analytical chemistry
Technical Note
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Dating Human Bone: Is Racemization Dating Species-Specific?

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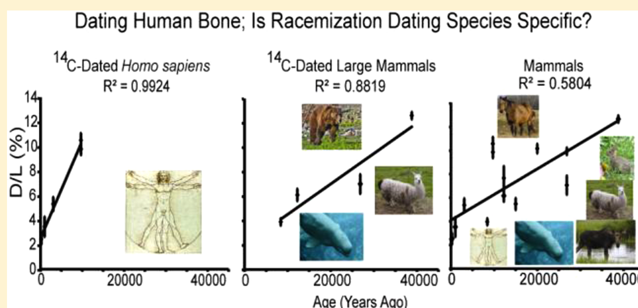
Supporting Information

ABSTRACT: Our recently developed dating technique based on the racemization rate of aspartic acid was applied to dating human bone, as well as that of other mammals, utilizing capillary electrophoresis mass spectrometry. First, several well-dated (mostly ^{14}C-dated and with strong archeological evidence) human bones ranging in age from 150 to ~10 000 years were used to develop a calibration curve for human bone. The D/L ratio of aspartic acid for these specimens ranged from 2.4% to ~10%, with a correlation coefficient of better than 0.99, indicating a strong linear relationship between the D/L ratio of aspartic acid and the age of the specimens. This calibration curve can now be used to date human archeological specimens of unknown age, up to ~10 000 years. However, when the technique was applied to well-dated mixed species of larger mammal bones such as bison, whale, llama, etc., the calibration curve showed a slower rate of racemization with a lower correlation (0.88). As additional large mammal bones with less certain age (i.e., using archeological evidence alone with no ^{14}C-dating) were dated the correlation coefficient decreased to 0.70. The correlation coefficient decreased further to 0.58 when the racemization data from all mammals (including human) were added to the calibration curve, indicating the importance of using well-dated, species-specific specimens for forming a calibration curve. This conclusion is consistent with our previously published calibration curve for a single species of silk (*Bombyx mori*), which followed the expected reversible first-order kinetics. These results support species specificity of amino acid racemization dating.



Determining the age of human bones is important in archeology, forensic science, and other disciplines. Currently, radiocarbon (^{14}C) dating (using both conventional and accelerator mass spectrometry) is the most common technique for dating bone. However, ^{14}C-dating has several disadvantages, including (i) a large sample (often >1 g of bone) is necessary, which leaves undesirable visible and morphologic damage to valuable specimens; (ii) the process is expensive and usually costs several hundreds of dollars per analysis; (iii) sample preparation is time-consuming and labor intensive; and (iv) ^{14}C-dating does not provide accurate dating for objects younger than ~500 years or older than 100 000 years.[1−6]

To address these shortcomings, decades ago, an amino acid racemization (AAR) dating technique was developed and applied to a wide range of archeological and historic samples, including shells, teeth, and bone ranging in age from modern to millions of years, using a variety of analytical techniques.[7−11] The main advantages of AAR are (i) a minimal amount of sample is required, and (ii) widely available technology, such as gas and liquid chromatography (GC and LC, respectively), can be used with a variety of detectors, such as flame ionization detectors (for GC), ultraviolet (UV) spectrometers (for LC), and mass spectrometers (with both GC and LC).[12,13] The use of GC and LC with chiral columns allowed individual laboratories to analyze their own samples, rather than outsourcing them to a commercial laboratory for dating. The use of AAR for dating has been controversial, because of reports of unreliable dates for bones.[14,15] To re-examine the use of AAR for dating bone, we have investigated several factors that could have contributed to the development of unreliable data in past experiments. The first factor is ambiguity in the age of the bones studied. To date an unknown bone, one must first develop a calibration curve for the bone using well-dated specimens.[14,16] However, in some previous studies, the bone calibration curves were obtained using bone dated solely from archeological evidence.[9] To eliminate this uncertainty, our study used mostly ^{14}C-dated bones with good archeological constraints to create the calibration curves. A second factor is the failure to address bone species as a possible variable in racemization.[17] In this study, the emphasis is on species-specific dating. Specifically, a calibration curve was developed exclusively for *Homo sapiens* bones. Additional mammal species were subsequently added to the curve in order to examine the effect of mixed species on the slope and variance of the calibration curve. A third factor is the use of older technologies such as HPLC-UV or GC-FID to identify and quantify amino acids and their D/L ratio.[18−20] These non-mass-spectrometric techniques are unable to identify overlapping peaks and other impurities hidden within the peak, which could lead to inaccurate quantification of the D/L ratio. Tremendous advances in chiral chromatography have provided

Received: September 4, 2013
Accepted: October 24, 2013
Published: October 24, 2013

 © 2013 American Chemical Society dx.doi.org/10.1021/ac402917z | Anal. Chem. 2013, 85, 11211−11215

Table 1. Sample Information for Analyzed Bones[a]

sample	age (yrs)	species	bone type	location	collection[b]	designation
1	150[c]	*Homo sapiens*	femur	Glorieta Pass, NM, USA	NMNH	GLO-099-2A
2	182−232[c]	*Homo sapiens*	femur	Griswold, CT, USA	NMNH	6CT58-5-AMM05
3	1000[c]	*Homo sapiens*	unidentifiable	Mongolia	NMNH	N/A
4	3074 ± 49[d]	*Homo sapiens*	femur	Hovsgol, Mongolia	NMNH	N/A
5	9690 ± 50[d]	*Homo sapiens*	limb segment	Horn Shelter, TX, USA	NMNH	41BQ46-AR-100
6	9710 ± 40[d]	*Homo sapiens*	rib	Horn Shelter, TX, USA	NMNH	41BQ46-AR-200
7	8400 ± 50[d]	*Cetacea*	vertebra	unknown	N/A	N/A
8	12 250 ± 90[d]	*Bison antiquus*	humerus	Aucilla River, FL, USA	FLMNH	UF 175237
9	26 850 ± 590[d]	*Hemiauchenia macrocephala*	metatarsal	McKittrick Brea, CA, USA	LACM	151883
10	38 800 ± 120[d]	*Ursus sp.*	unknown	Thistle Creek	N/A	N/A
11	12 000−15 500[e]	*Equus sp.*	rib	Aucilla River, FL, USA	FLMNH	UF 176248
12	15 000−20 000[e]	*Cervalces sp.*	calcaneum	Saltville, VA, USA	NMNH	23704
13	15 000−20 000[e]	*Bootherium sp.*	rib	Saltville, VA, USA	VMNH	92-58W-21S-1
14	20 000−30 000[e]	*Leporidae*	humerus	McKittrick Brea, CA, USA	LACM	151887

[a]N/A denotes that this information was not available. [b]NMNH, Smithsonian National Museum of Natural History; FLMNH, Florida Museum of Natural History; LACM, Natural History Museum of Los Angeles County. [c]Date determined from historical and archeological evidence. [d]Date determined by direct ^{14}C-dating and associated archeological evidence; ages are listed in ^{14}C radiocarbon years. [e]Date determined by bracketing of ^{14}C-dates within fossil site.

much improved separations and, when combined with mass spectrometry (MS), provides for much more accurate quantitation.[21−23] In addition, in a majority of these studies, the chromatograms that show D and L separation were not provided, to allow independent assessment of the data. In this study, capillary electrophoresis in conjunction with mass spectrometry (CE-MS) has been used for baseline separation and information-rich detection of amino acids and their D/L mixtures. Moreover, an ion electropherogram (raw data) for aspartic acid of each specimen is provided to demonstrate baseline separation between D- and L-isomers for accurate quantitation. MS is required for D and L quantitation of amino acids, because spurious overlapping peaks with different m/z can be separated by MS, thus providing more-accurate D/L ratios. In addition, CE-MS is almost 1000 times more sensitive than ^{14}C-dating (meaning that 1000 times less sample is required) and consumes a fraction of the sample quantity that GC or LC techniques consume. CE-MS also provides reduced analysis time and reduced cost per sample, since it uses a bare fused-silica capillary in conjunction with a microliter-scale quantity of a (+)-tetracarboxylic acid 18-crown-6 as a chiral CE background electrolyte. The final factor that we have considered for more-accurate AAR dating is testing the specimens for amino acid and protein contamination. In previous studies, protein impurities were seldom investigated; however, it has been shown that even exposure to a laboratory atmosphere, which can contain proteinaceous dust particles, can contaminate samples.[24] In the present work, analysis of the sample for other impurities was an integral part of the dating technique. The identification and quantitation of amino acid or protein impurities was easily checked by comparing the CE-MS electropherogram of the specimens' HCl digest solution with that of other pure bones, as well as by proteomics analysis of the enzymatic digestion of bone proteins extract.

■ METHODS AND INSTRUMENTATION

Sample Preparation. Detailed information regarding the samples used in this experiment can be found in Table 1. After bone pieces were washed with water and methanol to remove surface contaminations, a small sample was taken from the interior of the bone. One to five milligrams (1−5 mg) of the bone was digested in 6 N HCl at 110 °C for 2 h. The solutions were centrifuged at 14 000 rpm, and the supernatants were removed, dried to complete dryness, and resuspended in 0.1 N HCl (amino acid solution) for CE-MS analysis of D/L ratios. A single sample (Sample 1) was digested for 2, 4, 8, and 24 h to examine the effects of prolonged digestion on racemization. Samples in this time trial were analyzed by nano-LC-MS/MS to determine the presence/absence of peptides. This same sample was also examined for the effects of a more-traditional collagen extraction on racemization. A modified method of Longin was used to extract collagen; this procedure is common in stable isotope analysis and carbon dating.[25−27]

Capillary Electrophoresis Mass Spectrometry (CE-MS). The CE-MS analyses were performed using a Beckman Coulter ProteomeLab PA 800 capillary electrophoresis (Fullerton, CA) interfaced with a Finnigan LCQ Duo mass spectrometer (San Jose, CA), using a porous tip.[28] *NOTE: When etching the tip with hydrofluoric acid (HF), rubber gloves and eye protection should be worn and all work should be performed inside of a designated fume hood.* Fused-silica capillaries, ranging in length from 90 to 110 cm with inner diameters of 15 or 20 μm and outer diameter of 150 μm, were used to obtain baseline separation of aspartic acid. The separation occurred under forward polarity mode (positive 25 or 30 kV applied to the inlet electrode) utilizing pressure-assisted CE with an inlet pressure of 0.5−2 psi.[29] Samples were injected using voltage injection mode (−2 to −4 kV) or pressure injection mode (0.1−1 psi) for a duration of 2−5 s. A 30 mM solution of (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid (18-crown-6-TCA) in water was used as the background electrolyte to separate the amino acids D- and L-isomers.[30,31] Only the D/L values for aspartic acid/18-C-6-TCA complexes (m/z of 574) were reported here, since aspartic acid was the only amino acid in which the L-to-D conversion was fast enough that its ratio could be quantitated for the bone ages that were used in this study. Alternatively, other chiral selectors could be used for the separation of D- and L-amino acids.[32,33] Moreover, the use of a triple quadrupole mass spectrometer could improve quantitation of the D/L amino acids and thereby increase the accuracy of the dating. For each specimen reported here, the D/L ratio was measured at least three times and the average value of these three measurements was reported. Proteomics analyses were per-

formed using LTQ Orbitrap Velos (Thermo-Fisher, USA), in conjunction with Ultimate nano-LC (LC-Packing, USA). Bone proteins were extracted using the modified method of Buckley et al.[34,35] and digested with trypsin, and analyzed by Discoverer proteomics software package (Thermo-Fisher, USA). Please refer to ref 36 and the Supporting Information for detailed information on other experimental parameters.

■ RESULTS AND DISCUSSION

Sample preparation is an important first step for accurate D/L measurement of the aspartic acid.[36] Contamination must be minimized by washing the specimens and taking samples from the interior of the bone. Racemization must be minimized during sample preparation by minimizing the time required for complete HCl digestion. The high sensitivity achieved by using CE-MS allows ~2 mg of bone to be used for dating, which significantly reduces digestion time, thereby minimizing the L-to-D conversion during the sample preparation. Results from the time trial showed that a digestion time of 2 h was sufficient to completely digest a few milligrams of bone proteins to their amino acid (AA) constituents. This was verified by nano-LC-MS/MS analysis of the 2 h HCl digest in which no peptide in the m/z range of 400−2000 was detected. Also, the CE-MS of this solution in the m/z range of up to 800 showed no dipeptide or tripeptides. After the 2 h of digestion time, the aspartic acid D/L ratio for the 150-year-old human (date of death) bones was 2.4% (see Figure S-1A in the Supporting Information). Increasing the HCl digestion time from 2 h to 24 h increased the D/L ratio from 2.4% to 6.4% (see Figure S-1 (inset) in the Supporting Information). Therefore, for D/L measurement, all subsequent bone samples were HCl digested for 2 h. Similarly, the more-traditional collagen extraction procedure used for stable isotope, carbon dating, and traditional amino acid analyses, which require the sample to be heated at several stages to either 60 or 95 °C, increased the D/L ratio from 2.4% to 5.3% (see Figure S-1 in the Supporting Information).[25−27]

Another important factor in D/L dating of proteins is sample purity check. The CE-MS analysis for D/L measurement also provides a base peak electropherogram of the amino acid composition of the bone (AA fingerprint). The base peak electropherogram of AA could be used as a rapid and convenient means to check the purity of a specimen, since the presence of major protein or AA impurities will change the appearance of the AA fingerprint of the bone.[29,30,36] Figure 1 shows the electropherograms for four human bones listed in Table 1 ranging in age from ~200 to ~10 000 years old. Considering the ionization efficiencies of AA/18-crown-6-TCA complexes,[29,30,36] the relative intensities of the amino acids of the bone specimens are consistent with the theoretical amino acid composition of the *Homo sapiens* bone proteins (see Table S-1 in the Supporting Information), which confirm the purity of the bone proteins.[37,38] If a large difference is seen between the fingerprints of a bone specimen and that of Figure 1, it could represent the presence of other AA or protein impurities in the bone sample, such as bacterial contamination, or could be due to significant specimen degradation for very old samples (≫10 000 yrs). In the former case, the samples can be further analyzed by proteomics techniques to identify and quantify the protein contaminations. As shown in Figure 1, except for run-to-run or capillary-to-capillary peak intensity or migration time fluctuations that are common under CE-MS, no other major differences were observed in the fingerprints of these samples over time, indicating the bone samples analyzed were not contaminated by other protein impurities. Lack of significant contaminations were also confirmed by proteomics techniques using nano-LC-MS/MS. Figure 1 also demonstrates that in the bone age range of this study (up to ~10 000 years), there was no significant change in the relative intensities of bone fingerprints, i.e., the amino acid composition of the bone remained the same during the past 10 000 yrs. The result indicates that the proteins in bones are relatively pure and intact with amino acid composition similar to Type I collagen. For comparison, the electropherograms of silk, wool, and bone 2-h HCl digest are shown in Figure S-2 in the Supporting Information.

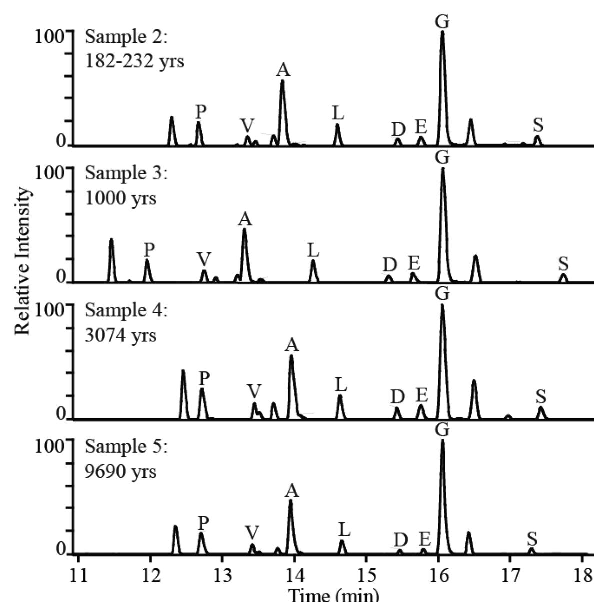


Figure 1. Base peak electropherograms of the HCl digests of *Homo sapiens* bones ranging in age from ~200 to ~10 000 years (Samples 2−5 described in Table 1).

For D/L dating, the development of a reliable calibration curve is an essential first step. The D/L ratios for the 150-, 207-, 1000-, 3074-, 9690-, and 9710-year-old samples were 2.4%, 2.5%, 3.5%, 5.3%, 10.5%, and 9.8%, respectively (see Figure 2). Based on the standard deviations, the precision of the dating is approximately ±5% of the age. The results show a strong linear correlation (R^2 ≥ 0.99) between the D/L ratio of aspartic acid and the age of the *Homo sapiens* bone. This is consistent with the theoretically expected D/L ratio for the reversible first-order reaction in which the beginning of the curve (D/L < 20%) is expected to be approximately linear, since the contribution of the reverse reaction (D-to-L conversion) is minimal.[36] Older *Homo sapiens* bone samples (>10 000 years old) would improve the applicability of this calibration curve; however, one Neanderthal sample that was analyzed (~50 000 years old from Shanidar Cave in Iraq, housed at the Smithsonian National Museum of Natural History) did not contain any detectable AA or proteins. The calibration curve presented in Figure 2 can now be used to date samples of unknown age that are suspected to be up to ~10 000 years old. Based on the current data and trend, aspartic acid racemization for *Homo sapiens* seems to be useful for specimens up to 30 000−40 000 years of age, the expected aspartic acid half-life, which is defined as when the percent of D-isomer is half that of the L-isomer, or when the D-isomer content is 33.3% of the total aspartic acid. Future work on *Homo sapiens* dating includes obtaining additional specimens to extend and strengthen this calibration curve.

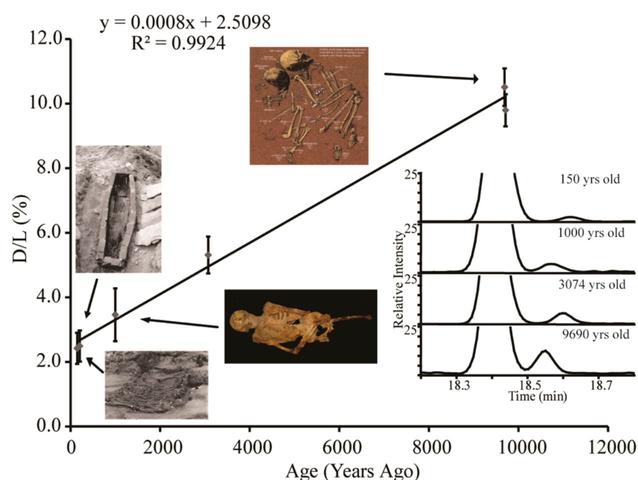


Figure 2. D/L calibration curve obtained from mostly ^{14}C-dated *Homo sapiens* bones (Samples 1−6 described in Table 1). Inset shows the corresponding ion electropherograms of the D- and L-isomers of aspartic acid. Image for Sample 1 is from *Having Fought and Died Together: Examining the Battle of Glorieta Pass Confederate Mass Grave* (www.lasvegasmuseum.org/pdf/Glorieta.pdf), reproduced with permission from Matthew Barbour, Nov. 2013. Image for Sample 2 reproduced with permission from N. Bellantoni (Nov. 2013), Bellantoni, Nicholas F., and David A. Poirier 1995 Family, Friends, and Cemeteries. CRM18(3):29-31. Image for Sample 3 obtained from Bruno Frohlich of the Smithsonian Institution, Sept. 2013. Image for Samples 5 and 6 are from *Their Skeleton's Speak*, Lerner Publishing Group, 2012, with permission of Laura Westlund/ Independent Picture Service.

The results obtained from *Homo sapiens* bone is consistent with our recent D/L analysis of a single species of silk (*Bombyx mori*) in which the calibration curve matched well with the theoretical reversible first-order kinetics.[36] Success of the new technique of D/L dating for *Bombyx mori* silk and *Homo sapiens* from this work support our hypothesis that D/L dating of aspartic acid works well when unknown specimens are dated based on the calibration curve obtained from the same species (i.e., species specific dating). More recent D/L studies of multiple foraminifera species, as well as coral species, also support this hypothesis.[39,40] To further test this hypothesis, the D/L ratio of aspartic acid was measured for a mixed species of large mammals using well-dated bones (Table 1, Samples 7−10). The calibration curve obtained from these species is shown in Figure 3, Panel A. First, as shown in Figure 3A, the rate of racemization for larger mammals is slower than that for humans [the D/L ratio for the 10 000-yr-old human is ~0.1 (10%), whereas that for the 10 000-yr-old large mammal is ~0.06 (6%)]. This slower rate may be a result of variation in bone composition between species, based on the amount of stress the bone must endure (due to the size of the animal).[41] Second, although there is a linear correlation, the correlation coefficient (0.88) is not as high as that of *Homo sapiens* (0.99), even when ^{14}C-dated bone was used. We propose that, although environmental factors could play a role in the lower correlation coefficient, mixed species used in this experiment also plays an important role. Also, the correlation coefficient systematically worsened when (1) the D/L ratio for large mammal bones in which their age were estimated based on geologic evidence and bracketing by ^{14}C-dates from other samples within the fossil site were added to the calibration curve (Figure 3B), or (2) when the D/L ratio of human bone was also added (Figure 3C). Therefore, it is possible that mixing species in

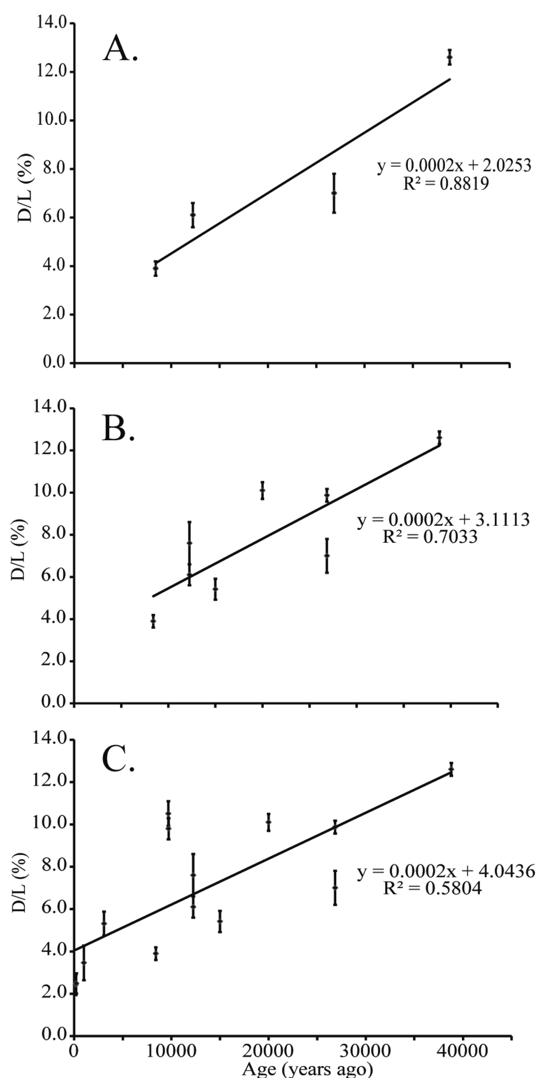


Figure 3. (A) D/L ratio of ^{14}C-dated samples of various large mammal species (Samples 7−10). (B) D/L ratios of Samples 7−14, including both ^{14}C-dated and archeologically dated samples (excluding *Homo sapiens* bones). (C) Average D/L ratios for all of the bones analyzed (all samples described in Table 1).

previous studies as well as unreliable age may have contributed to the variable results obtained from racemization dating.

■ CONCLUSIONS

Analysis of aspartic acid racemization rate for human bones, as well as several larger mammal bones, indicates that the racemization rate is species-specific. This result is consistent with our previously published dating technique for silk in which one species of silk (*Bombyx mori*) was used to develop the calibration curve. Dating based on the measurement of the racemization rate is especially useful when the calibration curve has been developed using the same species. In our study, as the species specificity decreased, so did the correlation coefficient. Moreover, the results of this study suggest that D/L analysis of aspartic acid is more accurate, taking into account several other important variables: (i) well-dated samples (^{14}C-dated) are used to develop the calibration curves; (ii) modern information-rich technology is used to obtain baseline separation of the D- and L-isomers, as well as accurate quantitation; and (iii) specimens are checked for impurities from other protein sources. This study has

produced a calibration curve that can now be used to date human bones of unknown age <10 000 years old. Future work will expand the range of applicability of this curve.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank the following individuals for assistance in procuring and processing samples: Drs. Doug W. Owsley, Bruno Frohlich, and Richard Potts (National Museum of Natural History); Richard C. Hulbert and Bruce J. MacFadden (Florida Museum of Natural History); Samuel A. McLeod (Natural History Museum of Los Angeles County); Dr. Nicholas C. Fraser (formerly of the Virginia Museum of Natural History); and Michelle L. Machicek (Smithsonian Museum Conservation Institute). We thank Dr. Thomas P. Guilderson at the Lawrence Livermore National Laboratory Center for Accelerator Mass Spectrometry for assistance with ^{14}C-dating. This material is based on work supported by the National Science Foundation (under Grant No. CHE 1241672). C.A.M.F. would like to acknowledge the National Science Foundation Research Experiences for Undergraduates (No. SMA-1156360), and Geologic Society of America Research (Grant No. 7747-04).

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