extraction and one such silica-gel kit (Qiagen Qiaquick preps) indicated PK digestion followed by Qiaquick column purification yielded better results than phenol-chloroform extraction alone (Yang et al 1998). The silica-gel spin columns act as a concentrator, removing pigmentation commonly left behind after phenol-chloroform extraction, but they are limited in the amount of solution that can be loaded into them at one time. This could be problematic because increased handling/processing of the DNA solution creates additional opportunities for contamination or sample loss. Although PK-based extraction methods may result in greater DNA yields, silica guanidinium protocols often result in higher amplification success rates and fewer PCR inhibitors (Cattaneo et al 1997).

SOURCES OF aDNA

Any substance of biological origin is a potential candidate for aDNA recovery, although some are more successful than others are.

Bone

Bone is generally considered an optimal aDNA source because the binding of DNA to hydroxyapatite slows DNA degradation. Experimental results support the notion that DNA yields from bone exceed those from soft tissue (Tuross 1994), although

extraction and amplification success appears independent of tissue type (Höss et al 1996; cf O'Rourke et al 1996). Hagelberg and coworkers (1991) observed a correlation between microscopic morphological preservation of bone samples and DNA recovery, but not with sample age. Because poor gross preservation is not always indicative of poor microscopic preservation (Shearin et al 1989), any ancient sample may potentially yield DNA.

Skeletal tissue can be sampled in several ways. Small fragmentary pieces can be used, or long bones can be sampled by drilling or mid-shaft sectioning. Skeletal elements without lesions should be chosen because lesions provide an avenue for contamination. Our practice has been to chose small fragmentary rib samples because they are numerous per individual, are of minimal morphological or paleopathological import, and are rarely missed from museum or archaeological collections. Additionally, spongy bones such as ribs can yield 10- to 20-fold more DNA than does compact bone (Lee et al 1991), although arguably not as reliably (Parsons & Weedn 1996; cf Parr et al 1996, O'Rourke et al 1996).

Teeth

Using teeth as sources of aDNA has the advantage of multiple, independent samples per individual. Teeth without caries should be chosen because dental caries allow contaminating DNA to enter the pulp cavity. Using unerupted teeth further reduces this risk. Two preparatory methods are commonly used for aDNA extraction from teeth. The first is simply powdering the tooth; the second requires sectioning the tooth prior to removal of the pulp cavity (e.g. Drancourt et al 1998, Merriwether et al 1994). The latter method permits gluing the tooth back together following pulp removal and, therefore, is less destructive. Powdered teeth yield either a white- or brown-colored powder, although the difference is unrelated to amplification success (Drancourt et al 1998). Powdering the entire tooth yields more DNA than does sectioning to access the pulp cavity, although the former produces more degraded DNA than the latter does (Smith et al 1993). A third method, endodontically accessing the pulp cavity (i.e. root canal), is not recommended (Smith et al 1993) because it is difficult to obtain the interior tissue efficiently.

Soft Tissue

If soft tissue is to be used as source material, subsurface tissue should be selected whenever possible to reduce contamination from handling. Desiccated soft tissue is likely to be the best source of aDNA from soft tissue because desiccation may protect DNA from hydrolytic damage [although it is still susceptible to oxidative damage (Pääbo 1989)]. Brain tissue from the Windover peat bogs [ca 7000–8000 years before present (BP)] yielded approximately 1 mg of DNA/g of tissue, only 1% of the expected yield from fresh tissue (Doran et al 1986). Realistically, the yield is even less because the majority of extract is likely

to be co-extracting peat. Nonetheless, human mtDNA-specific oligonucleotides hybridized to the blotted Windover samples. In general, aDNA yields from desiccated soft tissue are less than realized from bone samples, and the coextraction of inhibitors is greater.

Alternative Potential aDNA Sources

Hairs have been used to obtain DNA in forensic cases (Wilson et al 1995), although shed hairs generally contain only the shaft, not the DNA-containing root. The amount of DNA in human hair shafts in modern forensic samples is reduced to $\ll 1$ ng in only a few weeks and is often below the detection limit of PCR (Higuchi 1989; see also Allen et al 1998), reducing the prospects for aDNA recovery from ancient hair samples. Coprolites also hold potential for nucleic acid extraction (Chobe et al 1997). Poinar and colleagues (1998) report that use of N-phenacylthiazolium bromide is effective in releasing nucleic acids from Maillard products when using coprolites as a tissue source. This procedure, however, does not appear to increase the likelihood of success when dealing with extracts from skeletal material. Obtaining aDNA from plant macrofossils and pollen also holds considerable promise. Plants have large amounts of DNA in chloroplast, mitochondrial, and polyploid nuclear genomes. Reports of nucleic acids recovered from ancient (up to 5000 years old) samples of wheat (Brown et al 1994), corn (Goloubinoff et al 1993), and barley and radish seed (O'Donoghue et al 1996) suggest considerable future promise (Evershed et al 1997) in paleoecological studies.

Ethical and Legal Issues in Sampling

Opposition to skeletal analyses from around the world (e.g. United States, Australia, Israel) for cultural, religious, and political reasons impedes access to research samples and makes ethical, legal, and social issues paramount in aDNA research (Webb 1987, Ubelaker & Grant 1989, Jones & Harris 1998, Balter 2000). Researchers on ancient DNA need to be keenly aware of the local legal ramifications of, and restrictions on, their work (in the United States, the Native American Graves and Repatriation Act, PL 101-601), and no single strategy for gaining access to research materials can be offered. Nevertheless, we feel strongly that initiating open discussions, and obtaining appropriate permissions, prior to initiating aDNA research projects precludes adversarial conflicts later and facilitates future research access. We also believe that researchers have an obligation to use scientific principles to protect sample materials that are in the public trust, and which are of sufficient antiquity to not be reliably associated with modern populations. We should recognize, however, that results of aDNA analyses may be used to extend the time frame by which ancestral/descendant relationships may be plausibly demonstrated or inferred. These issues are likely to become more, rather than less, important or complicated and should be given equal attention as experimental design.

AMPLIFICATION METHODS

Amplification of target DNA sequences is now a routine laboratory technique (Erlich 1989, Innis et al 1990). Methods have become relatively standardized, although many techniques developed for use with modern samples do not work equally well with ancient ones. Accordingly, a variety of protocols for amplifying aDNA have been published (Pääbo 1989, 1990; Hagelberg et al 1991; Herrmann & Hummel 1994 and references therein). Many successful aDNA amplification protocols incorporate one or more of the following procedures.

Excess primer concentration in aDNA PCR reactions facilitates mispriming of primers to nontarget molecules, resulting in "laddering" effects as well as excess primer dimers on electrophoresis. Laddering effects can be especially notable in the presence of elevated primer concentration when the amount of target template is low, as is often the case with aDNA. Lowering primer concentrations well below standard protocol recommendations (e.g. $<0.2\ \mu M$) reduces primer-dimer formation and effectively eliminates laddering of gel lanes due to spurious amplification of nontarget molecules. This result is facilitated by maintaining stringent PCR conditions.

One of the easiest and cheapest methods to improve yield from an aDNA PCR reaction is the "hot start" procedure (Chou et al 1992). Hot starting a PCR prevents the primers and/or enzyme from annealing to DNA template prior to the sample reaching denaturation temperature at the first cycle (typically 95°C). This procedure increases amplification yield, sequence specificity, and precision by reducing the rate of mispriming and of creation of primer oligomers (Chou et al 1992). These nontarget products not only take up reagents (reducing PCR efficiency), they also interfere directly with the amplification of the target sequence. A hot start is achieved by keeping the PCR reaction mixture on ice until introduced into a thermal cycler that has been preheated to the denaturing temperature, or by using newly developed polymerases that remain inactive until heated to denaturing temperatures. Extending initial denaturing time (e.g. 5–7 min) and final extension time ($\gg 1$ min) may also increase yield and specificity (Pääbo 1989).

It is not uncommon to assume that the amplification of an aDNA sample failed because of lack of an appropriately sized band on gel electrophoresis. However, samples with low template concentration, or significant enzyme inhibitors, may simply result in such low amplification efficiency that insufficient product is generated to visualize on a gel. In such cases, increased amplification yield may be obtained by either "touchdown" PCR (Don et al 1992) or "booster' PCR (Ruano et al 1989). Touchdown PCR simply starts a PCR using stringent (high) annealing temperatures, and then steps down the annealing temperature during the first several cycles (~ 10) until the standard annealing temperature is reached, which is then used for the remaining cycles of the amplification. This results in low, and perhaps inefficient, primer binding during the early cycles, but mispriming is minimized, increasing the proportion of high-quality target sequence in each successive cycle. The method was originally developed for amplification of long

modern template molecules, but it may be effective in some aDNA samples despite the smaller fragment size. Booster PCR (Ruano et al 1989) amplifies a target sequence under increased stringency and reduced primer concentration for only a few cycles. The amplicons thus produced are then used as template in a regular PCR reaction. The principal concern with booster PCR is the additional opportunity of introducing modern contaminants when the initial, enriched samples are removed to "seed" the second round of PCR. Addition of bovine serum albumin to samples to bind nonspecific enzyme inhibitors or increasing enzyme concentration may also increase PCR efficiency and yield. Use of a high-fidelity polymerase active for the lengthened number of PCR cycles (typically 40) characteristic of aDNA analyses is also necessary.

Authenticity of Ancient DNA

Quality Control and Contamination Authenticity of aDNA is of paramount concern, and efforts to assure that research results reflect endogenous target sequences rather than modern contaminants have received considerable attention (Handt et al 1994a, Richards et al 1995). Handt and coworkers (1994a) recommend six criteria for evaluating authenticity of aDNA results: (*a*) Pre- and post-PCR activities should be spatially separated in the lab, or performed in different laboratories; (*b*) strict laboratory protocols should be adopted to prevent and monitor the introduction of modern DNA; (*c*) controls should be used routinely to monitor contamination; (*d*) replicate samples should be used to confirm initial results; (*e*) observed aDNA sequence data should make phylogenetic sense; and (*f*) an inverse relationship between fragment size and PCR efficiency should be observed. In recent years, as laboratory protocols for aDNA research have continued to develop, each has been modified and strengthened.

If possible, labs for aDNA analyses should be dedicated to this purpose. Eliminating research on modern samples in an aDNA laboratory eliminates a prime source of modern template. Even routine use of positive modern controls in aDNA amplification experiments should be minimized. In addition to a dedicated lab, different activities should be carried out in different rooms or different labs to minimize the possibility of contamination. Certainly PCR-preparation activities should be spatially separate from amplification and post-PCR areas. Increasingly, PCR-preparation areas are HEPA-filtered, positive-pressured areas that may be isolated from other laboratory procedures. Our lab performs PCR preparation in a sterile, positive-pressure bench-top enclosure with a HEPA-filtered air supply that is located in an isolated room in a separate lab that has its own HEPA-filtered, positive-pressure air supply and UV cross-linker. The enclosure also has an internal UV light that is routinely used to cross-link the work surface, tubes, racks, pipettors, and some reagents prior to each PCR setup. Even the polymerase and primers may be briefly irradiated to cross-link surface contaminants, but over-irradiation will inactivate the primers and kill the PCR reaction. All equipment should all be dedicated to aDNA research, and all benches should routinely be

cleaned with bleach or DNase, or cross-linked with UV-irradiation. We also cross-link the heating block of the thermal cycler 20–30 min prior to each amplification to help minimize carry-over contamination. All aDNA manipulations should be done by gloved (preferably double-gloved), masked, sleeved, and coated technicians.

Monitoring for contamination in individual experiments is accomplished by the use of multiple negative controls. These should be done for the extraction as well as each PCR experiment. Negative controls are simply tubes to which no DNA sample is added, but which are processed through all the steps of extraction and amplification as if they contained sample. The presence of amplified target in any of these negative control samples is evidence of contamination, and the experiment must be repeated. We advocate the use of multiple negative control samples, especially for PCR preparation, as the presence of contaminants may be subtle and nonuniform. Use of multiple controls increases the likelihood of detecting even small, random cases of contamination. We suggest routine use of both open and closed controls during PCR preparation. Open controls are tubes to which no DNA is added but that remain open throughout the PCR setup procedure, being closed only once all the regular sample tubes are closed and ready for transport to the thermal cycler. Closed tubes contain all the reagents for PCR (except for sample template), but they remain closed during PCR sample preparations. These two control types effectively distinguish contamination due to contaminated reagents versus that introduced during the PCR setup phase (i.e. carry-over contamination).

Replication of initial results is imperative. Ideally, replicates should be done by another laboratory (e.g. Krings et al 1997), but this is frequently not possible because of cost. Replicates in the same laboratory should be done by using separate extractions from a different skeletal element (or tooth) than was used for the original extraction and amplification. The replicate experiments should also be conducted at least several weeks or months apart. Without replication, aDNA results should be considered provisional. The results of aDNA research should also make phylogenetic sense (Handt et al 1994a), although this is more problematic for work with human aDNA. In human samples, the genetic similarity between the ancient sample and likely descendant populations should make sense. All laboratory personnel should be typed and sequenced for all markers for which the sample is being examined to facilitate identification of modern, laboratory-introduced contaminants. Others who have handled the sample(s), such as museum personnel, excavators, etc, should also be typed if they can be identified. Finally, if initial template copy number is low (<100), PCR errors may accumulate and contribute a substantial amount to the final PCR product (Handt et al 1996). These amplified sequences might be recognized as sequence heterogeneity, but they can be detected by directly sequencing cloned PCR amplicons, which can also detect contamination (e.g. Handt et al 1994b, Krings et al 1997, Kolman & Tuross 2000).

Sample Composition and Provenience Analysis of a single ancient specimen presents few problems with respect to sample composition other than correct

provenience—reliable dating of the sample. Analysis of several ancient samples as a "population," however, is more problematic. Most ancient population samples are composed of several individuals separated by varying periods of time in a restricted geographic area, and therefore they do not conform to standard definitions of a population. If the samples come from a geographically and temporally restricted prehistoric horizon, however, and are associated with a uniform material culture, it seems reasonable to treat them as representing multiple, related, continuous lineages, unless archaeological evidence indicates otherwise. It should be recognized at the outset that this is not properly a population in the traditional sense, and assumptions of standard population or genetic analyses may well be compromised by such sample composition. It also means that reliable temporal provenience is essential for such samples. Dating samples directly increases the cost of aDNA analyses, but absent direct dating of typed specimens, provenience of the samples is compromised. Dates for archaeological sites or horizons merely associated with remains used for aDNA analyses may not be reliable indicators of sample age, or at least they may leave the temporal provenience of samples an open question (cf Santure et al 1990). With the exception of the Fremont samples from the Eastern Great Basin (Parr et al 1996, 1998), dating of samples for aDNA research has been neither widely nor uniformly practiced.

An additional problem with aDNA research is less than uniform success in obtaining marker typings on all samples. For example, when using discrete marker data, such as those used to infer Amerindian haplogroups (haplogroups are collections of related DNA haplotypes that share one or more key markers. Mitochondrial haplotypes are identified by the co-occurrence of restriction sites, control region sequence variants, and insertions/deletions. mtDNA haplogroups common in native populations of the Americas are defined by Schurr et al 1992, Torroni et al 1993, Forster et al 1996, Smith et al 1999), not all primer sets are likely to be successful on every sample. This complicates the computation of haplogroup frequencies and results in haplogroup and marker frequencies that are discordant. This is an unfortunate reality in working with degraded nucleic acids, and it makes comparisons across samples, both ancient and modern, difficult. This is being relieved somewhat by the increasing reliance on sequence rather than discrete marker data.

Finally, despite all the precautions noted above, contamination will inevitably occur in any aDNA lab. It is a constant threat, and an inevitable result. Equally inevitable are PCR failures due to enzyme inhibitors, failures that may be overcome by repeated attempts at cleaning the samples and altering PCR profiles. This reality, too, makes reporting success rates difficult because one failed PCR does not mean that data will not ultimately be forthcoming. However, these difficulties mean that other than a final proportion of samples that yielded amplifiable and scorable DNA, no standard procedure exists for investigators to reliably report success and failure rates on individual experiments or data sets. No such standardization seems imminent. Nonetheless, many aDNA studies have been successfully conducted and have addressed a variety of problems in physical anthropology, population

history, demography, and evolution. For a recent review of aDNA studies and application in other areas of biology, see Wayne et al (1999).

APPLICATIONS OF aDNA RESEARCH IN PHYSICAL ANTHROPOLOGY

Europe

Analyses of ancient human DNA in physical anthropology falls conveniently into two categories: studies on single, individual specimens, and studies on archaeologically derived skeletal collections, prehistoric "populations." Of the former, the most widely known and important is the molecular analysis of the Feldhofer Cave Neandertal-type specimen by Krings and colleagues (1997, 1999). DNA was extracted from the Neander Valley specimen using the proteinase K/phenol-chloroform method followed by silica suspension. Amino acid racemization results indicated the ratio of D- to L-enantiomers of aspartic acid in the Neandertal samples was consistent with nucleic acid survival (Poinar et al 1996). mtDNA hypervariable sequences were then amplified and cloned into a plasmid vector. The complete first hypervariable region (HVRI) sequence of mtDNA [nucleotide position (np) 16,023–16,400] was determined from multiple clones and multiple extracts of the Neandertal sample. [The full human mitochondrial genome sequence was established in 1981 (Anderson et al 1981). This original mtDNA sequence is known as the Cambridge Reference Sequence (CRS) and resulted in the systematic numbering of each np in the molecule to facilitate comparisons with other mtDNA sequences. Two mitochondrial control region segments are known to accumulate substitutions at a particularly high rate, due primarily to the absence of DNA repair mechanisms in mitochondria. These sequences, known as hypervariable regions (HVR) I and II, therefore evolve at a very rapid rate and are particularly useful for studying relatively recent evolutionary events.] A small subset of cloned sequences (3/30) was indistinguishable from the CRS (Anderson et al 1981, Andrews et al 1999), whereas the remainder were distinct from the CRS and of presumed ancient origin. The authenticity of the Neandertal sequence was confirmed in a separate laboratory. The Neandertal HVRI sequence exhibits 27 nucleotide differences from the CRS (24 transitions, 2 transversions, and 1 insertion). The average number of nucleotide differences between the Neandertal sequence and 994 modern human sequences from around the world was 27.2 ± 2.2 (range 22–36). Alternatively, the average number of substitutions between pairs of the modern human samples was 8.0 ± 3.1 (range 1–24). The smallest observed difference, then, between the Neandertal sequence and nearly a thousand contemporary human sequences was only two fewer than the maximum difference observed among contemporary samples. For context, the mean number of substitutions between chimpanzee and modern human mtDNA lineages was 55.0 ± 3.0 (range 46–67) (Krings et al 1997).

Although the distribution of observed differences between the Neandertal sequence and a collection of modern human lineages overlaps slightly, the average difference between the Neandertal sequence and modern humans is triple that observed among modern mitochondrial lineages and about half that seen between modern humans and chimps. This distinction between the Feldhofer Neandertal specimen and modern human mtDNA sequences was confirmed by sequencing the HVRII region (Krings et al 1999). HVRII in the Feldhofer Neandertal exhibited 11 transitional differences from the CRS, in addition to a 3-base insertion. Combining the Feldhofer HVRI and HVRII sequences for comparison with modern human hypervariable sequences resulted in approximately triple the number of observed differences between the Neandertal specimen and modern humans (35.3 nucleotide differences). Moreover, the Neandertal sequence was equally divergent from modern African and Asian sequences as from modern European (Krings et al 1999).

More recently, Ovchinnikov et al (2000) obtained DNA from a Neandertal infant excavated from the Mezmaiskaya site in the northern Caucasus and directly dated to 29,000 years BP. The 345-base sequence in the HVRI of this sample yielded nearly twice as many differences between it and modern human sequences (22 differences, comprising 17 transitions, 4 transversions, and 1 insertion) as between it and the Feldhofer Neandertal (12 differences, comprising 11 transitions and 1 transversion). It is important that the two Neandertal HVRI sequences share 19 nucleotide substitutions that are different in the CRS. The magnitudes of observed sequence differences between the two Neandertal specimens are equivalent to those seen among modern human populations, despite the fact that they are geographically, and possibly temporally, distant (the Feldhofer specimen remains undated). Phylogenetically, the two Neandertal sequences form a separate clade distinct from modern human HVRI sequences (Ovchinnikov et al 2000). Moreover, both Neandertal sequences are equally divergent from all modern human groups. Like the Krings et al (1997, 1999) analyses, the new Neandertal sequence data were independently confirmed in a second laboratory.

The Mezmaiskaya Neandertal aDNA results corroborate Krings et al's (1997) initial assessment that Neandertals are unlikely to have been directly ancestral to modern humans and, therefore, support the out-of-Africa model of human origins (cf Ward & Stinger 1997; see also Kahn & Gibbons 1997, Hoss 2000). The two Neandertal aDNA sequences indicate a date for their most recent common ancestor of approximately 150,000–300,000 years ago, but an evolutionary divergence between Neandertals and modern humans well in excess of 300,000 years (range, 365,000–853,000 years ago; Ovchinnikov et al 2000), before the appearance of the earliest Neandertals. Because of the implications for modern human origins, the recent research on mtDNA variation in Neandertal specimens is a particularly important application of aDNA analyses in physical anthropology.

Another single specimen that has received considerable attention is the Tyrolean Ice Man (Handt et al 1994b). This late Neolithic individual was cryo-preserved in a high-altitude glacier. Despite the cold environment, the DNA of this sample

was degraded, and no nuclear amplifications were successful, including attempts at molecular sexing. mtDNA was accessible, and the HVRI region was sequenced from clones. Heterogeneity of observed sequences indicated the presence of modern contaminants, but authentic, ancient mtDNA of the sample could be identified as a result of confirming a consensus sequence from multiple clones. Although labor intensive, this strategy is effective in authenticating ancient template (Handt et al 1994b). The sequence variants observed in this individual were most consistent with those common in modern populations living north of the Alps (Handt et al 1994b).

Similarly, the heterogeneous HVRI sequence variation observed in seven skeletons (~1100–1850 BP) excavated in the Netherlands represented most of the major mtDNA haplogroups of Northern Europe (Colson et al 1997) and is consistent with the highly variable HVRI sequences that characterize modern island Frisian speakers. Fily and colleagues (1998) report on HVRI and HVRII sequence variability in four Bronze Age (3700 BP) skeletons from the Basque region of southern France. The results are notable because a maternal relationship could not be ruled out for three of the samples but could be discounted for the fourth. It is, thus, possible that these four cave burials were members of a family unit, although the inference is based only on the HVRII sequence data. More important was the demonstration that the HVRI sequences obtained were not human but murine in origin. This emphasizes the need to assure specificity of primer design in aDNA research (Fily et al 1998), especially if nonhuman material of comparable age to human samples is being used as positive control samples (Richards et al 1995).

The Americas

Stone & Stoneking (1993, 1998, 1999) obtained DNA from skeletons of the relatively recent Oneota archaeological complex of western Illinois. mtDNA haplogroup diversity (Torroni et al 1993, Ballinger et al 1990, Schurr et al 1992) in the Oneota samples indicated 31.5% were haplogroup A, 12.0% haplogroup B, 42.6% haplogroup C, and 8.3% haplogroup D. Six specimens (5.5%) were inconsistent with any of the Amerindian haplogroups. Two of these were subsequently determined to be of exogenous origin, whereas the remainder represented a fifth founding haplogroup. Of the samples, 52 were sequenced for the HVRI region and found to have a high proportion of singleton mtDNA types (73.9%). This is higher than typically observed in modern Amerindian populations. It may reflect loss of rare lineages due to drift in small populations (perhaps as a result of population declines at contact), or it may be a characteristic of ancient samples in general [due to sampling of lineages through time (Stone & Stoneking 1998)]. Insufficient sequence data on other ancient populations are available to distinguish between these alternatives.

Kaestle (1997, 1998) characterized a series of skeletal samples (~300–6000 BP) from Pyramid Lake and Stillwater Marsh in the Western Great Basin. These samples were genetically indistinguishable based on mtDNA haplogroup analysis.

They also proved to be genetically similar to modern Paiute/Shoshone and California Penutian samples, with low-to-moderate frequencies of haplogroups A and B, low frequency of haplogroup C, and high frequency of haplogroup D.

We assayed mtDNA variation in the Northern Fremont of Utah (Parr et al 1996, O'Rourke et al 1999) and Anasazi of the US southwest (Carlyle et al 2000). Of 43 Fremont samples, 40 were directly dated, whereas 8 of 40 Anasazi specimens have been directly dated so far, with both sets of samples dating to approximately 1000–2000 BP. The latter samples are distributed over a larger geographic area and a slightly longer time frame than are the Fremont materials. Nevertheless, the haplogroup profiles of these two geographically proximal ancient samples are similar. Both are characterized by low to absent frequencies of haplogroup A, moderate-to-high (>50%) frequencies of haplogroup B, and low (<15%) frequencies of haplogroups C and D. Both the Anasazi and Fremont are also characterized by a few samples that do not conform to the traditional four founding haplogroups and are presumed to represent haplogroup X (Smith et al 1999), or an as-yet-undetected contaminant. Further molecular characterization is required to identify these haplogroups.

Modern North Amerindian mtDNA variation is strongly geographically patterned (Lorenz & Smith 1996), and ancient samples studied to date appear to exhibit the same geographic structure (O'Rourke et al 2000). Thus, the Oneota (Stone & Stoneking 1993, 1998) are most similar to modern populations currently inhabiting the central plains and eastern woodlands of North America, as well as an archaeologically recovered Fort Ancient sample from West Virginia (Merriwether et al 1994, 1997). The Western Basin samples (Kaestle 1997, 1998) share greatest similarities to modern populations in Northern California and the northwest Great Basin, whereas the Fremont and Anasazi share mtDNA haplogroup profiles in common with modern southwestern populations. Thus, aDNA analyses confirm that the observed geographic structure of modern North American mtDNA variation has been temporally stable (>2000 years) and apparently little affected by the dramatic disruptions attendant to contact (Stone & Stoneking 1999, O'Rourke et al 2000). The observed geographic and temporal stability of mtDNA discrete markers needs to be confirmed with a greater number of ancient samples and hypervariable-region sequence data.

Fewer ancient samples have been molecularly characterized in Central and South America, but among those that have been studied, the geographic and temporal structure noted in North America appears to be lacking. Merriwether and colleagues (1994, 1997) examined mtDNA haplogroup diversity using discrete marker data in two ancient samples from Northern Chile (Chinchorro and Inca) and the Copan Maya of Honduras. The deletion marker was absent in both Chilean samples, although it is found at high frequencies in the modern populations of the region. The Copan Maya skeletal samples were uniformly haplogroups C or D, whereas the modern Mayan populations of the region are characterized by high frequencies of haplogroups A and B. However, partial typing of some specimens indicates that additional haplogroups are present in the Copan skeletal series

(Merriwether et al 1997). These results are consistent with earlier observations of geographic structure of genetic variation in North, but not South or Middle, America, based on classical markers (O'Rourke et al 1992, O'Rourke & Suarez 1986).

Merriwether and colleagues (1994, 1995) have argued that the ubiquity of Amerindian haplogroups in antiquity argues against multiple migrations of Amerindian founders to the Americas. In contrast, it has been suggested (Lalueza Fox 1996a,b; Lalueza et al 1997) that the absence of mtDNA haplogroups A and B in dental samples of extinct populations of southern Patagonia /Tierra del Fuego indicates separate founding events for different haplogroups. However, only two of the 60 samples are of any appreciable antiquity (4000–5000 BP), the remainder dating to the past two centuries. The authors note that the nineteenth century in southern South America is the "extinction period" (Lalueza Fox 1996a,b; Lalueza et al 1997). It is not obvious that samples obtained from populations undergoing decimation and extinction would be representative of precontact groups. Indeed, reduced population size during this period would be expected to be accompanied by reduced genetic variability. In contrast, haplogroup B (as well as lineages A and C) is present in a small series of artificial mummies from Columbia (Monsalve et al 1996), whereas HVRI sequence data indicates a diversity of haplogroups in 18 Amazonian skeletons dated between 500 and 4000 BP (Ribiero-Dos-Santos et al 1996). In addition to all four of the primary founding Amerindian haplogroups, Ribiero-Dos-Santos et al (1996) found a heterogeneous group of sequences that appeared related to haplotypes observed in modern Amerinds and Asians. The authors suggest that this indicates substantially greater mitochondrial lineage diversity in Native Americans prior to the effects of European contacts (Ribiero-Dos-Santos et al 1996, O'Rourke et al 2000).

Oceania

Hagelberg and colleagues (Hagelberg & Clegg 1993, Hagelberg et al 1994) assayed mtDNA variation in prehistoric Polynesian samples to address questions of Polynesian origins. Using 200- to 2500-year-old samples from throughout Melanesia ($N = 5$), Micronesia ($N = 3$), Polynesia ($N = 7$), and the Central Pacific ($N = 6$), Hagelberg & Clegg (1993) showed that the 9-bp deletion was present in recent Polynesians but absent in older Melanesian and Central Pacific specimens from Lapita archaeological sites. Coupled with a diagnostic HVRI sequence motif that is defined by three nucleotide substitutions at specific positions relative to the CRS (16,217 T $\rightarrow$ C, 16,247 A $\rightarrow$ G, 16,261 C $\rightarrow$ T), these authors suggest a Melanesian origin for modern Polynesians in contrast to a mainland Southeast Asian origin, as suggested by other genetic data (Bellwood 1991, Melton et al 1995, Redd et al 1995). Alternative scenarios also exist (e.g. Richards et al 1998). Hagelberg et al (1994) also assayed 12 prehistoric skeletons from Easter Island for the 9-bp deletion marker and HVRI variation. All the samples were found to possess the deletion, as well as the Polynesian motif of substitutions in the control

region, thus confirming the Polynesian origin of the original inhabitants of Easter Island.

East Asia

In Japan (Horai et al 1989, 1991), HVRI data in a series of Jomon (3000–6000 BP) and "early modern" Ainu samples (200–300 BP) reveal sequence similarity to modern Japanese, and some similarity to modern Southeast Asian lineages, which the authors take to indicate a mainland origin for early Japanese. This inference must be considered preliminary because the number of ancient samples examined is only six and the age range large. Although some studies on one or two ancient samples are available (e.g. Francalacci 1995), until recently no systematic investigation of prehistoric patterns of genetic variation in China had been undertaken. Oota and colleagues (1995, 1999) analyzed 2000-year-old mtDNA from 58 dental and bone samples recovered from the Yixi site in Shandong Province, China, and obtained sequence data for both HVRI ($N = 23$) and HVRII ($N = 16$). In comparison to a variety of modern Asian sequences, both phylogenetic analysis and pair-wise sequence similarity measures indicate greatest similarity between the ancient Yixi samples and modern Taiwan Han Chinese. Inclusion of the Jomon sequences reported by Horai and colleagues (1989, 1991) showed that one of the Jomon samples shared an mtDNA type with individuals in Southeast Asia and Oceania, whereas the remaining four Jomon samples shared mtDNA sequences with the ancient Yixi and many modern circum-Pacific people. Thus, the dual-structure model of Japanese origins proposed by Hanihara (1991) receives little support from these aDNA data because the Jomon samples were genetically more similar to the ancient Yixi rather than to mtDNA lineages that typify Southeast Asian or Oceanian populations (Oota et al 1999).

Paleopathology

Mitochondrial DNA is not the only high-copy-number genome found in ancient samples. Genomes of pathogens are also present in multiple copies in affected individuals, and this has proven beneficial to paleopathologic studies. Traditional paleopathological methods for inferring the presence of disease rely on diagnostic skeletal lesions. Unfortunately, many disorders leave either no, or no diagnostic, skeletal traces. Accessing genomes of infectious agents circumvents this difficulty. Several recent research groups have now identified DNA from *Mycobacterium tuberculosis* in ancient samples from the Middle East (Donoghue et al 1998), Europe (Spigelman & Lemma 1993, Taylor et al 1996, Baron et al 1996), and North America (Salo et al 1994, Braun et al 1998). These samples range in age from historical pathology specimens to specimens from 1400 BP, with *M. tuberculosis* DNA being isolated from bone (Baron et al 1996, Taylor et al 1996, Braun et al 1998), lung tissue from a naturally desiccated mummy (Salo et al 1994), and calcified pleural material (Donoghue et al 1998). Similarly, Drancourt et al (1998) report obtaining diagnostic sequences for *Yersinia pestis*,

the organism responsible for septicemic plague epidemics, from dental pulp. Eight unerupted teeth were obtained from victims of bubonic plague epidemics in 1590 and 1722 in France. All specimens yielded sequences identical to the RNA polymerase b-subunit–encoding gene (*rpoB*) and the virulence-associated plasminogen activator-encoding gene (*pla*) of *Y. pestis*. Several control teeth from burials in a separate cemetery, which showed no signs of plague, yielded no evidence of the disease-causing microbe. Both gene sequences were identical to modern *Y. pestis*. Several species of *Clostridium* were detected by sequence analysis of 16s rRNA gene amplicons in colon samples taken from a thousand-year-old Andean mummy (Ubaldi et al 1998), whereas Chagas disease was diagnosed by species specific DNA of *Trypanosoma cruzi* isolated from a 4000-year-old mummy from Chile (Guhl et al 1999).

Despite small samples, such studies hold considerable promise for identifying the agents responsible for numerous reported epidemics in ancient history, for confirming specific diagnoses of paleopathological lesions in skeletal material, and, through comparison of ancient and modern microbial sequences, for providing insight into the evolution of infectious diseases at the molecular level.

Ancient Nuclear DNA

Although mtDNA is the molecule of choice for aDNA analyses due to its high copy number, single-copy nuclear DNA has been obtained in a few well-preserved specimens. Often such efforts provide insight into the history, distribution, or evolution of disease. Filon et al (1995) identified a frameshift mutation in codon 8 of the β-globin gene in a subadult skeleton with extensive pathology excavated at Tel Akhziv and dated to the sixteenth to nineteenth centuries. This mutation results in the β-null phenotype (β°-thalassemia) and usually results in early childhood mortality. Based on skeletal evidence, however, this individual lived until about age 8, substantially longer than most children did with the phenotype. The individual was also found to have a rare C $\rightarrow$ T transition in codon 2, which is associated with haplotype IV (Orkin et al 1982), and which alleviates the course of the thalassemia by maintaining elevated levels of fetal hemoglobin. Such precision of paleopathologic diagnosis is not possible without molecular genetic analyses.

Unrelated to disease diagnosis, Zierdt et al (1996) examined a short tandem repeat polymorphism (VWA31/A) in the human von Willebrand factor gene in a series of teeth and bone samples from skeletons dating between 1200 and 1500 BP. Although on amplification both the dental and skeletal samples yielded nuclear DNA products, the success rate was higher (36%) with DNA derived from teeth than from bone (22%). Either rate is substantially lower than that typically seen with mtDNA targets (>65%). The observed frequency for this marker in the archaeological samples was comparable to that seen in modern populations of the region. Despite the success in amplifying this marker in some specimens, the results were somewhat problematic. There was an excess of homozygotes observed,

which may relate to the difficulties of amplifying degraded aDNA, and attempts to amplify a second short tandem repeat resulted in substantially reduced success rates. Measurement error in measuring allele sizes for these repeat markers also proved difficult, which suggests that technical improvements of current methods may be necessary before regular screening of nuclear markers in ancient samples becomes commonplace (Ramos et al 1995). Doran et al (1986) and Hauswirth et al (1994a,b) recovered surprisingly high-molecular-weight DNA from 7000- to 8000-year-old brain tissue preserved in crania from burials in a neutral pH peat bog in Florida. The recovered nucleic acids were successfully probed for human mtDNA and *Alu* repeat sequences, and sequences specifying alleles of the major histocompatibility complex, $\beta 2$ microglobulin, and the mtDNA hypervariable region were determined in a few samples. The samples here are unique in apparently yielding very-high-molecular-weight aDNA, and in the high success rate of typing nuclear genetic markers.

By far the greatest effort at accessing nuclear DNA in ancient samples has been in developing methods for molecularly sexing skeletal material. The availability of molecular methods to determine the sex of skeletal material would be a tremendous benefit to paleodemographic studies because the sex of subadults could be reliably determined, something not possible with standard morphological sexing techniques. In addition, sex of fragmentary materials, lacking traditional landmarks used for sexing adults, would also be possible. A variety of molecular sexing techniques have been developed and applied to prehistoric samples (Hummel & Herrmann 1991, Pascal et al 1991, Lassen et al 1996, Stone et al 1996, Palmirotta et al 1997, Ovchinnikov et al 1998, Faerman et al 1998, Cipollaro et al 1998, Santos et al 1998).

FUTURE PROSPECTS

aDNA research remains a difficult, labor-intensive, and expensive enterprise. It has realized considerable success, however, and as sophistication of analytical techniques continues to advance, and as more analysts become experienced in the manipulation of aDNA and detection of contamination, it holds considerable promise for aiding in the resolution of numerous problems in human population history and evolution. Many such questions in population history and paleodemography will require analysis of larger samples sizes than has been typical to date (e.g. >50), as well as routine dating of most analyzed specimens. Recent work on zoological specimens (Greenwood et al 1999) suggests that greater genetic resolution of ancient samples will be possible by focusing on nuclear sequence variation. Increasing the number and types of markers available for aDNA analysis, especially longer sequence screens (e.g. Nasidze & Stoneking 1999), combined with greater utilization of museum collections (DeGusta & White 1996), promises to make significant contributions to primate (Bailey et al 1999) and human taxonomic and evolutionary studies.

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