2017

ANCIENT DNA EXTRACTION FROM STONE TOOLS

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UNIVERSITY OF MONTANA

2017

Abstract

Proteins and DNA can be trapped in the microcracks on the surface of stone tools, which can then be extracted and analyzed to aid in inferring the use of the tool (Shanks et al. 2001; 2005). This nondestructive method involves the use of sonication to release DNA from the microcracks, then amplification of regions of mitochondrial DNA that are species specific. This technique was applied to stone tools from the Bridge River site in British Columbia by researchers at UMT's Ancient and Modern Molecular Anthropology labs. Bridge River archaeologists have designated the tools as used in "food processing or tool manufacturing" (Prentiss 2014), yet our analyses could connect the tools with specific species such as elk or deer. While part of our project entailed perfecting the methodology for DNA extraction and combatting complications, the main hypothesis behind our research is:

Hypothesis: The potential for extracting DNA from archaeologically recovered lithics will enable identification of the species on which they were used.

We were able to amplify the 16S region of the mtDNA molecule from a slate hide scraper, which when compared to GenBank was an exact match for *Puma concolor* (North American cougar / puma / Mountain Lion). Other tools have yielded bacterial matches (*Paenibacillus* and a *Bacillus* simplex strains). Our preliminary results suggest that DNA is accessible from the Bridge River lithic assemblage at low frequencies. As this project continues, we hope our goals aid in understanding the Bridge River site lithic tool use based on future aDNA collection.

Acknowledgements

I would like to express sincere appreciation towards Dr. Meradeth Snow for her training, patience, dedication, and donation of her time and lab space. Thank you to Rachel Summers-Wilson, Kristy Pilgrim, and McKenzie Morgan for their help in the lab. Finally, I'd like to thank our partner, Xwisten, the Bridge River Indian Band for their significant role in facilitating this research. The project has been generously funded by the (USA) National Science Foundation (grant BCS-0713013 funded the 2007-2009 field seasons) and the (USA) National Endowment for the Humanities (grant RZ-51287-11 funded the Housepit 54 excavations 2012-2014 and grant RZ-230366-15 funded HP 54 excavations in 2016).

Introduction

There is often debate between archaeologists regarding what lithics recovered from ancient sites were used for. Ancient DNA (aDNA) analysis of stone tools, such as those described in this paper can begin to address if the tools were used to process specific species of animals. While methods for extracting aDNA from lithics were developed over ten years ago (Shanks 2001), this methodology has been used in only a handful of publications as opposed to more common methods like surface residue analysis (Langejans 2010). With the explosion in development of more efficient, cheaper methods of DNA analysis and aDNA analysis in particular, it is probable that more studies concerning the extraction of aDNA from stone tools will follow suite, especially since lithic technology is an optimal source of aDNA when it seems other sources are unavailable (Shanks 2001).

We in the Modern Post-PCR and Ancient DNA labs at UMT's Anthropology department have attempted to extract aDNA from micro-fissures in stone tool technology from the Bridge River Excavation site in the Middle Fraser Canyon, British Columbia. The site was occupied periodically from 1800 years ago to the mid-19th century (Prentiss et al. 2008). Excavation and analysis were completed by UM Professor Anna Prentiss and has been an ongoing project. The protocol to extract and amplify any aDNA locked in the cracks involved treating the tools with chemicals and sonication (Shanks et al. 2005). While part of my project entailed perfecting the methodology for DNA extraction, the main hypothesis behind my research was:

Hypothesis: The potential for extracting DNA from archaeologically recovered lithics will enable me to identify the species on which they were used.

Bridge River researchers have designated the tools as used in "food processing or tool manufacturing" (Prentiss 2014), yet our analyses could connect the tools with specific species such as elk or deer, and provide an interesting new avenue of investigation. Our major goals for the project were to improve aDNA extraction and amplification protocols for lithic tools from archaeological contexts and to explore how possible results of this methodology could contribute to understanding the Bridge River site.

To date, we have worked on over twenty stone tools from the Bridge River site. We extracted bacterial DNA from two of the samples and *Puma concolor* from a third. Though we cannot directly tie the bacterial DNA to ancient use of the tools, there is a chance that the *Puma concolor* aDNA was related to use of the tool and can be used in future interpretations of the site.

It is important to note the complications and limitations of extracting and interpreting aDNA, especially from such a lightly researched source as lithics. Contamination and aDNA taphonomy can mislead successful results (Leonard et al 2007, Kimura et al 2001), so it is essential that we interpret any and all results with the utmost caution, relying on combinations of other approaches, such as macro-fracture analysis, in conjunction with our own research. A well-rounded approach, heavily focused on the context of the archeological find is the best situation in which our methods can be used to increase understanding. Additionally, circumstances such as contamination, unsuitable preservation environments, and issues with primers can potentially limit the effectiveness of extraction and amplification initially, curbing us from beginning to interpret results at all. It must be understood that the process of extracting aDNA from micro-cracks in stone tools requires a large sample of tools and often a lengthy amount of time to produce results, if they are even possible.

Literature review

Why Use Stone Tools for aDNA recovery?

Stone tools have been used as source for aDNA before micro-crack extractions in the form of micro residue analysis (Langejams 2015). Micro residue analysis relies on collecting often residues from the surface of tools that is often invisible to the naked eye. Anything from plant fibers to blood to aDNA have been used to interpret sites. While residue analysis has been used for a wide array of archeological purposes without involving DNA, aDNA can also be extracted from the surface of tools to tie that tool to use on a specific species in the past. Without extraction of aDNA, archeologists can only interpret tools with their shape and usewear pattern and species specific aDNA can add to that interpretation. Loy and Dixon (1998) used micro residue analysis of megafauna aDNA from Beringian fluted points to settle debates over their age. A collection of fluted points were suspected to be related to the early Paleo-Indian traditions of Clovis and Folsom, yet in the absence of definitive contextual and temporal evidence interpretation was difficult. By connecting the tools to extinct megafauna such as mammoth using micro residue analysis and aDNA, the researchers were able to solidify the timeline of the tools (Loy and Dixon 1998).

The practice of extracting aDNA from microcracks *within* the surface of stone tools has even wider possibility than micro-residue analysis, while sharing all its advantages. Microcracks are byproducts of pressure and percussion from flaking techniques to shape fine ground cryptocrystalline rocks (Shanks et al 2005). aDNA extraction techniques described later push DNA molecules trapped in those cracks out to be amplified, sequenced and used in interpretation of sites and tools. Thus, even when the surface aDNA has been degraded or contaminated,

micro-crack analysis can retrieve preserved aDNA from within the tool.

Lithics are often the most preserved and accessible materials in the archaeological record, a benefit to both micro-residue and micro-crack aDNA extractions. Additionally, neither method destroys the artifact during extraction processes, a rarity in the world of DNA extraction (Shanks et al. 2004). As we will address later, the only change perceivable after micro-crack aDNA extraction is cleaner tools.

In addition to the advantages shared with micro-residue analysis, micro-crack extraction has a few more perks that enhance its usefulness in research. Some experimental research has provided evidence that aDNA may preserve better in micro-cracks than on the surface or in the surrounding environment (Shanks et al. 2001). This isn't that surprising considering the substantial protection from many processes that lead to DNA degradation that the micro-cracks provide. Micro-crack extraction been used successfully on samples up to 35,000-65,000 years old (Hardy et al. 1997)). Though micro-residue analysis has been used on such ancient relics, it is more commonly used on more recent artifacts. There is simply less chance that ancient artifacts will contain surface residue.

Another reason micro-residue analysis is often less effective than aDNA extraction from micro-cracks is that it frequently relies on protein, lipid, or fiber residue and thus preservation of them to be available for testing. DNA is more stable and smaller than proteins, etc, and fits better in the small, protective micro-cracks. Accordingly, micro-crack methods may be useable when immunological tests searching for proteins are not (Kimura et al. 2001).

Other experimental research on the effectiveness of micro-crack DNA extraction using modern DNA found that DNA is trapped in the micro-cracks within minutes of use when

butchering involving blood, though other uses for the tool where liquid blood is not in contact with the artifact take prolonged use to trap DNA (Shanks et al. 2004). While we will cover the complexity of connecting extracted aDNA to tool use later on, this research gives clear evidence that some DNA related to tool use is effectively trapped and preserved in micro-cracks. Overall, aDNA extraction from micro-cracks in stone tools is a viable method for learning more about past cultures when there are no other organic remains available. It may be usable when the more common micro-residue analysis is not and will likely become more widely utilized as DNA sequence technology becomes cheaper and more available.

aDNA Contribution at Bridge River

The Bridge River site is a large housepit village located several kilometers from the confluence of the Bridge River and large Fraser River, near Lillooet, British Columbia. The village is one of several in the Middle Fraser Canyon context that have received intensive archaeological studies during the past several decades. The Bridge River site was occupied periodically from 1800 years ago to the mid-19th century (Prentiss et al. 2008). Excavation and analysis was completed by UM Professor Anna Prentiss and has been an ongoing project with UM students. So far, robust archaeological methods have been used to interpret anything from the cultural and economic evolution of the site to focus on specific house pit utility.

Much of Dr. Prentiss and other researcher's work have targeted understanding patterns of subsistence and technology changes (Prentiss et al. 2011). This interest is perfect for the application of aDNA extraction to aid in interpreting the site as it encompasses tool use, and we hope that future research will include our contributions. Additionally, our work could help assess site function, mobility, the range of activities undertaken at the site or aid in reconstructing and evaluating behavioral hypotheses (Langejans et al. 2015).

Challenges

Before we dive into methodology and exactly how our work contributed to understanding the Bridge River site, we need to address the limitations of working with aDNA in this context. Ancient DNA studies are characterized by low-quality DNA from degradation of the DNA molecules, a process which correlates loosely with factors such as time, temperature, presence of water, etc. Degradation leads to post mortem mutation processes such as deamination, which cause the wrong "letter" or base to be substituted in the sequence (Burger 1999). Additionally, the very structure of the DNA begins to degrade, leaving us with short chunks of sequence, instead of the longer sequences modern DNA can produce. This is why amplification of aDNA is a crucial step, to have enough DNA to sequence. Interestingly, extraction of shorter "chunks" of DNA is how many researchers designate that their sequence is indeed not modern contamination. If results contain a long, beautiful sequence of DNA with few mutations, it is indicative that the sample has been contaminated with modern DNA (Pääbo et al 2004).

The nature of DNA degradation over time means that few sites are suited to good DNA preservation and thus successful aDNA extraction studies. Extreme environments that are waterlogged, very dry, oxygen depleted, contain extreme pH levels, extreme high/low temperatures, heavy metal interaction, and/or antibacterial/fungal conditions are the best for DNA preservation (Langejans et al. 2015). Unfortunately, these environments are few and far between and the Bridge River site did not really fall into any optimal preservation category.

There have been a few studies using the methodology we based our research on in much younger experimental conditions that resulted in an average of only 10% of tools yielding DNA (Kimura et al. 2001). Our results at UMT have yielded an even lower percentage of successful extractions, as is expected using actual ancient test subjects. Another obstacle to getting results

was the fact that our main subject, scrapers, may not be best at preserving aDNA due to resharpening and loss of microcracks containing DNA. The optimal tools for preserving aDNA in micro-cracks is actually churt knives (Kimura et al. 2001).

As we experienced, contamination is also a huge complication. Modern DNA is exceptionally more robust and easy to amplify than its degraded aDNA competitor and thus, it takes a lot less DNA sneezed or shed off of the researcher to completely overshadow any aDNA present in a sample (Deguilloux et al 2011). We take many steps to generally prevent contamination in our labs. Access is restricted and uni-directional from the post-PCR lab. PCR amplification in the post-PCR lab sends millions of copies of amplified DNA into the air and onto all researchers in the vicinity; thus a researcher who has spent time in the post-PCR lab is barred from the clean Ancient Lab. All members must wear full-body coveralls, booties, arm guards, hair-nets and hoods, face masks, and gloves when in the clean Ancient Lab. All surfaces are bleached daily, and materials are also exposed to UV light for at least 15 minutes prior to use. Additionally, we followed any specific protocols based on that in Shanks et al.(2001) aimed at preventing contamination, and we seemed to do it quite effectively.

It is possible to identify sequences that do not belong to expected species, but there is always the possibility of false positives due to contamination in DNA amplification reagents themselves. The reagents that allow us to amplify DNA are derived from DNA and thus sometimes create false positives. For example, Taq is derived from bacteria and many primers from livestock. They result in false positives 2-5% of the time (Leonard et al. 2007).

Finally, once aDNA is successfully extracted, it can be difficult to prove it was a result of use. Many events may happen to tools in the years between use and recovery and many opportunities for contamination unrelated to tool use. Experimental studies in which researchers

used tools and then buried them for a year in separate deposits revealed that as much as 50% of positive result species were unrelated to the species the tools were used on (Langejans 2010). The context of the species discovered on tools must make sense in light of the site, species in the region, and potential for their use with the tool. As stated previously, aDNA extractions should be a small addition to a diverse investigation when interpreting a site and tool set.

Materials and Methods

Methodology ended up being the largest focus of this project considering how long it took to get results to interpret and theorize about. Many months were spent adjusting and fiddling with each step in the process until we found what worked. For the most part, we followed a procedure developed in Shanks et al (2001). Orin Shanks was without a doubt the pioneer in developing a set procedure for extracting aDNA from micro-cracks in stone tools; indeed, there are few papers on the subject in which he was not involved. All of the following was conducted in our clean Ancient Lab in full anti-contamination gear. From extraction, to amplification, to electrophoresis and sequencing, the entire process took about three to four days.

Our first step involved lightly scrubbing the tools in water, then rinsing them twice in ultrapure water to eliminate surface contamination from handling. The tools were then placed in sealed polypropylene bags and 4mL of 5% ammonia hydroxide was added and left to soak for 30 minutes. Ammonia hydroxide permeating the tools created a pH that is more conducive for DNA to free itself from the micro-cracks and into our solution. Our next step was sonicating the tools at 50 hrtz for 5 minutes. This created small bubbles, which entered the microcracks on tool surfaces and forced out the DNA into solution. Sonicators are used in a similar way to remove soil and residue from crevices in jewelry and soil was in fact readily removed from the micro-

cracks in our tools as well.

After rocking the tools for 30 minutes, we cut a small hole in each bag, drained them into filter tubes, and spun them in an Amicon Ultra-4, Centrifugal Filter Device. This removed the ammonium hydroxide and isolated DNA, which was caught in a filter while the ammonium was spun through. Solutions of EDTA and SDS were then added and spun through, standard procedure for removing DNA from a filter and collecting it into low-bind 2 mL collection tubes. These tubes are specifically designed to prevent DNA from sticking to the sides.

The next step was to add proteinase K to the solution and incubate for 6 hours at 56º C. Proteinase K is an enzyme that cleaves the peptide bond in proteins next to the carboxyl group of hydrophobic amino acid residues. We use it in our procedure to degrade any nucleases that could contaminate our sample. Following this, the samples were cleaned to remove further contamination following the QIAGEN MiniElute PCR Purification kit specifications, which is basically a series of steps involving adding buffers, eluting, and creating an optimal pH for extraction using sodium acetate. The pH change turned out to be one of the most challenging parts of the procedure since our sample had been soaked previously in ammonium hydroxide. We had to work hard to reach our target pH, repeatedly adding sodium acetate and checking the pH. It's important to note that these extraction techniques did not harm the tools; the only changes perceivable after the procedures were cleaner tools.

Finally, samples were amplified with primers to target the Cytochrome B and 16S regions of the mtDNA molecule. These regions contain mutations that are specific to different species and allowed us to designate between them during sequencing. Primers are short strands of RNA or DNA that serves as a starting point for DNA synthesis in a polymerase chain reaction (PCR) that amplifies a small amount of DNA into a sequenceable amount. Our samples were amplified

in the post-PCR room and then checked to see if our extraction and PCR had yielded any DNA.

To see if our extraction and PCR amplification worked, we conducted a process called electrophoresis. Electrophoresis involves mixing a small amount of the amplified DNA with a UV fluorescent dye, placing it in an agarose gel, and running a current through it. Since DNA is negatively charged, if present it will run down the gel in a predictable and detectable way and form a "band", the position of which tells us how long the strand of DNA we've extracted and amplified is. Longer sequences of DNA cannot run as far down the gel during electrophoresis simply due to their extended size. The band fluoresces when placed under a UV light, allowing researchers to identify and record it.

If we saw a band, we sent our sample across campus to the biology labs be sequenced using a Sanger Sequencer. We would compare any results sent back to us to a database, GenBank. GenBank is a resource of other sequences of the same mtDNA region in which the species is known. If regions of our sequence matched with a species in GenBank, we could assume that species was present in our sample.

Results

We conducted the previously described methods on twenty different stone tools, often multiple times. The entire process took about seven months and many hours every week from a variety of researchers. First, only bacterial matches were extracted (Paenibacillus and a Bacillus simplex strains), which are difficult to distinguish as contamination or not. Bacteria is (and has been) so universally present in the environment that these common strains could have come from anywhere and even if they are aDNA, they reveal little about tool use. *Finally*, a week before we presented at the annual meeting for the Society of American Archaeologists (the SAAs), we

amplified the 16S region of the mtDNA molecule from a slate hide scraper, which when compared to GenBank was a strong match for *Puma concolor* (North American cougar / puma / mountain lion).

Since the sequence of mountain lion DNA was relatively short and contained indicative mutations of degradation, we could postulate that it was ancient DNA. We were quite surprised to recover mountain lion aDNA and immediately conferred with Dr. Anna Prentiss to discover if the species was common at the site and whether the people living there could have been using the scrapers on them. It turned out that lions were common to this region of British Columbia and their hides were highly valued. In particular, they were important commodities for dance/ritual purposes and the hide was potentially being processed for such reasons. Additionally, we inquired about the age of the particular scraper that had yielded aDNA. The scraper was found on a floor at the Bridge River site that dated to 1300 years ago.

Discussion

Currently, our preliminary results suggest that DNA is accessible from the Bridge River lithic assemblage, albeit at low frequencies. We have not confirmed the *Puma concolor* sample. From the tool that has yielded aDNA, it is possible to start suggesting that the slate hide scrapers may have been used to process *Puma concolor* hides. Because it is such a unique species, it is unlikely that the *Puma concolor* aDNA could have resulted from contamination from our lab or from DNA amplifying reagents. Additionally, the nature of the sequence we extracted was robust enough to provide strong evidence for a likely match in GenBank, yet mutated and short enough to provide evidence that it was likely aDNA as well. Future work will hopefully yield insight into how frequently scrapers were utilized for this purpose, as well as what species other tools in

the assemblage may have been involved with. We will continue to perfect our methodology for extracting aDNA from lithics to see if we can perhaps increase our prevalence of successful results.

Combating degradation, contamination, and other complications are all issues we have addressed and will continue to challenge us. As this project continues, we hope our goals aid in understanding the Bridge River site lithic tool use based on future aDNA collection. Additionally, based on interest in our work at the SAAs we may be using our extraction methodology to help interpret other sites soon.

Conclusions

Ancient DNA extraction from stone tools and other sources is a relatively recently developed mechanism for aiding in the interpretation of archeological sites. Since the development of PCR in the late 1980s, the field has rapidly expanded and with it the possibility for expanding knowledge about many anthropological questions concerning anything from pathogens to Neandertals (Pääbo et al 2004). Although it can contribute great information to a project, aDNA analysis is usually a difficult and tedious process requiring numerous samples and a long period of time. It's also important for researchers considering adding an aDNA analysis to their greater projects to note that while considerably cheaper than in the past, aDNA analysis is expensive and doesn't guarantee results.

Ancient DNA gives us insight into the molecular makeup of an individual in a species at a certain point in time. However, extreme caution must be used in any aDNA study, considering its highly degraded and contaminable nature. Before any conclusions can be drawn from our or any other aDNA researcher's results, they must be confirmed by other researchers, and firmly

placed in context with other forms of archeological and anthropological study. It is all too easy to rely too heavily on any one aspect of an archeological study and come to incorrect or biased conclusions. An inclusive, cautious, and scientific approach is always the best way to include ancient DNA into any anthropological or archeological study. Despite all of the challenges and limitations, when conducted and supported correctly, aDNA analysis from stone tools and other sources can contribute great information to appropriate studies and should be widely integrated into many aspects of anthropology.

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