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Ancient DNA from Texas Pictographs

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Three to four thousand year old DNA in Pecos River style pictographs was subjected to polymerase chain reaction (PCR) amplification and phylogenetic analysis. We sequenced a 106 base-pair (bp) fragment from the highly conservative histone 4 gene, assessing potential contamination by using negative controls (no DNA) and positive controls (known DNA). The pictograph sequence was compared to sequences from vegetable and animal ''carriers'' that could have been used to bind the pigment to the rock. The analysis revealed the use of an organic binder/vehicle derived from a mammal, probably an ungulate of the Order Artiodactyla.

Keywords: DNA, HISTONE 4 GENE, PHYLOGENETIC ANALYSIS, PICTOGRAPHS, POLYMERASE CHAIN REACTION, DNA SEQUENCING.

Introduction

Rock art is found on every continent and comprises one of the more abundant, informative, yet neglected, of all archaeological artefacts. Prior to the 1980s, no means existed for dating the art chronometrically, and therefore for integrating it into the archaeological record. However, dating techniques are now available for both rock engravings or petroglyphs (Dorn, 1994; and references therein) and pictographs (Van der Merwe, Sealy & Yates, 1987; Loy *et al*., 1990; McDonald *et al*., 1990; Russ *et al*., 1990; Valladas, Cachier & Arnold, 1990; Watchman, 1991).

There has been a resurgence of interest in prehistoric symbolic, religious and artistic systems (Hodder, 1982; Renfrew, 1982) of which prehistoric rock art is an essential component. For example, a sophisticated ethnohistorical approach for interpretation of southern San rock art was provided by Lewis-Williams (1981, 1982). He then demonstrated how such symbolic interpretations may be treated as scientific hypotheses and tested with empirical evidence (Lewis-Williams, 1983). More recently, Lewis-Williams & Dowson (1988) developed a functional/analogical model for mental imagery resulting from altered states of consciousness, that may be graphically portrayed in rock art. Whitley & Loendorf (1994) suggest that rock art may be at the theoretical and methodological forefront in archaeological studies of hunter–gatherers.

An empirical iconographic interpretation of Pecos River style pictographs by Turpin (1994) concluded that some of that art is ritualistic. Materials selected for preparation of paints probably had religious significance. It is therefore of interest to determine what materials these were. Unfortunately, we have neither ethnographic nor chemical data to guide us in the Lower Pecos River region of Texas. The area near the confluences of the Pecos, Devils and Rio Grande rivers, and their associated watersheds has been inhabited by Native Americans from at least 11,000 years ago up to the historic era (Hester, 1988; Turpin, 1990). Prehistoric inhabitants used limestone cliff overhangs for shelter and frequently painted pictographs on the walls. Figure 1 shows an example of a Pecos River style pictograph. Dark and light red, black, yellow and orange pigments prepared from naturally occurring iron and manganese oxides and hydroxides are common. White pigment also is present, but rare.

We have undertaken a three-part study of ancient polychrome Pecos River style pictographs of Texas. First we developed a technique for dating pictographs, applied it to five Pecos River style pictographs, and found them to be ~2950 to 4200 years old (Russ *et al*., 1990; Russ, Hyman & Rowe, 1992; Chaffee, Hyman & Rowe, 1993; Ilger *et al*., in press); that work continues. One of us is involved in X-ray diffraction analysis of pigments to determine the minerals used and accretion compositions (Hyman, Turpin & Zolensky, in press).

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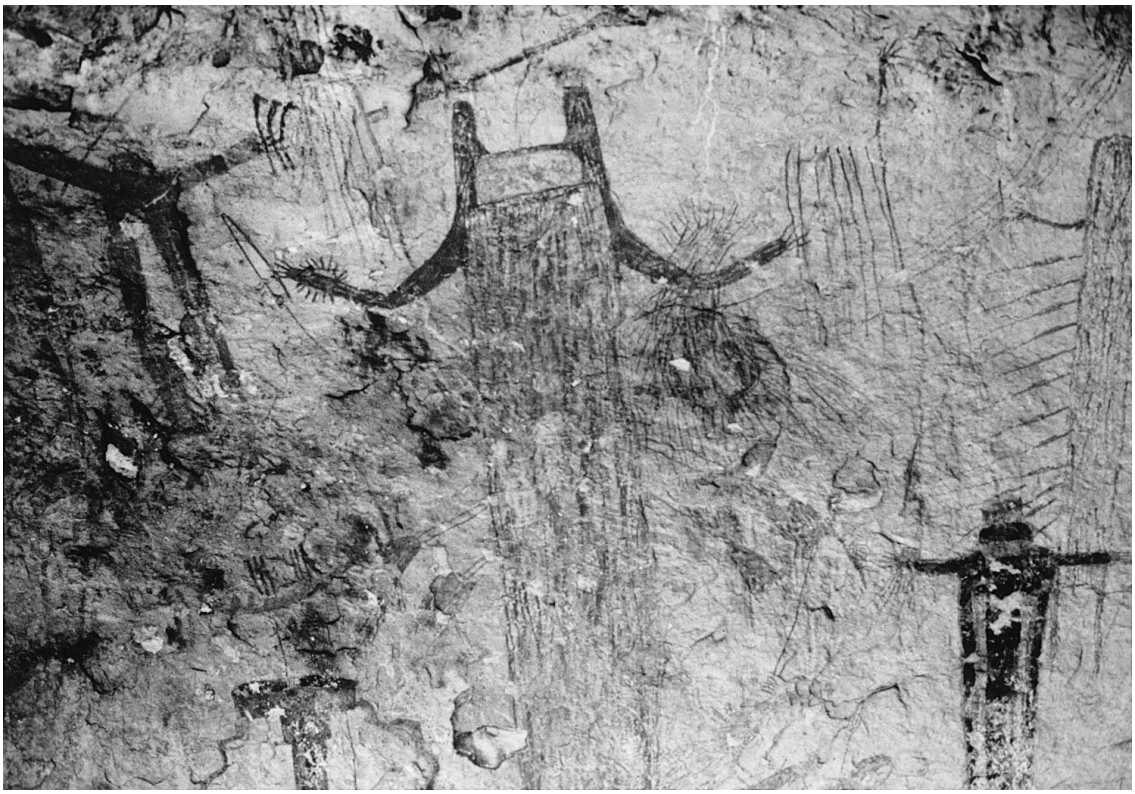

Figure 1. Pecos River style pictograph of an anthropomorphic motif from Panther Cave, 41VV83, in the Lower Pecos River region.

In addition we investigated the organic components of the binder/vehicle(s) to determine what materials were added to the paint. Many easily available organic materials such as blood, urine, milk, eggs, vegetable juices, animal fats, etc., were suggested as pictograph paint binder/vehicle(s). However, no chemical or biochemical analysis were reported and origins of the organic binder/vehicle(s) used in prehistoric Native American paints were speculative until now.

We collected one sample from each of two different Pecos River style pictographs located in Seminole Canyon rock shelter site 41VV75. That site was chosen since it has undergone severe natural exfoliation of pictographs painted on the limestone walls. Both pictographs we sampled were well over 50% spalled from the wall. An outer accretionary mineral coating on the pictographs may protect DNA from the effects of the atmosphere and weather. The microstratigraphy of a pictograph on a rock surface is illustrated by the polished section shown in Figure 2. There principal layers are apparent: (1) limestone substrate, (2) pigment layer, and (3) naturally occurring accretion layer. Zolensky (1982), Silver (1985) and Hyman, Turpin & Zolensky (in press) identified whewellite (calcium oxalate), calcite (calcium carbonate), and gypsum (calcium sulfate) in accretion layers on pictographs in the Lower Pecos River region. Sample 41VV75A was taken from a red pigmented area; sample 41VV75B was from a black painted area on a different motif about 1 m away. To minimize sampling impact, we selected only pictograph fragments that were on the verge of spalling. Samples were taken high on the walls to reduce possible contamination from human or animal sources subsequent to the painting of the pictograph. Precautions were taken to avoid contamination during and after collection. We extracted ancient DNA from the two Pecos River style pictographs. We then used polymerase chain reaction (PCR) (Mullis *et al*., 1986; Mullis & Faloona, 1987) to amplify ancient DNA fragments. Phylogenetic analysis was used to decipher its origin.

DNA extraction and PCR amplification of archaeological samples is more challenging than routine PCR amplifications of modern DNA. This is due primarily to diagensis and the increased chance of *in situ* contamination. In some instances, ancient DNA samples contain unknown PCR-inhibiting substances (Pääbo, 1990; Brown & Brown, 1992). Adaptation of PCR amplification techniques to ancient DNA provided genetic information for archaeological studies which have been difficult or impossible until recently (Erlich, Gelfand & Sninsky, 1991). PCR has been performed on human and animal DNA thousands of years old (Pääbo, Gifford & Wilson, 1988; Pääbo, 1989; Pääbo, Higuchi & Wilson, 1989; Pääbo, 1990; Cherfas, 1991; Hagelberg & Clegg, 1991; Hummel & Herrmann, 1991; Lawlor *et al*., 1991; Sykes, 1991; Richards *et al*., 1993), on maize $\sim$1000 years old (Rollo, Amici & Salvi,

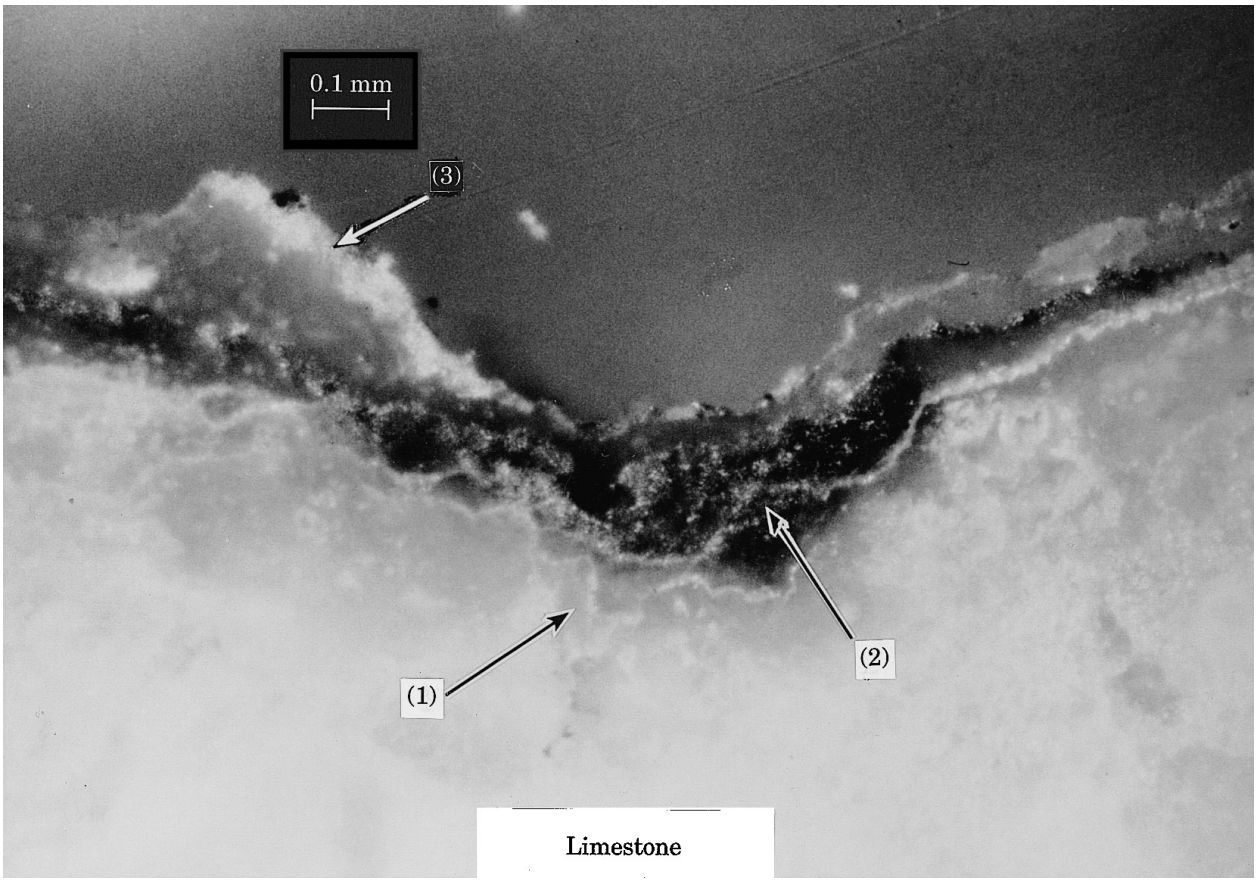

Figure 2. Polished section for the red-pigmented sample 41VV75A, taken from a different motif than the black pigmented sample 41VV75B. There are three principal layers visible: (1) the limestone substrate, (2) the red pigmented layer (hematite), and (3) the naturally occurring mineral layer (calcite and whewellite) which probably protected the DNA from total degradation.

1988), on fossilized plant DNA millions of years old (Golenberg *et al*., 1990; Poinar, Cano & Poinar, 1993) and insect DNA over 25 million years old inside amber (DeSalle *et al*., 1992; DeSalle, Barcia & Wray, 1993). Thus PCR amplifications using Taq polymerase produce faithful copies of ancient DNA in sufficient quantities for phylogenetic analysis.

Experimental Method

DNA extraction

A sodium citrate buffer of pH 4·1 was prepared in which 47·4 g of guanidine thiocyanate, 0·8 g N-lauroyl sarcosine sodium salt, and 0·7 ml of 2-mercaptoethanol were dissolved to give 100 ml of extraction buffer (Davis, Dibner & Battey, 1986; Bartlett & Davidson, 1992). This extraction buffer was sterilized by autoclaving.

Two ancient painted rock samples were placed in petri dishes with extraction buffer and gently rocked on an orbital–mechanical shaker. We used whole pieces of pictograph samples rather than scraping off the pigmented surface to limit the chance of laboratory contamination. All containers used throughout were sterilized and only deionized, distilled, autoclaved water was used. After 5 days of agitation, the pictograph extraction solution was pipetted into tubes. The samples were rinsed with H_2O which was also transferred to the tubes. Next, TE buffer (10 mM tris-HCl, 1 mM of EDTA, pH 7·5) was added and each sample tube was centrifuged at $2000 \times g$. Supernatant liquid was pipetted into clean tubes and phenol saturated with 1 M Tris-HCl, chloroform, and isoamyl alcohol in the ratio (v/v) of 25:24:1, pH 8·0 (PCI solution) was added. After gently mixing and centrifuging at $2000 \times g$, the aqueous layer was pipetted to new tubes. This was extracted twice with chloroform and isoamyl alcohol in the ratio (v/v) of 24:1 (CI solution), in the same manner as with the PCI solutions (always retaining the aqueous layer).

The aqueous layer of each tube was placed into centrifuge tubes along with 1/20 volume of 3 M NaCl and 2·5 volumes of cold absolute ethanol which precipitates the DNA. The tubes were agitated and stored at -20°C, then centrifuged at $1300 \times g$ and 4°C. Afterwards, we decanted the solutions and added H_2O and TE buffer to each tube which was vortexed to aid

pellet rehydration. DNA was reprecipitated by adding isopropanol, then stirred and cooled to −20°C, centrifuged at 28,400 × *g* at 4°C and the liquid discarded. Pellets were dried and then rehydrated. Supernatant containing the DNA was removed and filtered through a Millipore 30,000 MW filter by centrifuging at 3500 × *g* followed by wash H_2O. DNA was rehydrated in 50 µl of H_2O, removed via pipette and stored at 4°C awaiting further treatment.

Selection of PCR primers

Primers are short segments of DNA needed as the starting point for polymerase to begin synthesis of the targeted DNA template. Primers exactly complementary to the precursor region of targeted DNA fragments are desirable, but imperfect primer–template matches can work if the first six to nine bases are compatible. Typically, knowledge about the genetic target sequence to be amplified exists. In our case having neither ethnographic evidence nor previous knowledge as to the source(s) of ancient organic binder/vehicle(s) made primer selection more difficult. To circumvent this problem, we constructed universal eukaryotic primers that would detect and amplify ancient DNA of either plant or animal origin. The universal plant/animal primers chosen bracket a 106 base-pair (bp) region of the histone 4 gene. We chose the histone gene family for several reasons. (1) Histone genes are highly conserved, i.e. they are similar from species to species. By using eukaryote specific primers, bacterial (prokaryotic) contamination was excluded since histone genes are absent in bacteria. (2) Histone genes occur in multiple copies, increasing the chance for a desired DNA template to be present. (3) Finally, the histone 4 family was chosen over other histone genes because there is information on histone 4 of numerous species. Using GenBank data, we selected both forward and reverse primers complementary to the histone 4 gene regions of all histone 4 DNA sequences reported. MacVector 3.5 Sequence Analysis software (IBI/Kodak, New Haven, CT) was used to find the best fit alignment for the histone 4 primer construction by comparing every published sequence of histone 4 genetic region sequence to human histone 4.

The histone 4 primers selected were: Forward-5′-CGC ATC TCC GGC CTC ATC TAC GAG GA-3′; and reverse-5′-GTA GAG TGG GCG GCC CTG GCG CTT GA-3′. We made the 26 bp primers on an Applied Biosystems, Inc., Oligonucleotide 392 RNA/DNA synthesizer.

PCR amplification, isolation and extraction of DNA fragment

PCR reproduces DNA *in vitro* very efficiently (Mullis *et al.*, 1986; Mullis & Faloona, 1987). We used the usual procedure which includes three cycles: (1) denaturation at 93°C, (2) primer annealing at 50°C, and (3) extension at 72°C of primed DNA using polymerase extracted from *Thermus aquaticus* bacteria. Typical amplifications consisted of 35–40 cycles.

PCR amplifications of *Bos taurus* (bovine), *Lycopersicon lycopersicum* (Better Boy tomato), *Homo sapiens* (human), and *Gallus gallus* (chicken) DNA demonstrated the primers' efficacy as universal plant/animal primers. To verify PCR amplification success, products were subjected to electrophoresis on 1·5% agarose gels with TE running buffer. Approximately 15 µl of each PCR product was pipetted to a designated sample lane. We used bacteriophage lambda molecular weight standard to monitor separations. After electrophoresis, the gel was stained with ethidium bromide. Excess ethidium bromide was removed with H_2O. UV irradiation of ethidium bromide–DNA complexes produced visible DNA fragment bands. Having verified PCR amplification, we proceeded to isolate DNA fragments by gel electrophoresis using a 4% low melting temperature agarose gel at 4°C. The new gel was also stained with ethidium bromide and washed in H_2O. Bands of interest were exposed by UV light and removed with sterile razor blades quickly to minimize UV damage to DNA fragments. We placed excised DNA bands in tubes and stored at −20°C. PCR products were purified by using the Magic PCR Preps DNA Purification System kit (Promega).

Ligation, transformation and purification of plasmid DNA

We used vector–bacterial cloning to provide ample single strand primer-templates for DNA sequencing. First we inserted the unknown DNA strand into a modified bacterial plasmid by ligation. Cloning required that the plasmid vector be cut and the PCR amplified DNA spliced into the cut site. Plasmid DNA was compared on a 2% agarose gel with uncut vector plasmid to verify that it was really cut. We modified cut ends of the plasmid vector by adding thymine bases. This was covered with mineral oil and incubated in the PCR thermal cycler at 70°C. After incubation, the reaction mixture was extracted once with PCI and once with chloroform, centrifuged at 2500 × *g*, precipitated with absolute ethanol at −80°C, and resuspended in TE buffer. Next, the DNA fragment of interest was inserted by ligation using T-4 DNA ligase (a polymerase enzyme) added directly into this solution. After centrifuging at 110 × *g*, the reaction solution was incubated at 12°C. Immediately at the conclusion of the incubation period, the transformation procedure was performed on the DNA/plasmid ligation extract.

Extracts were added to *Escherichia coli* cells and kept on ice. Next, samples were heated to 43°C for 1 min and then placed back on ice for 2 min. This thermal shock forces the plasmid into *E. coli* cells. After adding L-broth culture media to each sample, they were incubated at 37°C to optimize growth

conditions. Some of each cell sample (150 µl) were pipetted onto agar gel plates. After incubation, gel plates contained both white and blue bacterial colonies. White bacterial colonies contain plasmids with DNA inserts; blue bacterial colonies do not. White colonies were touched with sterile toothpicks, streaked across a fresh agar plate and then dropped into tubes containing L-broth with ampicillin added. These tubes were shaken in an orbital shaker–incubator, also at 37°C. Plates corresponding to tubes of white bacterial colonies were placed back into the incubator. These plates serve as a control to monitor the colonies to ensure that they continue to retain the DNA fragment.

We used a mini prep protocol to purify plasmid DNA from other bacterial cell components. Only culture cells that corresponded to initially white colonies that remained white on the second set of streaked plates were used for plasmid isolation. This eliminated plasmid–bacterial host cells that did not contain the DNA segment. This white/blue screening process has two discrimination criteria. (1) Inclusion of the plasmid confers antibiotic resistance to the host bacterial cell. (2) Non recombinant plasmids (no DNA insert) produce a blue colour in the presence of X-GAL and ITPG. In recombinant plasmids, presence of foreign DNA inserts render β-galactosidase genes non-functional and they remain white. Plasmid denaturation prepared the plasmid DNA for sequencing reactions, which work most efficiently on single strand DNA templates (Kusukawa *et al.*, 1990; Allard, Ellsworth & Honeycutt, 1991).

Sequencing reactions

DNA sequence information was determined by the dideoxy chain termination method (Sanger, Nicklen & Coulson, 1977). We incorporated radioactively labelled ^{35}S into the DNA fragments using a Sequenase® Version 2·0 DNA sequencing kit (United States Biochemical Corporation). DNA sequencing protocol consisted of: (1) plasmid primer annealing, (2) template labelling-extension, and (3) reaction termination. Sequencing reaction products were stored at −20°C until they were loaded onto a polyacrylamide sequencing gel.

Sequencing separations were performed using high resolution polyacrylamide gel electrophoresis. The final four dideoxy-sequencing reaction products for each sample were heated to 75°C and 15 µl from each sequencing reaction was pipetted into designated sample lanes. Separation of sequencing products on the gel usually proceeded at 50°C with running buffer (1·7 M Tris, 1·7 M boric acid, 0·2 M Na_2EDTA). After 20 min, 2 M sodium acetate was added to sharpen sequence bands (Sheen & Seed, 1988). We monitored migration of DNA bands using tracking dye contained in the sequencing reaction mixtures. Gels were affixed to paper by drying under vacuum. Dried gels were placed next to Kodak XAR-5 film in a developing cartridge and stored at −80°C. Typical exposure times were 36 h; afterwards negatives were developed in an X-OMAT automatic film processor. DNA sequences were read from developed autoradiographs.

Controls

Modern DNA contamination was a concern, so we took precautions (Kwok & Higuchi, 1989; Lindahl, 1993) to ensure that we amplified ancient DNA and not a DNA contaminant. Negative controls (no DNA added) and positive controls (known DNA added) were incorporated to maintain the integrity of every PCR experiment. We demonstrated that unpainted limestone background rocks taken near the pictographs contained no eukaryotic DNA. They were subjected to the same extraction, purification, and amplification procedures as the painted rock samples. No amplification of DNA occurred in unpainted background rock. The feasibility of extracting either animal or plant DNA from limestone was demonstrated by applying fresh chicken blood and tomato plant extract to limestone that was left in direct sunlight for 5 days. These were subjected to the same extraction, purification, and amplification procedures as other samples. These gave PCR amplification products using the histone 4 primers. It appears that no substance is present in the limestone that seriously inhibits PCR amplification. During each PCR experiment, we used a no-template (negative) control which contained every reagent needed for PCR amplification except addition of DNA. Detection of PCR products in these mixtures would have indicated contamination DNA in the reagents. We also used positive DNA controls (PCR amplifications of modern bovine or tomato DNA) during every round of PCR amplifications to assure both that PCR reaction mixture components were efficacious and to avoid false negative results.

Phylogenetic analysis

Once ancient DNA sequences were determined, they were treated as character data and compared to other known sequences of the same genetic regions obtained either from published sequences (GenBank) or sequences we determined from known sources. Phylogenetic analysis of ancient DNA data provides inferences about taxonomic kinship (Higuchi *et al.*, 1984). We used to software, PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1993), to perform phylogenetic analysis, striving to achieve optimal tree(s) which best describe the data. PAUP has three options which allow evaluation of possible trees: exhaustive search, branch and bound search, and heuristic search. We used branch and bound search and exhaustive search methods (Swofford, 1989, 1993; Swofford & Olsen, 1990) to evaluate DNA sequence data. Experimentally determined DNA sequences

were compared to each other, as well as to reported sequences of similar histone 4 regions from the GenBank database. Additional details on the experimental procedure beyond that presented above can be found in Reese (1994).

Results and Discussion

The gel for ancient 41VV75A and 41VV75B DNA sequences obtained by the Sanger method are shown in Figure 3. Nucleotide sequences (the region labelled S) for the two samples are given in Figure 4. These two sequences from two different samples separated by about 1 m differ by one base. They were subjected to phylogenetic analysis using PAUP 3.1.1. Pictograph ancient DNA sequences were compared to the same genetic regions of fish, fungus, bird, amphibian, mammal, plant examples contained in GenBank (the first eight groups contained in Table 1). We obtained a parsimony tree for phylogenetic analysis of amplified ancient histone 4 gene DNA fragment sequences compared to some representative GenBank histone 4 sequences as shown in Figure 5. Both exhaustive and branch and bound tree search methods (based on strict, majority rule and bootstrap criteria) gave the same tree. This initial analysis demonstrated that ancient DNA sequences 41VV75A and B share more characteristics with mouse and human than with other vertebrates, plants or fungi. Thus, we tentatively concluded that pictographic DNA obtained from two Pecos River style pictograph samples (41VV75A and B) was of mammalian origin.

Given that premise, we attempted to narrow the potential sources of organic binder/vehicle(s) with additional studies. For that, we selected several Lower Pecos mammal fauna candidates that were not contained in GenBank and sequenced the same histone 4 region. Species chosen were: *Sylvilagus floridauus* (rabbit), *Odocioleus virginanus* (white-tailed deer) and *Bison bison* (American bison). In addition, we sequenced *Bos taurus* (cattle) and *Capra hircus* (goat) DNA to exclude the possibility of contamination from those sources, as their DNA is frequently encountered in this laboratory. Each of these samples was subjected to similar PCR amplifications, cloning and sequencing steps discussed above. These Lower Pecos fauna sequences (Figure 4), two sequences obtained from 41VV75 samples and aligned GenBank database sequences previously used to construct the PCR primers, were subjected to phylogenetic analysis using PAUP 3.1.1.

The branch and bound method was used to compare the ancient DNA fragment to that from selected Lower Pecos River fauna, as well as groups selected from GenBank. This analysis gave the strict consensus tree shown in Figure 6. A bootstrap majority-rule consensus tree using the branch and bound method yielded the same tree. Based on this analysis, the binder/vehicle used in two samples taken from 41VV75 contains material from a mammal closely related to two species of Lower Pecos prehistoric fauna—bison and deer. Order Artiodactyla contains ungulates which are

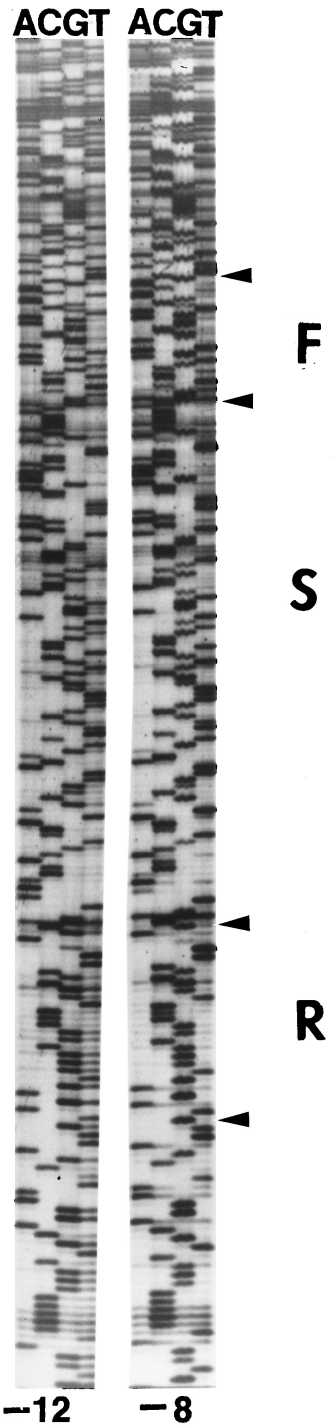

Figure 3. DNA sequencing gel for the two ancient DNA 41VV75 sequences. The region labelled F is the forward primer, S is the DNA fragment sequenced and R is the reverse primer. Similarity of the two sequences is apparent, with a difference at only one bp position.

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41VV75A   5'- G ACT CGT GGG GTG CTG AAG GTG TTT CTG GAA AAT GTG
                ATC CGG GAC GCG GTC ACC TAC ACG GAG CAC GCC AAA
                CGC AAG ACT GTA ACC GCT ATG GAC GTG GTT TAC - 3'

41VV75B   5'- G ACC CGT GGG GTG CTG AAG GTG TTT CTG GAA AAT GTG
                ATC CGG GAC GCG GTC ACC TAC ACG GAG CAC GCC AAA
                CGC AAG ACT GTA ACC GCT ATG GAC GTG GTT TAC - 3'

BISON     5'- G ACC CGT GGG GTG CTG AAG GTG TTT TTG GAG AAC GTG
                ATC CGG GAC GCG GTC ACC TAC ACC GAG CAC GCC AAG
                CGC AAG ACT GTC ACC GCC ATG GAT GTG GTG TAC - 3'

CATTLE    5'- G ACC CGC GGG GTG CTG AAG GTG TTC CTG GAG AAT GTG
                ATC CGG GAT GCA GTC ACC TAC ACC TAG CAC GCC AAG
                CGC AAG ACT GTC ACC GCC ATG GAC GTG GTC TAC - 3'

GOAT      5'- G ACC  CGC GGG GTG CTG AAG GTG TTC TTG GA? AAT GTG
                ATC CGG GAT GCA GTT ACC TAC ACA GAG CAC GCC AAG
                CGC AAG ACT GTC ACC GCC ATG GAC GTG GTC TAC - 3'

RABBIT    5'- G ACC CGT GGC GTG CTC AAG GTC TTC CTG GAG AAC GTC
                ATC C?C GAC GCT GTC ACC TAC ACG GAG CAC GCC AAG
                CGC AAG ACG GTC ATG GC? ATG GAC GTG GTG TAC - 3'

DEER      5'- G ACG CGC GGC GTC CTG AAA GTG TTT CTG GAG AAT GTG
                ATC CGG GAT GCA GTC ACC TAC ACC GAG CAT GCC AAG
                CGG AAG ACT GTC ACC GCT ATG GAT GTG GTG TAC - 3'
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Figure 4. Aligned histone 4 gene sequences for the ancient DNA fragments extracted from the two pictograph samples (41VV75A, red pigment, and 41VV75B, black pigment), from *Bison bison* (American bison), *Bos taurus* (cattle), *Capra hircus* (goat), *Sylvilagus floridauus* (Eastern cotton-tailed rabbit) and *Odocioleus virginanus* (white-tailed deer). The only difference between the two pictograph DNA fragments is shown in underlined bold face type.

Table 1. Histone 4 genes from GenBank and from our work used in this study

Common name	Scientific name	GenBank accession number
Human	*Homo sapiens*	X00038
House mouse	*Mus muscalus*	J00422
Fish	*Tilapia niloticta*	X54078
Maize	*Zea maize*	M13370
Wheat	*Traiticum aestivum*	X00043
Neurospora	*Neurospora crassa*	X01611
Frog	*Xenopus laevis*	X00224
Chicken	*Gallus gallus*	J00866
American bison	*Bison bison*	This study
Cattle	*Bos taurus*	This study
White-tailed deer	*Odocioleus virginanus*	This study
Better Boy tomato	*Lycopersicon lycopersicum*	This study
Goat	*Capra hirus*	This study
Rabbit	*Sylvilagus floridauus*	This study

hoofed mammals with even toes on each foot such as cattle, sheep, goats, deer, bison, antelope and more esoteric members such as elephants, camels, gazelles, hippopotamuses, etc. However, identification to species is not possible with our histone 4 gene segment because the highly conserved 106 bp fragment does not have sufficient phylogenetic resolution. This is not surprising as the primers and genetic region were

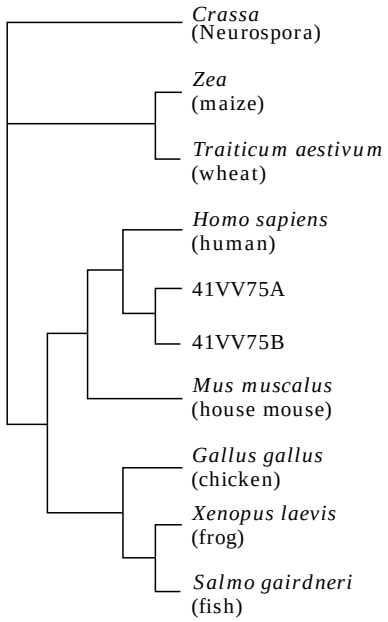

Figure 5. Parsimony tree for phylogenetic analysis of 41VV75 pictograph PCR amplified ancient histone 4 gene DNA fragment sequences compared to some representative GenBank histone 4 sequences using the branch and bound method. Neurospora was used as the outgroup. The length of this tree is 72 steps; consensus index (CI), excluding uninformative characters, is 0·69 and the retention index (RI) is 0·679.

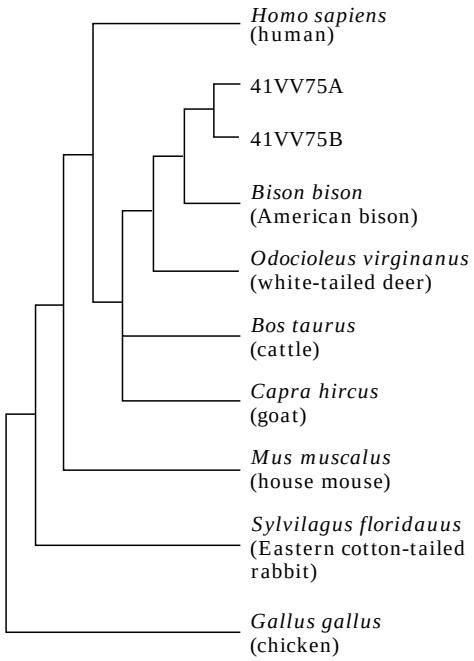

Figure 6. Strict consensus tree for a portion of the histone 4 gene sequence comparing pictographs 41VV75 to selected Lower Pecos River region mammal fauna and selected GenBank histone 4 sequences using the branch and bound method. Chicken histone 4 was used as the outgroup. This consensus is based on three equal length trees of 53 steps, CI, excluding uninformative characters, is 0·674 and the RI is 0·651.

chosen to amplify any species of either animal and plant DNA. We plan to use other more variable DNA regions to make lower level taxonomic determinations. This is possible if a genetic region bordering a more polymorphic region can be chosen and amplified.

Conclusions

Ancient DNA was extracted and PCR amplified from two different Lower Pecos Style pictographs samples, one red and one black, taken from site 41VV75 located in Seminole Canyon, Texas. DNA recovered from Pecos River style paint binders is closely related to DNA from deer and bison. This study shows that ancient people of the Lower Pecos River region used animal products of Order Artiodactyla *c.* 3000 to 4200 years ago in the manufacture of rock shelter paints. Bone marrow is a good source for DNA residue that survived for several thousand years. Mammalian red blood cells are questionable sources for ancient DNA as they have no nuclei, and hence no DNA. Any residual DNA in blood is from bone-produced white blood cells. Preservation of DNA in these pictographs was probably due in part to several factors. (1) A mineral accretion forms over the paintings after they are painted that offers some protection against the weather. (2) The pictograph samples were located within a shelter that affords protection from direct rainfall and (3) their location in the shelter also places them in shade for a portion of the day, lessening their exposure to deleterious effects of direct sunlight. The lack of detection of plant DNA in the paint does not eliminate the possibility that plant matter was used, but it does strongly suggest its absence; over time added plant DNA may have degraded to the extent that it was not amplified.

We have implemented additional studies to look at other genetic regions in order to further delineate binder's origins/sources. Phylogenetic analysis of more variable genetic regions common to Order Artiodactyla should be more definitive. One possible approach, now that ungulate DNA has been demonstrated to be present, is to use a different set of conserved primers such as those that amplify cytochrome b regions found in mitochondria DNA (Kocher *et al.*, 1989). Cytochrome b regions have more base pair variability than the histone 4 gene region used in this study. These continuing studies should allow the species level discernment needed.

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