Detecting Dinosaur DNA

The fact that DNA sequence can be obtained from fossil organisms has opened new windows of opportunity for research in organismal and molecular evolution (*1*). Among these is the possibility of obtaining genetic information from major groups of organisms now extinct. Recently, S. R. Woodward *et al.* sequenced DNA from a portion of the mitochondrial cytochrome *b* gene from Cretaceous bone fragments apparently from a dinosaur that lived 80 million years ago (*2*). However, the likely source of those DNA sequences appears to be human contamination.

In addition to experimental controls, a major line of evidence normally used to support a finding concerning ancient DNA is the phylogenetic relationship of the putative ancient sequence to those from the closest living relatives of the fossil organism (*1*). In the case of a possible dinosaur sequence, there is strong evidence from morphology that birds represent the closest living organisms to dinosaurs, and morphological and molecular evidence indicate that crocodilians are the closest living relatives of birds (*3*–*4*). Also, the fossil record indicates that, after splitting with mammals, at least 100 million years of evolution occurred on the lineage leading to dinosaurs and birds before the latter groups diverged (*3*). Therefore, a putative dinosaur sequence would be expected to cluster with birds and crocodilians in a phylogenetic analysis of amniotes.

Woodward *et al.* (*2*) do not present an evolutionary tree, but discuss their sequences in terms of percent sequence difference, noting that these cytochrome *b* sequences differed from all others in the databases. We also performed a BLAST search using the majority rule consensus sequence [figure 6 in (*2*)] and obtained matches to 130 cytochrome *b* sequences of vertebrates (*5*). As reported by Woodward *et al.* (*2*), the consensus sequence differs by about 30% (26% to 52%) from those vertebrate sequences in the databases. However, 87 of the most similar sequences (closest matches) are mammals, including all nine eutherian orders represented, whereas birds, amphibians, and fish comprise nearly all of the remaining sequences and have the lowest similarity to the consensus sequence. Among the mammal sequences, the closest matches are to whales (99/133 = 74% similarity). However, among the nucleotide sites showing similarity to the human sequence (93/133 = 69%), four are rare variants in the other 129 vertebrate sequences (*6*).

A phylogenetic analysis (*7*) with all tetrapod sequences obtained from the BLAST search joins the putative dinosaur DNA sequence (*2*) with human (Fig. 1). Although statistical support for most nodes in the tree is low as a result of the short length of this region (133 base pairs), bootstrap support for this cluster (91%) is relatively high. Furthermore, a consensus sequence of the nine bone sequences which maximizes similarity to human (118/133 = 88% similarity) clusters with the human sequence at a statistically significant bootstrap *P* value of 100%. Consensus sequences with similarity maximized to each of the other taxa yield considerably lower (0 to 46%) probabilities for clustering with the taxon to which similarity was maximized (*8*).

Despite meticulous care, contamination of polymerase chain reaction (PCR) experiments with foreign DNA, often of human origin, is an ever-present aspect of ancient DNA research because of the sensitivity of the methodology and rarity of the target molecules (*1*). The suggestion by Woodward *et al.* (*2*) that variation among the nine sequences (seven from the same bone fragment) is a result of damaged template may be correct. However, our results suggest that the DNA template was not from a Cretaceous organism such as a dinosaur, but rather from an extant organism, most likely a human.

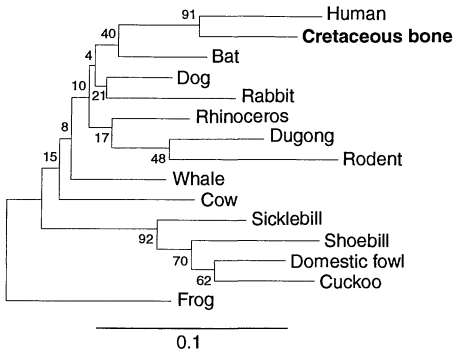

Fig. 1. Phylogenetic tree of partial cytochrome *b* DNA sequences in representatives of extant tetrapod groups and putative dinosaur DNA sequence (majority rule consensus) derived from Cretaceous bone fragments (*2*). Numbers on nodes are bootstrap confidence probabilities. Inclusion of all nine putative dinosaur sequences (*2*) resulted in an identical tree in which those sequences clustered together with human. A frog was included to root the tree. Tree shown is neighbor-joining with transversion distance; parsimony analyses (transversions only and weighted transversions) also clustered the putative dinosaur DNA sequence with the human sequence; 133 sites total, 88 variable, and 66 parsimony. GenBank accession numbers: human (V00662), bat (L28943), rhinoceros (X56283), dugong (U07564), cow (J01394), dog (L29416), rabbit (U07566), whale (X75581), rodent (L11902), sicklebill (X74253), domestic fowl (X52392), cuckoo (U09262), shoebill (U08937), and frog (U02890).

Determining the authenticity of an ancient DNA sequence often can be diffcult, and criteria for this have been discussed elsewhere (*1*, *9*). Two criteria that are important, and that were not fulfilled in the study by Woodward *et al.*, are phylogenetic context and independent replication. Although phylogenetic support has been presented for other findings of DNA surviving for millions of years (*10*), real advance in this field will come only when it is demonstrated that those studies can be replicated in independent laboratories.

S. Blair Hedges
Department of Biology and
Institute of Molecular Evolutionary Genetics,
208 Mueller Laboratory,
Pennsylvania State University,
University Park, PA 16802, USA
Mary H. Schweitzer
Paleontology Department,
Museum of the Rockies,
Bozeman, MT 59717, USA,
and *Department of Biology,*
Montana State University,
Bozeman, MT 59717

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6. Sites 15646, 15687, 15703, and 15706; S. Anderson *et al.*, *Nature* **290**, 457 (1981).
7. The DNA sequences were analyzed with MEGA version 1.01 [S. Kumar, K. Tamura, M. Nei, *MEGA: Molecular Evolutionary Genetics Analysis*, (Pennsylvania State University, University Park, PA, 1993)] for distance analyses and PAUP [D. L. Swofford, *Phylogenetic Analysis Using Parsimony*, Version 3.1 (University of Illinois, Champaign, IL (1993)] for parsimony analyses. Average pairwise Jukes-Cantor (T. H. Jukes and C. R. Cantor, in *Mammalian Protein Metabolism*, H. N. Munroe, Ed. (Academic Press, New York, 1969, pp. 21–132) corrected distances were large (0.3 to 0.5), and therefore a transversion distance (M. Kimura, *J. Mol. Evol.* **16**, 111, 1980) was used with neighbor-joining [N. Saitou and M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987)]; and transversion only, or transversions weighted 10 times transitions, were used with parsimony. The marsupial sequence was excluded from the phylogenetic analyses because of anomalous results. Statistical significance (>95%) was assessed with the bootstrap method [J. Felsenstein, *Evolution* **39**, 783 (1985)], with 2000 replications.
8. Bootstrap probabilities for clustering with the taxon-specific maximized consensus in neighbor-joining analyses (transversion distance) are as follows: human (100%), bat (17%), whale (8%), rabbit (46%), dog (6%), rhinoceros (31%), cow (22%), dugong (9%), rodent (26%), sicklebill (1%), domestic fowl (21%), cuckoo (0%), shoebill (1%), and frog (6%).
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11. We thank C. A. Hass, S. Kumar, and S. Pääbo for helpful comments.

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The comparisons reported by Woodward *et al.* (*1*) were limited to identity percentages, whereas more informative comparisons should be possible by scoring each aligned amino acid pair with the use of a log-odds substitution matrix based on homologous protein alignments (*2*). I compiled a database of all 223 cytochrome *b* segments from different species in the combined protein databanks (through 11/94). Each segment was scored for similarity to a consensus representing the seven long bone sequences, with the use of the most frequent predicted amino acid at each position. A range of BLOSUM (*3*) and PAM (*4*) substitution matrices was used for scoring. In addition, each segment was scored using position-specific scoring matrices (*5*) constructed from the seven long bone sequences and from the two rib bone sequences.

All tested scoring systems provided similar results (data not shown). Among the well-represented taxa, the highest mean scores were found for cetaceans and ungulates. In both cases the mean scores are significantly higher than the mean scores for birds. It is notable that all 15 alignments with cetacean segments outscored all 72 alignments with bird segments, even though both groups are diversely represented (*6*). Overall, scores for vertebrates were much higher than for arthropods, which in turn were much higher than for non-animals (plants, fungi, and bacteria), indicating that this method applied to bone sequences provides rankings consistent with known phylogenetic relationships. Moreover, similar results were found for rib sequences analyzed independently of long bone sequences, despite several nucleotide sequence differences (*1*).

I conclude that the bone sequences more closely resemble homologs in mammals than in birds, which are thought to be the closest living relatives to dinosaurs. Furthermore, the significantly higher scores for some mammals (cetaceans and ungulates) than for others (*7*) further suggest either a mammalian origin or convergence of this region of cytochrome *b*. The analysis also contradicts criticisms that the bone sequences resulted from microbial contamination or were seriously affected by PCR-generated errors. Therefore, further PCR-based analysis of the Utah bones is warranted. For such studies, most efficient synthesis should be possible with primers modeled on the mammalian taxa with high alignment scores. Reducing the high PCR failure rate (*1*) in this way should greatly increase the amount of sequence available for phylogenetic analysis.

Steven Henikoff
Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA 98104, USA

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Assuming that each of the published sequences (*1*) are representative of the study by Woodward *et al.*, we chose two for extensive analyses to assess the history of these molecules (3-37 from bone fragment one and 5-37 from bone fragment two). When alignments were determined by comparison against all of the sequences in a current issue of Entrez (NCBI, release 6.0 of GenBank) with the use of the MacVector program (version 4.1.4, Eastman Kodak, Rochester, New York), the best 30 alignments against fragment one were all mammalian cytochrome *b* sequences, with the first nine chosen from the order Artiodactyla (cattle, deer, antelopes, and their relatives). A similar result was obtained for alignments against fragment two, with the best four alignments each to human cytochrome *b* genes. Other vertebrates are not equally divergent from these purported dinosaur sequences. To the contrary, these unknown sequences have closest similarity to the mule deer (*Odocoileus hemionus*, accession number X56291) and to human cytochrome b genes (*Homo sapiens*, accession number V00662), respectively.

The best strategy for determining relatedness of an unknown sequence is not through a similarity search, but rather by a phylogenetic analysis using parsimony (*2*). While we agree with Woodward *et al.* (*1*) that their small fragment of cytochrome *b* sequence is inappropriate for use in a phylogenetic analysis, it is the only available evidence, and parsimony is still the best strategy for determining the closest relative and for identifying these new sequences. We aligned the unknown cytochrome *b* sequences to several mammals (human, cow, rat, and mouse), to chicken, and to clawed frog (*Xenopus*, our outgroup). The most parsimonious solution was one that grouped Cretaceous bone fragment two with the human and next with the other unknown fragment. This resulted when the characters for each codon were numbered and third positions were omitted and when we looked at the more conserved transversions. When amino acids were translated from the original nucleotide sequences and parsimony analysis was conducted, the unknown fragments were closest, then the chicken (supported by two characters). This pattern also resulted when all characters were examined.

Our most conservative and informative analyses point to mammals as the closest relatives to the available "Cretaceous" sequence, an unlikely relation if these are truly dinosaur remains. This contradicts with numerous morphological characters that support birds as the closest living relative to the dinosaurs (*3*). One might ask, how did mammalian DNA get into these samples? At the time that these coal beds were formed, all of the known mammals were smaller than the bone fragments described (*4*). Possibly, either ancient DNA of a smaller mammal was preserved along with these deposits and thus contaminated these bone fragments, or a more recent DNA sample contaminated these tissue samples. Fossil mammals are known from this geological formation, potentially supporting the former hypothesis. We prefer the latter hypothesis because of the great similarity of these sequences to living mammalian genes.

Marc W. Allard
Deshea Young
Yentram Huyen
Department of Biological Sciences, George Washington University, Washington, DC 20052, USA

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Our preliminary phylogenetic analysis of the putative dinosaur sequences in the report by Woodward *et al.* (*1*) showed them to be weakly related to the human cytochrome *b* gene, albeit quite distantly (earlier comment by Hedges *et al.*, data not shown). As nuclear insertions of mitochondrial DNA are known to occur (*2*), and as 12*S* ribosomal DNA sequences amplified from ancient monkey bones have been attributed to insertions of mitochondrial DNA into the human nuclear genome (*3*), the putative dinosaur cytochrome *b* sequences might represent ancient integrations of mitochondrial DNA into the human nuclear genome.

Mitochondrial DNA–free nuclear DNA was prepared by differential lysis of human spermatozoa, essentially as described (*4*). With the use of the primers and PCR conditions described by Woodward *et al.* and the human nuclear DNA as a template, we obtained a PCR product of the expected size of 174 base pairs and other, presumably unspecific, products. In order to elucidate whether this product was related to the putative dinosaur sequences, we synthesized four different oligonucleotides specific for the sequences 2-37, 3-37, 4-37, and 31-44 described by Woodward *et al.*, but different in at least two positions from the human cytochrome *b* gene. A mixture of these probes hybridized under stringent conditions to the amplification product of the expected size from the human nuclear DNA (data not shown).

The amplification product was cloned, and about 3000 individual clones were screened by the same mixture of probes. More than 300 clones were positive (data not shown). Among these, two sequences were found to predominate. A phylogenetic tree relates these two sequences to the nine putative dinosaur sequences, to human, rodent, and cow sequences as well as to sequences from two birds and a shark (Fig. 1). Although the bootstrap probabilities are low as a result of the small amount of sequence information available, the dinosaur sequences form a cluster. However, this cluster does not group with birds as might be expected for dinosaur sequences, but rather with the human cytochrome *b* sequence. Moreover, the two sequences amplified from the human nuclear genome fall inside the cluster of putative dinosaur sequences and are most closely related to sequence 4-37. The latter result is supported by 95% of bootstrap replications.

We can envisage several fascinating scenarios, that could account for these results. First, our preparation of human nuclear DNA, or other reagents, might be contaminated by dinosaur DNA. We find this alternative unlikely because we, to the best of our knowledge, have no such DNA in our laboratory. Second, dinosaur mitochondrial DNA might have penetrated the mammalian germ line by a hybridization event (or events) between mammalian ancestors and dinosaurs sometime before the end of the Cretaceous. We find this alternative implausible for reasons related to the biology of the organisms involved, and because the dinosaur sequences would subsequently have converged on the human mitochondrial sequence. The third, less stimulating alternative is that the dinosaur extracts, or other reagents, used by Woodward *et al.* were contaminated by small amounts of human DNA.

We find the last alternative plausible because DNA is not expected to survive over millions of years except, perhaps, under extraordinary conditions (*5*). Moreover, fragments of mitochondrial DNA have been found integrated in the nuclear genome of several vertebrates (*2*). Such sequences can exist in many copies (*6*) and might vary substantially in sequence when short amplifications with primers specific for conserved parts of the mitochondrial genome are performed (*3*). If single copies of such nuclear insertions are responsible for the sequences published by Woodward *et al.*, we expect PCR errors to contribute to the diversity among the sequences and to compound the phylogenetic analysis.

In conclusion, these results strongly suggest that Woodward *et al.* (*1*) accidentally amplified nuclear copies of human mitochondrial DNA. The fact that a sequence from an ancient specimen is not identical to any hitherto determined sequence cannot be taken as an indication for the ancient origin of that sequence.

H. Zischler
M. Höss
O. Handt
A. von Haeseler
Zoological Institute,
University of Munich,
P.O. Box 202136,
D-80021 Munich,
Germany
A. C. van der Kuyl
J. Goudsmit
Human Retrovirus Laboratory,
Academic Medical Center,
Meibergdreef 15,
1105 AZ Amsterdam,
Netherlands
S. Pääbo
Zoological Institute,
University of Munich

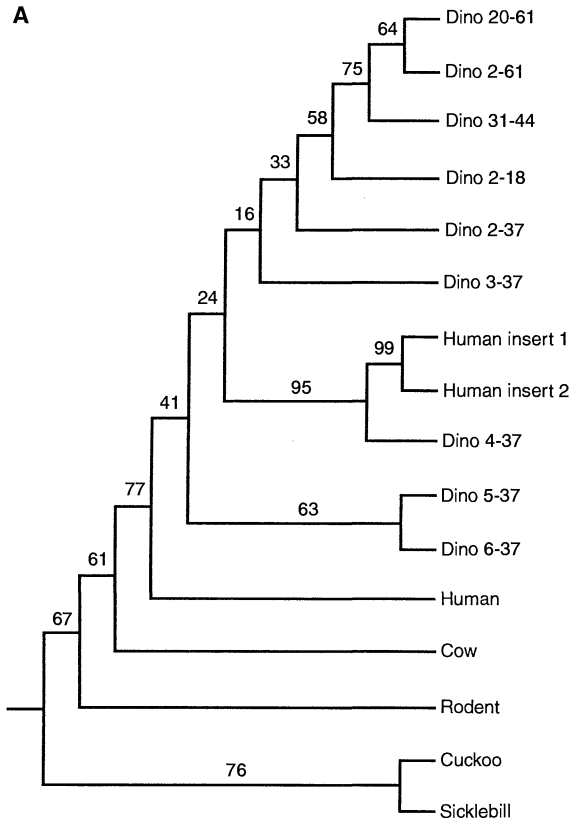

Fig. 1. Phylogentic tree (**A**) relating the dinosaur sequences from the report by Woodward *et al.* (*1*) to vertebrate mitochondrial sequences. (**B**) Two sequences integrated in the human genome. Numbers on internal branches refer to bootstrap probabilities. The tree reconstruction was performed using a neighbor joining algorithm, maximum likelihood distances with transitions weighted equal to transversions, and 100 boostrap replications. Sequences are available in GenBank under accession numbers: human (V00662), cow (J01394), rodent (L11902), cuckoo (U09262), sicklebill (X74253). A shark (L08035) sequence was used for rooting.

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Response: Heinikoff and Allard *et al.* correctly point out that an organisms genetic history is maintained and can be revealed from the information contained in a DNA sequence. This is one of the principal reasons for attempting to recover ancient genotypes. However, DNA from ancient sources is usually damaged as is reflected in the sequences obtained after amplification (*1*). In addition, the history of a DNA sequence may be convoluted, with a number of branches and rejoins through the history of its lineage (*2*). Therefore it is possible that not all DNA sequences will reflect a direct path or be equally informative. Sequences may give valuable information, but conclusions based on this alone may not be enough to guarantee that the source of the sequence is ancient.

We reported that the Cretaceous sequences were unique and differed from all others in the database, but not that they were unrelated to any of the database sequences (*3*). Henikoff's observation that the predicted amino acid consensus sequence is more closely related to existing mammal sequence and in particular cetaceans, matches the results that we obtained with the BLAST searches. Whales represent the most closely related mammals. There are more than 35 other mammals whose sequence are more closely related to the Cretaceous sequence than is the human sequence.

We constructed a consensus sequence from the nine different amplifications because of the high probability that each of the individual sequences was not a true representative of the original sequence, as damage was incurred over the 80 million years since the bone was deposited. Therefore, we would argue that these sequences should not be used independently and that the consensus sequence represents the original sequence more accurately. Likewise, the creation of a maximized sequence by choosing a single base in preference to the other seven or eight bases that match each other could lead to different phylogenetic results than would be obtained using the consensus sequence. This is reflected in some of the low bootstrap probabilities seen in these reports.

Hedges and Schweitzer use as evidence of human origin of the Cretaceous sequence the sharing of unique characters that are rare human nucleotide variants. Of the four that they cite, one (15706) is not rare, being present in greater than 50% of published mammal sequences. Also, there are seven other equally rare human sites not shared with the Cretaceous sequences. There are also nine sites that are totally invariant between the individual Cretaceous sequences yet differ from the human sequence. This argues for a source other than human contamination for the Cretaceous sequence.

Zischler *et al.* propose that the sequences we reported (*3*) could be a result of contamination by a mitochondrial insert into the nuclear DNA. This would require either multiple contamination events by different DNA's, each having a different sequence, or multiple insertions of a sequence into the same genome, which then must have diverged from the original mitochondrial sequence by 30%, yet only differed by 8%. The contaminating DNA would also have to be free of all mitochondrial DNA. All protocols that we used involved the isolation of total DNA, both nuclear and mitochondrial. Mitochondrial DNA is orders of magnitude more abundant in these preparations than nuclear DNA. This should, at the least, produce a mixed or ambiguous sequence, resulting from the amplification of mitochondrial and nuclear sequences. We did not observe this. We have been involved in many other attempts to recover ancient DNA from a variety of samples and organisms. Occasionally we have observed contaminant bands in negative control samples. In all cases, these sequences have corresponded to an easily identified source. In thousands of amplification attempts using the same protocols and primer sets used in our report, we have never obtained a sequence similar to the Cretaceous sequence that would suggest a possible contaminant source of the sequence. Contamination by human nuclear DNA is probably not the explanation for the origin of the Cretaceous sequence. A possible explanation for the similarity of the Cretaceous and mammalian sequences may be in the convergence of the cytochrome *b* gene on the basis of energy considerations in the organisms and selection pressures on the protein, or in this particular region within the protein.

Phylogenetic analysis can be a valuable tool in assessing the validity of an ancient sequence, especially in cases where there are indications of strong phylogenetic relationships between the ancient and modern samples, such as seen in cases of samples preserved in amber (*4*). However, there are some critical considerations. It is helpful to have sequences from known related taxa for comparison. It is easier to establish likely relationships between ancient DNA of taxa with extant species if the latter have left an easily followed line of direct descendants. In our study (*3*), the relationship between birds, reptiles, and dinosaurs did not fit into this category. There are still several difficulties in the record of phylogenetic origins within birds (*5*). Also, if the analysis is based on a single gene or locus, any selective pressures that may be exerted on the resulting protein must be taken into consideration. In this case, such selective pressures have not yet been adequately addressed. Analysis based on partial sequences such as that from the Cretaceous would be even more problematic. Mitochondrial DNA is known to evolve at a higher rate than nuclear DNA (*6*), and differences between two homologous sequences reach a plateau around 30% divergence within 30 to 40 million years (*7*). It is possible that the analysis of the 174 base pairs of Cretaceous sequence is being stretched beyond its expected usefulness. We would not eliminate the possibility of the ancient origin of a DNA sequence on the basis of hypothetical relationships between taxa for which the evolutionary history is not yet proved.

Scott R. Woodward
Department of Microbiology,
788 Widstoe Building,
Brigham Young University,
Provo, UT 84602, USA

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