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Ancient DNA in Archaeologically Charred Zea Mays L: Prospects and Limitations

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Arts

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ANCIENT DNA IN ARCHAEOLOGICALLY CHARRED *ZEA MAYS L.*:
PROSPECTS AND LIMITATIONS

by

Chelsey Geralda D. Armstrong

Graduate Program in Anthropology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Arts

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
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Abstract

Plant remains are an integral part of any archaeological investigation given the large role they play in ancient subsistence economies, medicinal practices, technologies and folklore. However, despite new developments in ancient genetics, research in *plant* ancient DNA (aDNA) is a relatively young and untouched discipline accounting for less than 7% of all aDNA analyses published in academic literature. As a result, paleoethnobotanists, archaeologists and geneticists have not understood the feasibility and limitations of each other's field. Few are aware that DNA extraction from charred plant remains is rare and without any kind of standard or working protocol. The possibilities of retrieving aDNA from charred *Zea mays* L. (maize) is considered in this study using modern maize for polymerase chain reaction (PCR) optimization and combining purification methods on ancient samples (1150-1250 AD), resolving the question of whether or not archaeologically charred plants are a viable source for genetic material. The confirmed positive results generate questions about the added-value of maize and how knowledge of genetic attributes can contribute to the growing field of archaeology and ethnobiology while demonstrating the value of these findings as they pertain to the treatment of charred floral remains by archaeologists and First Nation communities.

Keywords

Ancient DNA (aDNA), paleoethnobotany, botany, archaeobotany, ethnobiology, *Zea mays*, heritage conservation

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List of Abbreviations

aDNA	Ancient DNA
AFLP	Amplified Fragment Length Polymorphism

AMS	Accelerator Mass Spectrometry
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
cpDNA	Chloroplast DNA
CRM	Cultural Resource Management
C _T	Cycle threshold
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dUTP	Deoxyuridine-triphosphatase
EDTA	Ethylenediaminetetraacetic acid
ESTs	Expressed sequence tags
GC	Guanine/Cytosine
GISH	Genomic in situ Hybridization
GuScN	Guanadinium Thiocyanate
HMW	high-molecular-weight subunit
MEGA	Molecular Evolutionary Genetics Analysis
mtDNA	Mitochondrial DNA
NIP	Nearly Identical Paralogs
nDNA	Nuclear DNA
OUT	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction

qPCR	Quantitative Polymerase Chain Reaction (previously “real-time” or RTPCR)
R _n	Normalized Reporter
RAPD	Random Amplification of Polymorphic DNA
<i>rbcL</i>	Ribulose-bisphosphate carboxylase gene
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
TBE	Tris/Borate/EDTA
UNG	Uracil-DNA glycosylase

Chapter 1

1 Introduction and Background

For thirty years, ancient DNA (aDNA) analyses have been a promising part of studies in anthropology, paleoecology, biology and medicine. However, aDNA molecules are a precarious material and recovery techniques are still in an emerging and exploratory period. Organic materials do not preserve as well as non-biological matter. Nevertheless, some cultural and natural processes allow for better preservation of organic materials, leaving archaeologists hopeful for genetic analyses of a variety of plant, animal and human remains. Since the retrieval of a 229 base pair (bp) sequence of mitochondrial DNA from a species of extinct *Equus quagga* (Higuchi et al. 1984), archaeologists and geneticists have sequenced thousands of archaeologically preserved ecofacts.

Unfortunately DNA analyses on ancient plants has not drawn the same attention as mammalian research for many reasons, some of which include: poor preservation of easily degraded plant tissues compared to vertebrate endoskeletons; the inter-kingdom variability of plant composites (seed, leaf, rinds, charcoal) which limits universal protocols such as that available for isolating DNA from bone; the limitations with plant DNA markers (discussed further in Chapter 3); inhibitor compounds such as carbohydrates and polysaccharides which can disrupt the amplification process; and a perpetuated notion that plants are not as exciting or as important as other archaeological finds (Archer and Hastorf 2000). These circumstances are unfortunate given the wealth of knowledge that has been gained as a result of other forms of paleoethnobotanical analyses.

The term paleoethnobotany was first introduced by Helbaek (1959) and grew out of the field of ethnobotany (Pearsall 2008). During the 1970s and 1980s paleoethnobotanists were mostly concerned with the analysis of macroremains (with some exceptions, e.g. Hastorf and Popper 1988) such as seeds, charcoal, pollen, and pericarps. Later on investigators became increasingly interested in conducting micro-analyses on diatoms, phytoliths, and ancient DNA (e.g., Hart 2000; Piperno and Pearsall 1993; Schlumbaum et al 2008). With the development of flotation initiated by Patty Jo Watson (1976), the recovery of botanical materials from archaeological sites became a staple of any good excavation. As demonstrated by the remarkable preservation of the Ozette site in Coastal Washington, some ancient peoples relied on plants for the majority of their material culture (Butler 1995). As well, no matter how botanical remains are preserved, plant and human interactions need to be studied to understand past lifeways. Wade Davis (2001) and other ethnobotanists have found that there exists a close correspondence between the earth's biodiversity and cultural diversity (Nolan and Turner 2011). Datasets amassed from plant remains is therefore a critical element in understanding a people's reference point in a taskscape (Ingold 2011). Ignoring or overlooking plants and human experiences as they relate to the natural world means losing a complex composite of information on the cultural diversity in a lived landscape.

Many have now recognized the value of paleoethnobotanical analyses on archaeological sites (e.g. Hastorf & Popper 1998). A cohesive publication is available on how to recover, identify and analyze plant remains (micro and macro) making the potential for plant analyses more feasible and accessible (Pearsall 2008). While many

experts maintain that paleoethnobotanical analyses are important, professional archaeologists, (commercial and academic) rarely agree on which plants are, and are not, valuable cultural materials. It should be acknowledged that this could be the case for *any* material culture. But the general lack of discussion on process compounds, and influences how materials are treated after excavation such as the type of storage preferred, and level of analysis conducted. These kinds of issues shape perceptions about what is or is not valuable archaeological material, and in the case of paleoethnobotanical material, it enters into the psyche of practitioners of sub-disciplines such as aDNA analyses. For example, Gugerli et al. (2005) surveyed hundreds of aDNA articles and found that only seven percent were concerned with plants. Genetic studies relating to plants have often been ignored and as a result many academics and professionals are unaware of the potential for recovering DNA from, for example, charred materials. But the presence of DNA molecules in plant remains represents yet another value-added aspect of plant materials which means that improper recovery and storing of remains increases the loss of the already overlooked value of plants; in effect, given the potential for DNA research, there is now more to lose.

While the focus of early paleoethnobotanical studies was centered on agricultural production – when and how populations developed a culture of plant manipulation (e.g., Anderson 1954; Braidwood 1952; Childe 1952; Cohen 1977) – plants play an important role in understanding much more than past subsistence economies, and can contribute to the understanding of technologies, art, medicine and folklore. After all, paleoethnobotany grew out of ethnobotanical studies of the mid 20th century, which

focused on the cultural relationships and worldviews between Indigenous peoples and plants (Anderson 2011a). In ethnobotanical studies, agriculture is but one element of the human-plant interaction spectrum. We know, for example, that hunter-gatherers alter vegetation through the use of fire (Hallett et al. 2003), sow wild seeds (Nabhan 1989), plant tubers (Lepofsky and Lertzman 2008; Turner 2005), and protect plants and tracts of land (Harlan 1992). These practices have lent themselves to archaeological studies that have, for example, inferred social status from plant offerings in burials (Newsom 2002). Even gender inequalities were explored using spatial analyses combined with an examination of ancient plant processing and consumption (Hastorf 1991). Botanical analyses also have technical field uses. For example, they can help reconstruct past environments, investigate site formation processes and provide relative and absolute dates for sites.

Here I suggest we consider the terms *archaeobotany* and *paleoethnobotany*. These terms are often used interchangeably and, while experts differ on definition, paleoethnobotany generally suggests that plants have an added ‘human’ value. Like all other material culture, plants in this context are more relational to human experiences; they are more valuable and carry more weight when the ‘humanness’ in them is evoked. Why then, are plants often overlooked, underrepresented and de-valued by both commercial archaeologists and ancient DNA analysts? Even in contemporary academia paleoethnobotanical analyses have been downplayed (Lepofsky et al. 2001). Some issues impeding plant DNA analysis have been discussed above, but a similar paradigm exists more broadly for floral analyses of remains recovered in CRM or academic contexts. In

all of these scenarios there is clearly a question of value. In the Kantian sense of the term, value is promoted based on the ends it provides. In archaeology this is often translated as 'knowledge.' But the idea that archaeology is only a noble quest for knowledge is erroneously removed from the socio-political contexts in which archaeology occurs. Rather, archaeology and particularly CRM is tacitly concerned with contemporary values of commoditization that are harmonized with industrial and commercial development. The value of archaeological material is regarded as a function of contemporary contexts; these contexts are most often associated with available finances, technocratic development (how government and other funding agencies value research in particular fields) and a discipline's understanding of the possibilities and limitations of technologies.

Genetic information can be used to trace the origin of plant species or populations and identify which traits (such as a larger seed or a tougher seed coat) were selected for and when. Ancient DNA analyses have the potential to further investigate intensively managed plants not recognized as fully domesticated cultivars (e.g., Bonhage-Freund et al. 2011; Gremillion 1993; Peacock and Turner 2000). It can also trace human origins, trade relationships and societal collapses. But how feasible is it to answer these questions? As mentioned above, the field of aDNA has grown extensively in the last thirty years but plants have been largely overlooked. The promise of extracting DNA from charred plants and reconstructing ethnobotanical relationships is challenging at best. If we are going to address anthropological questions about prehistory then the focus – at least for plants – should be on methodological issues that must be overcome in order to

effectively extract ancient DNA from archaeologically recovered plant remains. aDNA analyses should not only test hypotheses to such anthropological depths as desired by many, rather it should challenge positivist notions of value while confirming just ‘how much’ plant samples can tell us at this stage.

1.1 Research Objectives and Hypothesis

The purpose of this study is to test whether or not viable DNA can be retrieved and sequenced from archaeologically charred maize (*Zea mays L.*). It is important to test whether or not charred plants carry genetic value for interpreting the archaeological record given that this class of material is recovered from many sites, and retained in countless collections, worldwide. In Chapter 2 I discuss the various ways in which DNA from charred kernels can contribute to the culture history and diversity of the Great Lakes region as well as test bigger hypotheses asserted by positivist anthropologists, for example, that all agriculture is produced by sedentary peoples. If DNA can be recovered from readily available charred plant remains we would have access to a dataset of great potential.

I will test the null hypothesis that charred *Zea mays* is too inhibited to amplify during a polymerase chain reaction (PCR), by examining if it is possible to retrieve DNA if proper protocols are met and the purification of samples is based on a pragmatic evaluation of the quality of sample (scale of deterioration). This research will also reflect on the value of bridging the disconnections between archaeologists, paleoethnobotanists and geneticists to advance more robust research. Although there are some drawbacks to a

values-based approach to archaeology (e.g. Poullos 2010), by adding genetic value to plant remains there is more onus on archaeologists to properly excavate, store and protect these remains, especially if there is broad understanding of what can be lost otherwise (i.e. not just macro inventory and morphological information, but molecular as well).

The perceptive Deborah Pearsall (2008:2) noted, "...if the paleoethnobotanist is not trained as an archaeologist, then he or she must learn to think like one or at least communicate with archaeological field personnel and project directors". This should be the same for geneticists and other laboratory staff who are analyzing archaeological plant data. Geneticists working with archaeological material that "think like an archaeologist" can amass a better data set, and structure more relative research questions, by understanding the spatial and temporal contexts of the material being studied.

Archaeologists must also engage with the world of molecular science if only to better appreciate the feasibility and limitations of research in the lab; it is not uncommon for archaeologists and paleoethnobotanists to think that it is *impossible* to recover DNA from charred remains *or* that its incredibly easy and anyone can do it. The truth is that there has simply not been enough research undertaken at this point, and the literature on plant DNA that is available is usually of no interest to archaeologists because of the heavy scientific jargon, and because of the focus on methodological advancements in the lab that are seemingly of limited use to those on the ground.

1.2 Background

1.2.1 Preservation of Paleoethnobotanical Remains

How and why plant remains preserve on an archaeological site depends on a variety of cultural and environmental factors. The physical properties of a plant or plant parts such as density, surface characteristics, and size can all affect preservation (Hastorf and Popper 1988). Likewise cultural processing practices, such as charring wheat grains to remove the chaff, will affect preservation. For example, it is no wonder that the oldest date for the introduction of a cultivar to Northeastern North America is *Cucurbita* (cal. 5025 B.P.), whose tough and durable pericarp (rind) can endure many of the physical and chemical processes that succeed in breaking down other organic tissues. Archaeologists are also more likely to come across plant remains that fall beside a hearth or are stored in sealed pits, than those left exposed to elements that quickly break down organic materials. No matter how plants preserve, it is critical that the cultural contexts of excavated remains are defined and that there is an on-site assessment of all deposits, in order to inform proper sampling strategies (Hastorf and Popper 1988; Pearsall 2008). This will help specialists working in the lab to determine an expected rate of recovery and sampling strategies; design research protocols that are conducive to the kinds of material being worked with; and select primers and appropriate markers for research questions.

Plants preserve under a variety of conditions: notably by charring, waterlogging, desiccation or mineralization. Waterlogging occurs in anaerobic environments when a site is saturated over a long period of time. The stable environment (not fluctuating from

dry to wet) allows organic materials to retain their physical and chemical properties. On the Northwest coast of North America, for example, shell middens often create a vegetal layer in the strata because the basic environment created by the shell reduces acidity and waterlogged soils preserves plant remains (e.g. Croes et al. 2006). Desiccation, on the other hand, results from long-term extreme dry storing in arid environments such as cave sites or in desert ecological zones such as in the American Southwest. For example, some of the best preserved and earliest maize cobs found were desiccated (Piperno and Flannery 2001). Charring occurs when materials combust in a low enough intensity and frequency that hydrogen and oxygen molecules are removed and the material is converted mostly into carbon-based polymers. For example Pearsall's (1988:101-102) work in Panaulauca, Peru sourced an abundance of charred macro remains such as seeds which were deposited and charred as a result of the following processes: seeds were gathered for food and accidentally charred during cooking, parching and other preparation activities; seeds were brought in as part of plants gathered for food and discarded as waste; seeds were present in camelid dung or corral debris burned as fuel; seeds were present in sod burned as fuel or; seeds were blown or carried in accidentally and charred.

Archaeologically, plants are recovered in charred form because the carbonized structure intercepts destructive microbes, animals and other environmental factors from completely destroying the material. Charring can occur naturally or culturally (intentional or not). The context in which charring occurred to maize samples in this research is discussed in Chapter 2. While there exists plenty of literature on the successful isolation of DNA from waterlogged and desiccated plant materials (Oliveira et al. 2012; Manen et

al. 2003; Pollman et al. 2005; Schneerman et al. 2004), extraction and purification of DNA from charred materials is poorly understood, even though most plant remains found globally on archaeological sites are in a charred condition (e.g., Zohary et al. 2012). And while there have been several studies that have focused on charred plant aDNA (e.g. Schlumbaum et al 2008), these are not widely known in archaeological research.

1.2.2 Ancient DNA Analyses of Charred Plants

Extensive literature is available on modern and ancient maize genetics (Bennetzen & Hake 2009; Doebley 1990; Doebley et al. 1988; 2004; Gupta and Varshney 2004; Staller 2010; Staller et al. 2006; Weising et al. 2005). However, the use of aDNA analyses to better understand domestication events, the production, manipulation and spread of maize, is limited to extractions from mostly desiccated remains (Freitas et al. 2003; Lia et al. 2007; Schlumbaum et al. 2008; Schneerman et al. 2004). Genetic studies on ancient maize (and most ancient plants for that matter) fail to utilize charred specimens due to the broad assumption that DNA is not preserved in charred materials, underscored by unsuccessful attempts to isolate and amplify viable DNA from such remains (e.g. Oliviera et al. 2012). This produces a disconnection between archaeologists and geneticists, the former believing that charred plant remains are of no genetic value, while the latter do not bother with experimental purification methods that could be serviceable to charred plant remains. If the study of ancient plant genetics is going to make any headway in archaeology, then it is necessary to consider the possibilities and limitations of isolating and recovering DNA molecules from charred materials.

Giles and Brown (2008) tested purification methods on artificially charred grains of *Triticum aestivum* (wheat) and found variations of silica-binding methods worked best for isolating positively identified DNA (See Chapter 4). Moore (2011) also tested purification methods and was able to retrieve higher yields of DNA from artificially heated samples of modern maize using the Yang et al. (1998) modified silica spin, as opposed to the buffer solutions referred to in the CTAB/DTAB method.

Perhaps the only positive results for recovered aDNA from archaeologically charred plants are by Goloubinoff et al. (1993), Brown et al. (1994), Allaby et al. (1999), Schlumbaum and Jacomet (1998), and Fernandez et al. (2013). The first study by Goloubinoff et al. (1993) is contentious since results have not yet been replicated and are based on questionable protocols and contamination issues that were not addressed at that time. The second study by Allaby et al (1994) employed a CTAB extraction method, and the third study by Schlumbaum and Jacomet (1998) employing a silica-based method. Both studies targeted high-molecular-weight (HMW) and subunit genes of glutenin in various kinds of charred wheat from multiple sites. Although both studies were groundbreaking and set the course for plant aDNA studies, Schlumbaum herself noted that overall most topics in plant aDNA are often considered without much follow up (2008). Labs have not followed up on research and archaeologists have not maintained the collaborations needed for more development in the field.

The lack of clear success in recovering ancient aDNA from charred plant remains early on contributed to a general sense that efforts were not worthwhile. This created a

challenge when undertaking this present study, in that, when discussing my research with colleagues in archaeology, most assumed (very decisively) that charred plants are definitely not a source for DNA, or that they would be absolutely useless in that kind of molecular study. But the simple truth is that there has not been enough research. Extraction and purification methods used on other types of plants (modern and ancient non-charred) are certainly a starting place for analyses, but there is not enough understanding of inhibition, and the potential yield of intact DNA molecules in archaeologically charred plants. Fernandez et al. (2013) have recently followed up on studies of charred wheat and found that silica-based extraction methods and amplifying specific target regions is the best strategy for recovering ancient DNA from partially charred material.

This research is an attempt to reach beyond the assumptions and demonstrate that DNA is recoverable from charred remains. This will set the course for future analyses of plant remains while re-defining the nature of ‘value’ in archaeology, which confirms that value is constantly added and removed based on a number of socio-politico contexts, available resources and confounding assumptions of the experts producing the artifacts.

Chapter 2

2 Zea mays L. and Archaeological Contexts

“It is therefore advantageous to the agricultural interests, as land is becoming so valuable, to reserve as much of the soil of England as possible for the cultivation of wheat and more valuable products; and nothing will tend to promote this object more than the introduction of a copious supply of cheaper farinaceous for the poor and labouring classes”

– Dr. J.S. Bartlett, addressing a letter to Lord Ashburton in May, 1842 speaking of the debt owed to Americans, by the British for introducing corn as a cheap substitute.

2.1 Zea mays L.

2.1.1 Origins and Domestication

From whichever discipline you chose to explore it, maize is a biologically remarkable and culturally salient plant. How a tropical species came to occupy farmlands as far north as the Boreal Softwood Shield of northern Canada (Figure 2.1) is testament to the plant’s anthropological and biosystematic versatility.

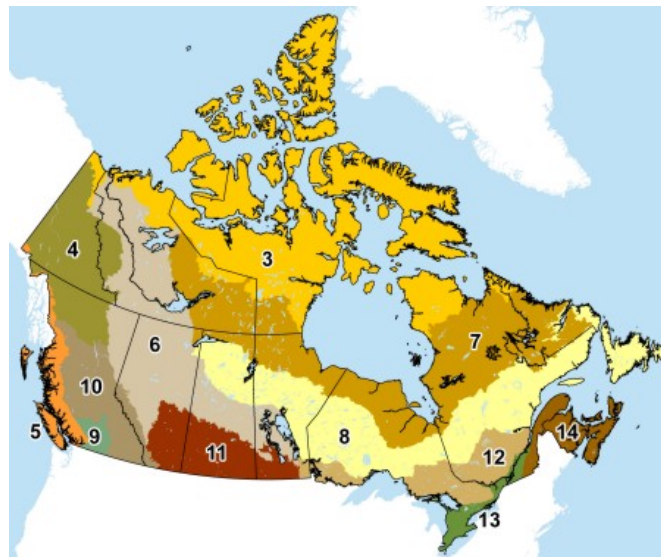


Figure 2.1: Bird Conservation Regions of Canada. Region 8 – the northern limit to maize expansion – indicates the Boreal Softwood Shield, which includes Northern Saskatchewan, Manitoba, Ontario, Quebec, eastern Labrador and all of Newfoundland. (Environment Canada 2011 www.ec.gc.ca)

Maize is a domesticate from the *Poaceae* family and it produces a dry indehiscent fruit; the kernel is a single seed leaf (monocot) and the ears (cobs) are female inflorescences. The skeleton or core of the cob is a woody structure that is made of three layers: a peripheral layer of coarse and fine glumes; a meso layer of a complex lignified system; and an inner-core or pith (Bozovic et al. 2004). The complete genome sequence of *Zea mays* was published in 2009 by the NSF-funded Maize Genome Sequencing Project (Schnable et al. 2009), and we now know that the wild progenitor is *Zea mexicana*, commonly referred to as teosinte (Sauer 1993). The *Zea* genus has four wild species found in Mexico and Central America including: *Zea perennis*, *Zea diploperennis*, *Zea luxurians* and *Zea mexicana*. Doebley (1990) suggests that a subspecies of *Z. mexicana* (*parviglumis*) is the primary forebear of modern maize, found on the Michoacan-Guerrero border of western Mexico. Throughout the process of domestication, teosinte went through relatively similar genetic and morphological enhancements as other domesticated cereal grains like wheat and barley, which resulted in an increase in grain size, a tougher seed coat (resulting in the retention of ripe grains) and a reduction in seed dormancy (Zohary 2004).

The origins and spread of maize and its impact on local cultures and plant diversity has been extensively researched (e.g. Anderson 1946; Bird 1980; Doebley et al. 1988; Staller et al. 2006; Upham et al. 1987; Yarnell 1976), but as Staller (2010:85) notes, “recent groundbreaking results from maize geneticists have indicated that earlier archaeological interpretations of plant domestication and the economic significance of maize need to be reconsidered”.

It is also worth noting that the United States is the biggest producer of maize worldwide, accounting for 40% of global production, yielding over 313 million tones in 2011 (Food and Agriculture Organization of the United Nations 2013). Of all the grain crops, maize produces the highest yield worldwide and grows on every populated continent even though it is mainly used for animal feed and ethanol fuel. This modern importance of maize underscores that the phylogenetic and phylogeographic histories of the plant are important to the current neo-liberal agribusiness context, and in relation to the cultural processes that led to this hyper and global obsession with a weedy tropical plant.

2.1.2 Spread and diversification

Human interactions and relationships with maize have long interested anthropologists, ecologists, botanists and many other scholars. Whether advancing our understanding of domestication, diffusion, status, art or wealth, the tropical grass has been of interest to a broad range of disciplines, particularly archaeology. The earliest evidence of maize domestication comes from cob remains at Guilá Naquitz in the Oaxaca Valley, Mexico. Cobs were AMS radio carbon dated to 5420 +/- 60 (Piperno and Flannery 2001). From this rugged valley, early maize precipitates simultaneously spread north into Sonora, Chihuahua, New Mexico and Arizona and south into Honduras, Ecuador and Peru (Blake 2006). Maize was diffused into the American Southwest sometime around 3500 B.P., marking the Early Agricultural Period of Southern Arizona and New Mexico (Huckell 2006). The earliest evidence of maize on the eastern side of North America comes from paleofeces at Fort Center, Florida dated to 2500-3000 B.P. (Kelly et al. 2006). The

movement north is spatially and temporally consistent, reaching the Ohio-Illinois regions and Central Atlantic Seaboard around the same time at 2000 B.P. (Lustek 2006; but note some exceptions, for example, Meadowcroft [Crawford et al. 1997]). Crawford's (et al. 1997) work reviews multiple dates for Northeastern maize and shows the movement of maize into the Great Lakes region in southern Ontario by around A.D. 260-660. Recent phytolith analysis published by Hart (2008; Hart and Morgan 2009) shows some disagreement with those earliest dates. Stable isotopic analyses from sites in this region shows that maize was not intensively grown or overwhelmingly part of the diet prior to A.D. 1100 (Hart 1999; Smith 1992), however phytolith evidence does show the appearance of cultivated remains from the Vignette site in the Finger Lakes region at 300 B.C. (Hart et al. 2003; Hart and Matson 2009). Nevertheless, disagreements among academics and the sparse research conducted on the topic means that the spread and genetic diversion of maize, particularly around the Great Lakes Region, has not yet been defined.

Our understanding of early maize agriculture in the Great Lakes region has been increased substantially by the works of Crawford (1997; 2006) and Hart (1999; 2000; Hart et al. 2003; Hart and Matson 2009) and Boyd and Surette (2010). However, the 'academic race' to identify the earliest entry dates for maize in the Great Lakes has overshadowed some of the more important anthropological and phylogeographic questions about maize diversity and cultural diffusion. For example, thousands or tens of thousands of maize samples from the Late Woodland period of southern Ontario are in need of analysis. As well a higher resolution study of ancient maize genetics can provide

more insight into what types of maize were grown and how intensively. For example, we know that based on temperature and moisture availability of different regions in the American Southwest, a variety of ecological areas adopted or modified different types of maize. It is thought that at least twenty-one races of maize were positioned to diffuse from the Southwest before European contact and seven of those were found in the twentieth century (Corral 2008).

Understanding the processes that shaped the adoption of maize in the Great Lakes region will benefit from an in-depth analysis of potential maize varieties. As Diana Greenlee (2006:215) has remarked, “Geographically, one could choose any of several subareas of the East as the setting for detailed research into why maize-based subsistence systems appeared when and where they did and in the forms they did.” By looking at the development of maize within the Great Lakes we have the potential to better understand ancient geopolitical boundaries, follow trade patterns and examine the development of subsistence patterns at regional or larger cultural scales. These kinds of anthropological questions are not new, but have important implications for my research as I try to recover genetic material from charred maize remains.

2.2 Archaeological Context

2.2.1 Culture History of Western Basin

The samples used for this project were excavated by Golder Associates in 2008 from the Bingo Village (AgHk-42) site in southwestern Ontario (Figure 2.2). The site dates to the Late Woodland period of Southern Ontario (ca. A.D. 900-1600). The site has been materially associated with an archaeologically defined Late Woodland tradition known as

the Western Basin, which extended through southwestern Ontario and around the western end of Lake Erie (e.g., Murphy and Ferris 1990; Figure 2.3).

The Western Basin Late Woodland Tradition consists of a series of chronologically specific phases, known as the Riviere au Vase phase (A.D. 500-900), the Younge phase (ends A.D.1200/1300), Springwells Phase (A.D.1200/1300-1400) and finally the Wolf Phase (ends around A.D. 1600) (Murphy and Ferris 1990). The Bingo site has been subjected to AMS radiocarbon dating, and calibrated dates would place the period of occupation late in the Younge phase, between ca. A.D. 1150 and 1250 (Ferris, personal communication, 2013).



Figure 2.2: Pin drop denotes Bingo Village near London, Ontario between Lakes Erie and Huron

The Western Basin Late Woodland in southwestern Ontario is characterized by an increase in population and aggregation of village sites with a higher frequency of

sedentism through this period, following a similar but distinct pattern of cultural development to that seen for the easterly adjacent Late Woodland Tradition known as the Ontario Iroquoian (e.g. Ellis and Ferris 1990). Analyzing the shifts in food procurement and food production are a key element in understanding how these Late Woodland populations developed in relation to one another.

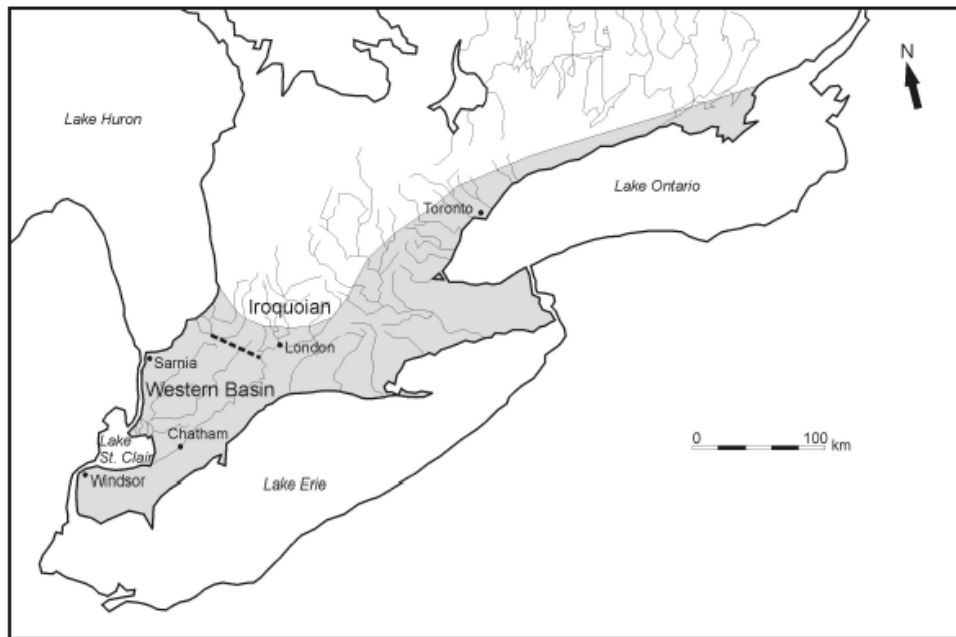


Figure 2.3: Western Basin Tradition and Iroquoian lands of Southwestern Ontario. The dotted line depicts the boarder regions between both groups from ca. A.D. 1000-1200 (Foreman 2011)

2.2.2 Southwestern Ontario Subsistence Strategies

The Western Basin subsistence strategy at the time of the Bingo site occupation consisted of a mixed economy of hunting and fishing of local taxa and intensive collecting of native *Chenopodium*, *Amaranthus*, *Polygonum*, *Phytolaca*, *Cyperus*, *Tilia* sp, and *Fagaceae*. Only recently have perceptions of Western Basin and neighboring Ontario Iroquoian economies shifted. It was presumed that stationary Iroquoian groups harvested

domesticated crops like *Zea mays* L., *Curcubita* L., *Helianthus* L., *Nicotiana* L. and *Phaseolus* L., while Western Basin peoples were seasonally mobile and less reliant on cultivars (Dodd et al. 1990; Murphy and Ferris 1990). Isotopic analyses now show that agricultural crops, particularly *Zea mays* L., was of equal importance in Western Basin and Iroquoian diets during the Younge phase (Dewar et al. 2010; Lennox and Molto 1994; Watts et al. 2011). While maize was an important feature of Western Basin diet, evidence still suggests moderate-to-high group mobility based on the seasonal distribution of other resources. This triggers a number of questions surrounding cultural diffusion and the intensity of interactions between both groups, the role of food production, the stereotypical perceptions of mobile foragers, and the cultural paradigm shifts associated with agricultural production.

The shift to agriculture in the Old World, termed the Neolithic (Childe 1952) and the Formative period (Willey and Phillips 1958) in the New World are habitually associated with shifts to sedentism and population growth and aggregation (Flannery 1973). This pattern is true for many cultures around the world but exceptions exist, most notably on the Northwest Coast where sedentism, population aggregation and complex social structures arose independent of intensive agricultural production. There is an inherent interest in the social repercussions of a shift from food procurement to food production because of the potential change in population demography and material culture. Because we know that mobile Western Basin people consumed just as much maize as the sedentary Iroquoian (Watts et al. 2011), it is hypothesized that 1) Western Basin persons, contrary to the rule that agriculture begot sedentism, were actively

harvesting agricultural crops while maintaining seasonal mobility through extensive caching technologies (either an exception to the rule or time-lapsed transition *in favour* of the rule); or 2) the Iroquoian and Western Basin cultures were actively participating in social and economic exchange.

However if there existed a genetic distinction between Iroquoian and Western Basin maize, presumably they were growing, sowing and maintaining their own stores of maize independent from one another. Although it would be uncharacteristic of mobile hunter/gatherers it would not be unheard of, and caching technologies would have ensured year round access to maize stores (critical to achieving the isotopic numbers for maize), despite cold weather dispersal (e.g., Dewar et al. 2010). Maize is a great candidate for adopting a more ‘mobile agricultural’ practice. There is a relatively low level of caloric investment and reliable harvest in rather unpredictable environments such as those with irregular soil or rainfall and, in this part of the world, fewer frost-free days in a year. Some landraces of maize in the Yucatan take only seven weeks to mature after planting (Nal t’eel or ‘rooster maize’), (Arias et al. 2000). Even with the contemporary paradigm shift to homogenize agriculture we see extensive diversity of maize varieties among modern Yucatan farmers who utilize multiple landraces locally adapted to different growth cycles and differentiated by colour (Table 2.1). It is therefore well worth exploring the subsistence economies of Western Basin and Iroquoian traditions. While there are extensive and relatively informative ethnohistoric ethnographic data, they are sometimes limited in their ability to interpret maize economies from early mid-late Late Woodland periods. Although they certainly have a place in this research (below),

archaeological and genetic investigations into the diversity and similarities between maize crops can tell us more about cultural transactions and subsistence economies.

Maize landrace	Mayan or Spanish name	Cycle length	Grain color	Mayan name(s) according to grain color
Nal t'eel	<i>Nal t'eel</i> ("rooster maize")	7 weeks	White	Sak nal ("white maize")
			Yellow	K'an nal ("yellow maize")
Nal t'eel x Tuxpeño	<i>X-t'uup nal</i> ("youngest child maize")	2.0 months	White	Sak nal ("white maize")
			Yellow	K'an nal ("yellow maize")
Nal t'eel x Tuxpeño	<i>X-mejen nal</i> ("young maize")	2.5 months	White	Sak nal ("white maize")
			Yellow	K'an nal ("yellow maize")
			Dark purple	X-éek' jub ("purple")
Tuxpeño	<i>X-nuuk nal</i> ("old maize")	4.0 months	White	Sak nal ("white maize")
			Yellow	K'an nal ("yellow maize")
			Yellow-red	Piix cristo ("knees of Christ")
			Scarlet red	Chak chob ("red hot")
			Dark red	X-granada ("pomegranate")
			Dark Purple	X-éek' jub ("purple")
Olotillo x Nal t'eel	<i>Ts'fit bakal, Bek'ech bakal</i> ("long thin cob")	3–3.5 months	White	Sak nal ("white maize")
			Yellow	K'an nal ("yellow maize")
			Dark Purple	X-éek' jub ("purple")
Locally adapted improved maize:				
V-527	Maíz mejorado	2.5–3 months	Yellow	
V-528	("improved maize"), Maíz		White	
V-532	híbrido ("hybrid maize")		White	
V-533			Yellow	
V-536			White	
Nal Xoy			Yellow	

Table 2.1: Varieties of maize grown in Yucatan adapted from Arias et al. (2000) from Tuxill et al. (2010)

2.2.3 Ethnohistory and Analogy: Features and Food Processing

In order to conceptualize the cultural context in which maize remains from Bingo Village were deposited, multiple lines of evidence are used to create a composite picture.

Ethnographic, ethnohistoric and archaeological knowledge can help reconstruct past lifeways by looking at the particulars of the spatial and temporal conditions of archaeological features like subterranean pit structures (Binford 1967). Elisabeth Tooker and Bruce Trigger's work on the Huron are excellent ethnohistoric studies that extracted baseline information from early European accounts about early agricultural practices in

the Great Lake regions, especially the narratives relating to the significance of maize, and the kinds of harvesting, processing and cooking patterns that may reflect to the kinds of material culture found at Bingo.

The sheer abundance of maize found in underground pits at Bingo may be understood in Tooker's (1991) recording of maize yields which, in a year were sometimes 100 grains per one stalk of maize. Trigger (1969) surmised that crop yields (maize being the most important) accounted for three-quarters of all the food that was eaten. Historic Huron Iroquoian practices included men clearing land by cutting trees and using the brush to start a fire to clear remaining stumps, and the woman, using wooden spades would dig round holes a pace apart and deposit 9 or 10 kernels per hole (Tooker 1991; Trigger 1969). Maize planted was able to ripen within 3 months (Tooker 1991). The Northern Flint variety of maize commonly documented in historic sources, and varieties of which are found on Late Woodland archaeological sites could grow over six feet tall and bore two to three ears.

The cultural practices that modified maize after harvest are fundamental in understanding why or how so many kernels preserved at Bingo for almost 1000 years. Different preparation and curing activities were used for cooking and processing different meals. Tooker noted that ears were tied in bundles and hung on poles forming a rack in the house to dry until storing and women and young girls would shell, clean and stock kernels in vats or casks. Trigger (1969) noted that after drying, kernels and ears were pounded into flour in a mortar, hollowed out from a tree trunk and a 6-7 foot long pole

(Figure 2.4). Interestingly this process is imbedded in the linguistic configuration of Iroquoian words for maize. Janice Longboat, a linguist and elder of the Haudenosaunee Iroquois First Nation shared with me, that the most used Iroquoian word for maize, actually means ‘to pound’.

There was over twenty ways of preparing maize flour or *ottet* and maize kernels for meals (Tooker 1991). Of most importance to this research is anything that may have involved roasting or the use of ashes, since samples from Bingo were removed from



Figure 2.4: Huron woman grinding maize, from Trigger (1969:63)

charred contexts presumably, such as roasting pits, ash pits, and as waste deposited in various cultural features. Although kernels were typically boiled with water or pounded,

roasting whole cobs or kernels in the ashes of a hearth has been recorded. Young, un-ripened cobs could be roasted whole and then boiled with fish or meat.

While there is no direct ethnohistoric information from southern Ontario that explicitly explains why so much maize might have been charred on site in the 13th century, it is worth noting that Dezendorf (2013) suggests hominy production (also known as *nixtamalization* in Mesoamerica) has a clear effect on kernel preservation after charring. In particular, hominy production or alkali processing requires cooking kernels with an alkali substance, such as wood ash, to extend storage life while increasing the value of essential amino acids lysine and tryptophan (King 1987). Studies by Goette et al. (1994) and King (1987) observed that most maize remains in the Andes and North America were boiled in wood ash. Dezendorf's (2013) experimental analysis concludes that many Native American groups used some kind of alkali processing method, the results of which will have contributed to the preservation of charred kernels at archaeological sites. Given the amount of preserved carbonized maize and the generally expansive nature of the kernel size (hominy tends to expand kernel width, see Appendix C), it would be worth testing the hypothesis that Western Basin and other southern Ontario peoples were treating kernels in alkali solutions, and when that practice may first have been adopted.

It is also worth comparing archaeological features like subterranean pits of other seasonal or semi-sedentary populations during the Late Woodland. Holman and Krist Jr. (2001) analyzed twenty-four cache pits from the Late Woodland in west central Michigan and found that pits were being used during a seasonal round to stock a range of edible

and non-edible resources. Cache pits may serve a number of functions related to food processing activities or storage but can also give clues to archaeologists about subsistence patterns and interpretations of sites. For example, the authors note that in the winter, when the ground was frozen, scaffolds were used for meat, which could mean that plants were not accessible in storage pits in the winter. If the thousands of maize kernels were not accessible in the winter at Bingo it may be possible that there were grain-stores stashed before the onset of snow and ground freezing.

Other ethnographic records from the late eighteenth century (notably Tanner [1956], who was captured and lived with Ojibwa and Ottawa in Northern Michigan and Minnesota), reports that food was stored in autumn at residential sites and returned to in the early spring. Blackbird (1896) saw that his parents would immediately uncover their caches of maize and beans when returning to their village in the spring.

One type of structure yet to be discussed are pit ovens. Although the Bingo site report makes no mention of this kind of feature it is worth exploring their use ethnographically in the Great Lakes region. The Iroquois dug pits in clay deposits or on the sides of banks and were first heated with coals then removed for roasting an assortment of vegetables and grains (Parker 1968; Waugh 1916). Kernels and whole cobs were roasted under ashes in a constructed earth-oven. Dunham (2000) mentions a number of ethnographies that recall the use of pit ovens to cook breads and roast kernels among the Ottawa, Nahma and Ojibwa. Particularly, Kohl (1985:300) wrote, “when the maize is

still quite young and unripe, they cut it down, husk it, and boil or bake it in red hot pits. These pits are first filled with burning wood and hot stones, heated and then cleaned out”.

As with all analogies contemporary or historic, the evidence is to be used as a guideline and not surreptitiously superimposed onto archaeological interpretations. For one, seventeenth century Huron maize production may have been much more intensified compared to twelfth and thirteenth century cultivation. Furthermore, the pit features at Bingo are not diagnostic enough to corroborate with other archaeological and ethnographic evidence. Pits filled with burnt kernels are hardly edible – were they garbage pits? If they are cache pits or grain stores left by mobile people, what kind of events led to post-depositional carbonization? This kind of contextual information is important to the archaeo-geneticist trying to understand molecular taphonomies. How and why plants preserve molecular data allows us to better inform archaeologists of the potential for aDNA analyses and in the future ask more anthropologically significant questions through the use of DNA.

2.3 Bingo Village

All samples from this study were recovered during excavations between 2006-2008. Most samples are from the 2008 field season. Excavations consisted first of 300 one meter square units being hand excavated and topsoil screened through six millimeter mesh (Figure 2.5). The site was then stripped of remaining topsoil, and sub-surface features identified and excavated. Features were typically excavated in a manner similar to topsoil units, though feature fill was bagged for subsequent flotation, carried out in 2009 and

2010 at the Museum of Ontario Archaeology by means of a double bucket method, recovering separate light and heavy fractions.

The Bingo site was found to consist of a dense scatter of Western Basin Younger Phase (A.D. 900-1200) materials with 4 house structures and multiple features within two encircling palisades (Figure 2.6). Excavations recovered over 250,000 chipped lithics (50% of assemblage); 150,000 flora and fauna remains (30% of assemblage); 76,000 fragmentary ceramic sherds (15% of assemblage); 250 stone and clay pipes (0.05% of assemblage); a variety of modified faunal remains including beads, harpoons, awls; ground and rough lithics; and personal adornment artifacts such as stone and copper beads and pendants and a ceramic disk.

2.3.1 Floral Analyses

The Bingo Village is one of many Younger phase sites excavated in the Arkona area (collectively termed the “Arkona Cluster”) of southwestern Ontario. This particular locale is also referred to as a “borderland” (Cunningham 2001; Watts 2008), because of the shared material culture and proximity between contemporaneous Western Basin and Iroquoian groups (see Figure 2.3). The high frequency of charred maize in many of the Bingo Village features coincides with new data on Western Basin diet (Dewar et al. 2010; Watts et al. 2011), and initiated my research question – that is to test the viability of genetic material in charred maize. Much more research on the data collected from Bingo Village is in need of analysis as little is still known about the Younger phase Western Basin Tradition and is especially true of floral analyses.

Golder Associates does not have an archaeobotanical protocol. Samples of maize were collected from pits where kernels were visible in abundance. In addition, carbonized plant remains were sorted out from soil flotation heavy and light fractions and bagged collectively. In the final report for this CRM project, maize remains were not subdivided by anatomy (e.g. cupules, kernels, glumes, embryos). There were no sampling methods or strategies employed other than recovery of pit fill for flotation, and the report consisted of the following, in its entirety on the subject of plants:

“A total of 150,142 flora and faunal remains (unmodified) were recovered during the Stage 4 excavations at the Bingo Village. This includes 146,536 faunal remains, 3,413 pieces of carbonized corn, 186 carbonized plant remains and seven carbonized nuts. This total does not include faunal remains with deliberate modification; these artifacts will be discussed in detail below” (Golder Associates 2012:78).

The report does not include a full summary of the materials recovered by flotation. To my knowledge I am the only paleoethnobotanist to examine the material and while going through the thousands of samples at Golder Associates and Sustainable Archaeology, it was obvious that the samples were not cared for. Ziploc bags containing floral remains were completely pulverized, likely due to being boxed with heavy artifact classes such as fire cracked rock. Whether pulverized remains included maize fragments is not possible to determine. As well, several flotation samples were labeled with the words “no tag,” indicating that the process of taking soil samples, transport, and duration

(B)

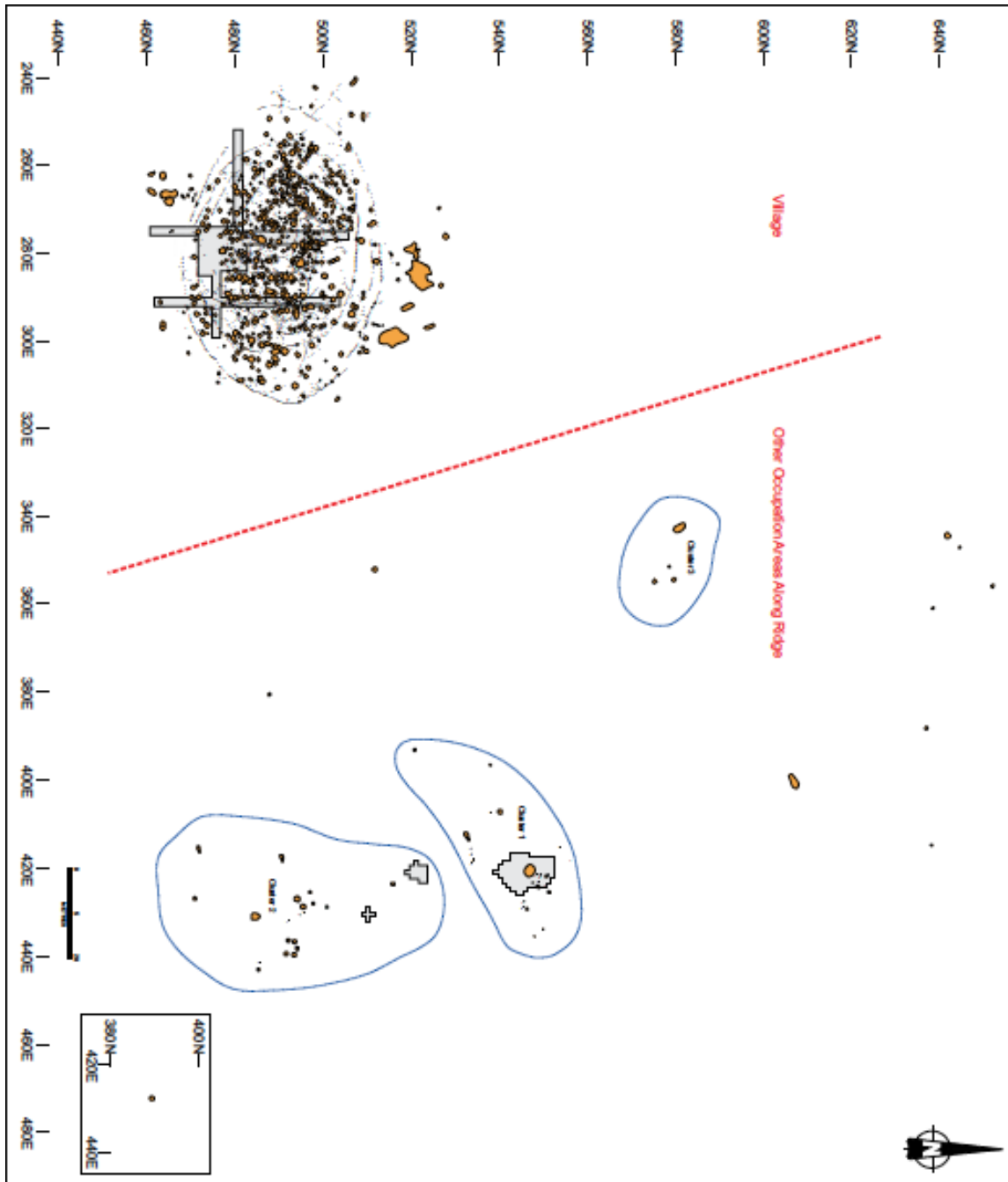


Figure 2.5: (A) Bingo Village 2008 Block Excavation. Shaded squares are stage 3 and white squares stage 4 units excavated. Below (B) Units excavated overlay village site. All maize

samples are from the Western portion block excavation. See Figure 2.6 for Village inset. Both figures from Golder Associates (2012)

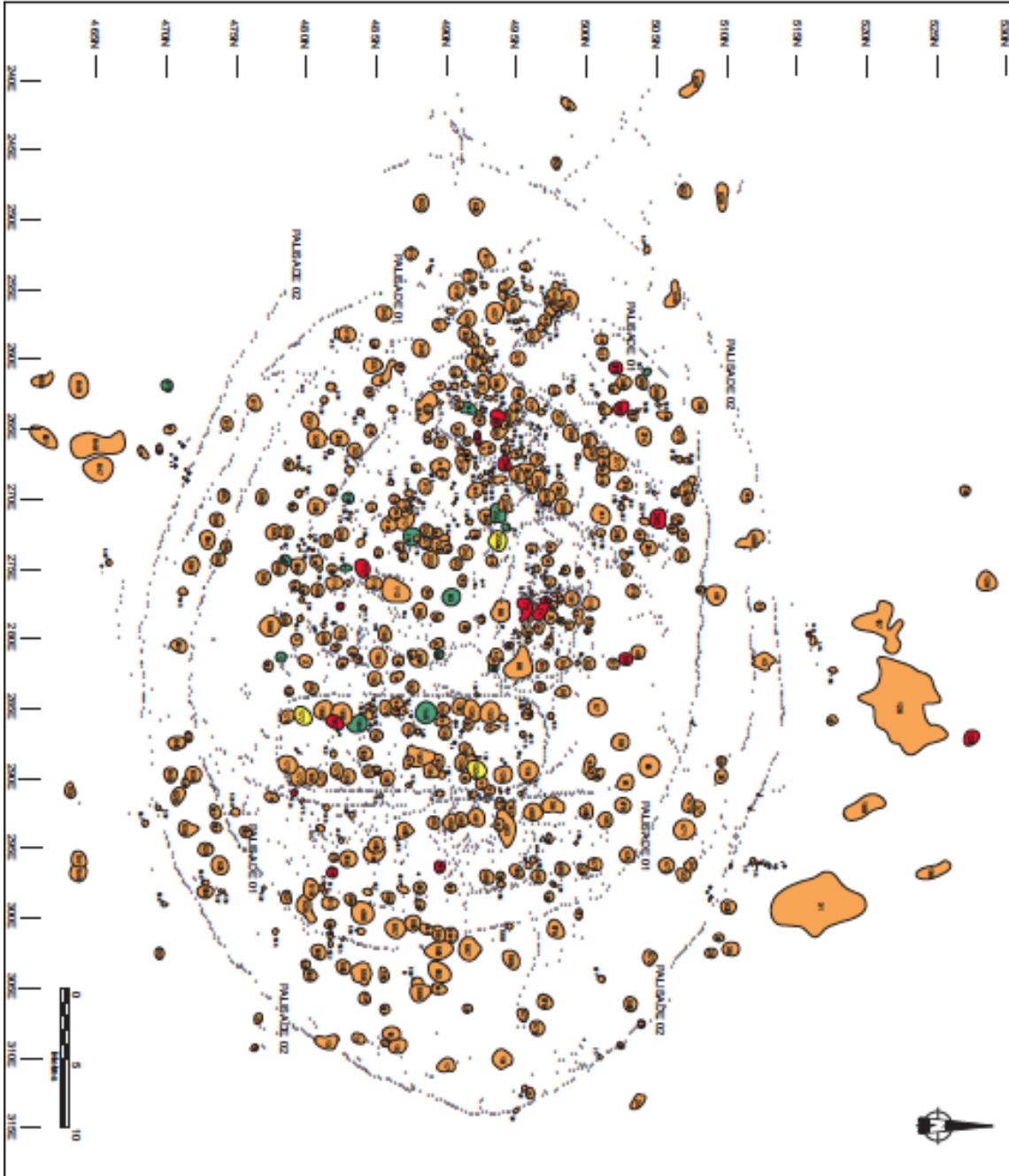


Figure 2.6: Bingo village settlement pattern from Golder Associates (2012:174). Note the four house structures and multiple features within two encircling palisades. See inset of Western most house structure in Figure 2.7

say, only samples with satisfactory labeling, morphological distinction and adequate distinction of provenience were chosen for genetic analyses.

2.3.2 Sample Context

A detailed account of sample size, counts and weights is available in Chapter 3. The following is an overview of the archaeological context from which the samples were chosen. Although no in-depth paleoethnobotanical analyses have been done, I have been told the maize from most contexts of the site were recovered from multiple underground ‘storage facilities’ (Shane McCartney, personal communication 2012). This is also based on the pure frequency of kernels found in similar contexts. Most features encountered at the site are *pits* (527 total), *ash pits* (28) *hearths* (21) and *burials* (14). Pits used for storage (and later waste) and ash pits are typically found near hearths. The ash pits and hearths differ in profile and shape from storage pits and are on average smaller and basin shaped while hearths have reddened oxidized soil (Golder Associates 2012). The pits from where maize remains were taken for this study had a mean length of 94.6 cm X 80.3 cm width and 37.6 cm depth and made up 88% of overall features types at the site. Figure 2.7. shows from which pits maize samples were used for ancient DNA analyses. Table 2.2. provides a brief overview of individual pits.

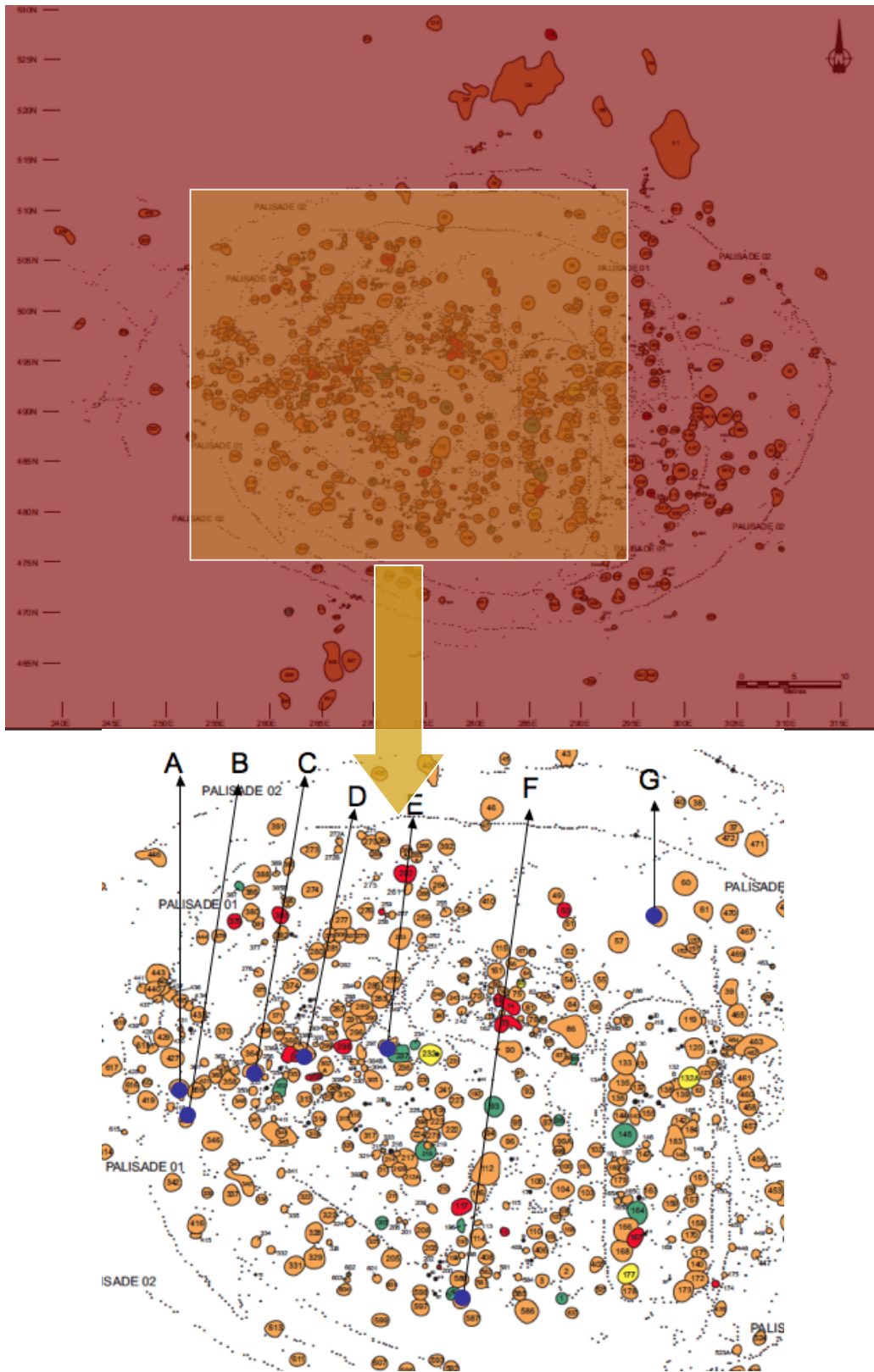


Figure 2.7: Bingo Village western portion of site. Inset shows pits where maize was excavated and used for aDNA analyses (see Table 2.2). Images modified from Golder Associates (2012)

Figure 2.7 Reference	Pit Feature	Associated Artifacts	Additional Notes	Additional Photos
A	420	3 bifaces, 163 body sherds, 2605 charred maize kernels, 779 faunal remains, 3 modified bone artifacts	n/a	n/a
B	417	1 pipe bowl, 2 bifaces, 25 body sherds, 7 charred maize kernels, 970 faunal remains, 2 projectile points	n/a	n/a
C	357	2 pipe bowls, 3 bifaces, 105 body sherds, 1 bone awl, 552 charred maize kernels, 451 faunal remains	Pit feature within household	Figure 2.8
D	301	9 bifaces, 1 ceramic pot, 3163 faunal remains, 8 projectile points, modified turtle shell, ? charred maize kernels	Pit feature within household	Figure 2.8
E	238	10 bifaces, 1 anvil stone, 7 charred maize kernels, 1013 faunal remains, 7 projectile points	Adjacent to feature 237 with human remains	n/a
F	588	2 pipe bowls, 2 bifaces, 120 body sherds, 1 bone bead, 57 charred maize kernels, 551 faunal remains,	n/a	n/a
G	59	5 kettle point chert projectile point tips, 1 Onandaga chert ovate base, antler projectile point, antler harpoon point, 2 bone awls, modified deer phalanx, modified bone artifact	n/a	Figure 2.9

Table 2.2: Overview of pit feature contexts. Refer to Figure 2.7 for spatial distribution of pit features. Data from Golder Associates (2012)

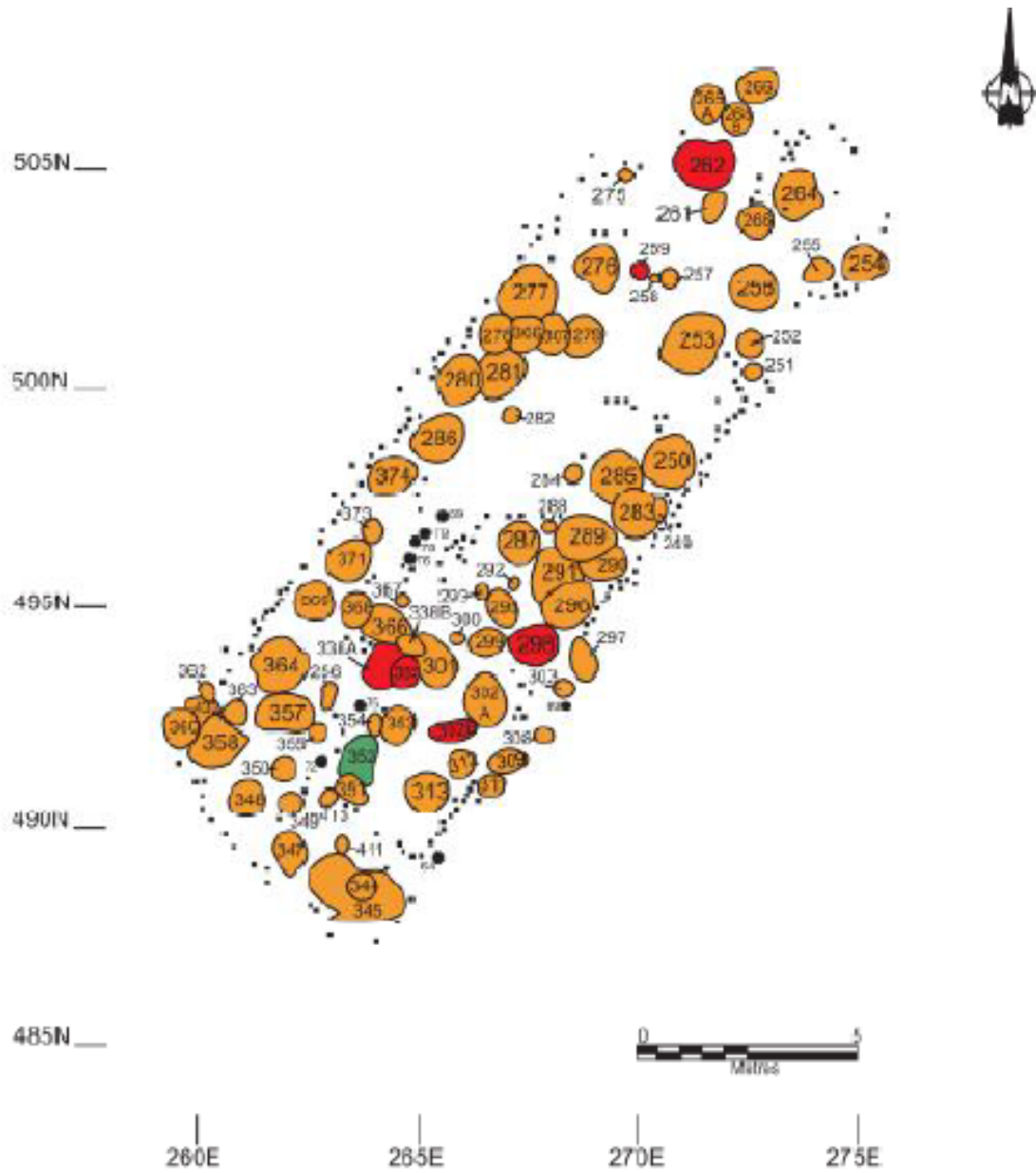


Figure 2.8: Household 3 plan view. Red features denote hearths, green contain human remains and yellow contain possible human remains. Note Pits 357 and 301 containing maize kernels used in this research.



Figure 2.9: Pit Feature 59, plan view, facing south. Photo from Golder Associates (2012)

2.4 Conclusion

Because of severe distortion to kernel morphology due to charring, roasting, or naturally occurring oxidization or combustion, measurements of kernel shape and size were inaccurate and did not provide reliable results regarding how processing affects carbonization and preservation. As a result, past studies were unable to make direct comparisons between modern carbonized kernels and archaeological carbonized kernels to determine the processing techniques used (Dezendorf 2013). However, the results from this study are still conditioned by the cultural and ecological contexts within which each kernel lived, died and preserved.

Chapter 3

3 Ancient Plant DNA and Plant Physiology

Deoxyribonucleic Acid (DNA) is a molecule that codes the genetic information of all living organisms. The information is stored and transcribed into proteins and other compounds that make up all things that are or were at once living. The four base nucleic acids that code for DNA are adenine (A), guanine (G), cytosine (C) and thiamine (T), together they pair up (G to C and A to T) and run along two bridges of phosphate deoxyribose. In all organic material, senescence and post mortem processes lead to the disintegration and decomposition of DNA. As the molecules get older the bonds that connect nucleic acids break down and interrupt the genetic integrity of the organism. Therefore retrieving ancient DNA has many challenges that do not arise in modern genetics such as, inhibition, low amounts of template DNA and potential contamination from natural and cultural conditions.

3.1 Studies in Ancient DNA

The development of modern genetics and molecular systematics began in the 1940s and 50s with the pioneering research of Chargaff, Watson and Crick who engineered, for example, research on DNA base composition leading to taxonomic interpretations on the percentage configuration of GC presence in an organism (Hua and Naganuma 2007). The discovery of DNA amplification by the polymerase chain reaction (herein PCR) led to a surge in modern genetics and subsequently the development of ancient DNA analyses. PCR has often been compared to a “photocopy machine” where the original copy (DNA template, oligonucleotides or primers, polymerase taq and other ingredients, depending

on the purpose of the experiment) is amplified two-fold at every heating (denaturation) and cooling (annealing) cycle in the lab (Appendix 3.1). The generation of PCR in genetics allowed for the development of ancient DNA analyses owing to the exponential copying of small or highly deteriorated fragments of DNA, which then allowed for the preservation, sequencing and analysis of that remnant sequence. Because morphological and modern genetic markers can only provide indirect evidence of evolutionary history (Willerslev and Cooper 2005), and with the success of *E. quaagga* DNA amplification (Higuchi et al. 1984), the field of paleo molecular genetics took off in the late 1980s, becoming especially relevant for archaeologists, paleoecologists and paleontologists. For the first time, aDNA (typically defined as DNA older than 100-200 years) allowed scientists to record genetic changes and evolutionary histories in real time and over short geological time-scales (Willerslev and Cooper 2005). However gene-sequencing took a hyperactive life of its own and soon laboratories all over the world were gene coding for a variety of organisms. Hofreiter (2012:1) recalls this period of ‘set backs’ in the early and mid-90s as something paleo geneticists are still dealing with today: “several high-impact publications that reported amplifications and analyses of DNA from many million year old samples [...] later on turned out to have been based on contamination with modern DNA.”

Today a number of important publications address the issues of contamination and the necessity for clean labs, blind tests and the replication of results, both within and between labs (Cooper and Poinar 2000; Mulligan 2006; Pääbo et al. 2004). The result of this strict inter-disciplinary scrutiny makes for a more credible field but also means that

constant re-evaluation of standards and limitations of approaches are met with a hermeneutic philosophy that allows many scholars to voice their concerns and take different directions with their research. There are a number of ways to now ‘study’ DNA, especially via molecular systematics and our understanding of species/variety designation and other elements involved in plant domestication and ancient gene coding. The next section of this chapter looks at the proper protocol for archaeologists to follow when seeking potential DNA analysis on ancient specimens, the value of aDNA information, studies of maize genetics and the challenges and limitations involved in plant paleogenetics, particularly how it relates to plant physiology.

3.1.2 How-to Plant DNA

Chapter 1 noted that plants preserve in a variety of ways based on the context of deposition, the climate and ecological factors affecting the site and the physical and chemical properties of the materials deposited (leaf, seed, rind, phytolith, etc.). The degree of preservation for plant remains on most sites pales in comparison to other materials found archaeologically, but that does not mean that these plant remains are any less significant to interpretive potential than the lavish stone tools or hardy deer femurs found at a given site. Floral analyses are rare at most archaeological sites in Canada (Lepofsky et al. 2008) however, there is now a general understanding in the discipline that plants can give us substantial information about the past and are important constituents of ‘heritage’ (See Chapter 6). The initial discovery that DNA could be retrieved from ancient samples (Rollo et al. 1987) has expanded the types of knowledge that could be amassed by archaeobotanical materials, but protocols and best practices for retrieving DNA are continually being recreated. Although there will never be one single

protocol for retrieving plant DNA, there is a general consensus bridging the various practices for DNA recovery that are based on how to treat and store a variety of archaeological samples.

Although the threat of contamination from human and to some degree animal DNA is of large concern in processing and recovering plant DNA, being focused on targeting plant DNA markers means that exogenous DNA is not as troublesome. This is because plant markers are incompatible to mammalian ones, especially when considering that 42% of aDNA plant studies target chloroplast DNA (Gugerli et al. 2005). However, cross-contamination from contextually similar samples and reference collections is still an issue. For example, during my first year working in an aDNA lab my colleagues and I used universal plant primers when working with ancient soybean from China and continually amplified Douglas fir DNA – this was likely contamination from the tissue paper the samples had been sent in. This potential contamination can also be remedied through the kind of primers being used; if a very specific primer is employed to target a single species, contamination threats decrease. While inhibition is undoubtedly the biggest challenge to aDNA analyses, contamination can still cause many problems – problems that, often, can be minimized and controlled.

Similar to conventional paleoethnobotanical analyses, sampling strategies are the single most important part of an analysis. If sampling is done poorly all subsequent steps are futile. The same is true when sampling plants for their genetic material. Typically sampling strategies are concerned with how much soil to collect and where to collect it.

While it may seem reasonable to collect large amounts of soil for future studies (i.e. better safe than sorry) this type of principle will be confronted with more of the same problems surrounding the over-consumption of archaeological heritage and collections management (Ferris 2002). As such, a conservative but explicable amount of soil should be sampled. For more on sampling consult Pearsall's second edition (2008) *Handbook of Procedures* and Hastorf and Popper's edited volume (1988) of *Current Paleoethnobotanical Analytical Methods*. Typically genetic labs will process 10-20 samples in a sitting. If material is scarce is it best to collect it all, however depending on the research question, a small sample population will suffice. For example, if the goal is to identify a species 3-8 specimens might do, however one might chose to be more rigorous and expand their data set if the goal is to conduct an analyses on population genetics.

A critical issue that needs to be considered when sampling is that when pulverizing plant tissues for extraction, a single seed may not produce enough raw material to work with. A 'bulk sampling' strategy may need to be used whereby seeds from the same context (i.e. excavated from the same feature) are integrated into a single sample (See Chapter 4). Once exposed, archaeobotanical remains must be recorded, handled and stored properly. Waterlogged samples need to be stored in similar anaerobic conditions and dried samples should be confined to dry storage. Preservation of genetic material is linked more to temperature and environmental consistency of a site rather than its age (Parducci and Petit 2004; Mulligan 2006). Storing samples in cold freezers and

controlled environments should typically ensure that no further damage or deterioration of nucleic acids occurs.

Finally, clean laboratories are the substrate of a satisfactory or reputable aDNA analysis. The authenticity of aDNA sequences relies on a physically isolated work area, dedicated to ancient DNA analyses only. All laboratory work for this research was completed at the Paleo-DNA Laboratory at Lakehead University in Canada, and a review of the facility is provided in Chapter 4.

3.2 Plant Physiology

Plant architectures can present obstacles to DNA isolation especially since most protocols for extraction and purification found in the literature are primarily for bone materials. Plant cells and the type of targeted DNA (nuclear, mitochondrial, chloroplast) have very different properties that can inhibit PCR and make the extraction/purification process inconsistent, if not difficult. Some problems encountered with modern plant DNA include: DNA degradation by endogenous nucleases (enzymes that cleave phosphodiester bonds like the restriction enzyme found *Escherichia coli*); coisolation of polysaccharides (also inhibiting PCR); and coisolation of soluble organic acids, polyphenols, latex and other secondary materials (Weising et al. 2005). These problems are worsened when working with ancient samples especially due to cross-contamination (with reference collections or samples from close archaeological contexts). Lastly, because the biophysical structure of plants is different from species to species and even from seed to leaf, no single protocol can be used universally.

3.2.1 *Plant Cells*

Many materials found in plant cells can inhibit and prove confounding for DNA isolation and amplification. During photosynthesis plants convert carbon dioxide, water and photons into simple sugars and oxygen. The first step of this process is a set of reactions that produce Adenosine-5'-triphosphate, a stored energy which produces sugars and carbohydrates and are then converted into amino acids, and which finally form into one of four major classes: Nucleic acids (DNA and RNA coding and storing information for synthesis of cells); Lipids (fats, oils, glycerol); Proteins (responsible for many properties of life such as nitrogen metabolism and hormone biosynthesis); and Carbohydrates (the most abundant of all the molecules). Plant cells contain an aqueous collection of chemicals called protoplasm surrounded by a plasma membrane and a cell wall. The cell wall varies from plant to plant but is typically composed of hardy compounds forming varying proportions of cellulose, xylan and lignin and additional proteins and enzymes. Mammalian class organisms do not have cell walls (Figure 3.1), which will alter the DNA isolation process, perhaps adding a step or requiring those in the lab to be mindful of the extra materials when purifying plant DNA. Kistler (2012) shows that by adding extra steps to commercialized plant extraction kits, such as utilizing overnight incubation, pulverizing tissue in an extraction buffer and removing tissue after centrifuging the sample, a more efficient extraction is possible when working with lignified tissues like gourd rinds.

Carbohydrates (polysaccharides) are especially problematic in DNA amplification. The two most common forms found in plants are starch and cellulose (in the cell wall). They are difficult to break down so that DNA is not fully pure (isolated)

and the PCR is inhibited or the purification breaks down carbohydrates while simultaneously breaking down DNA. Jobes et al. (1995) used a high molar mass concentration of sodium chloride to interrupt co-precipitation of polysaccharides and DNA, while Ahmed et al. (2009) used hydrated ether. I would warn against using these methods too intensely or too often considering the authors were working with modern samples. Ancient DNA is much more vulnerable to these reagents and therefore purification methods should be used sparingly.

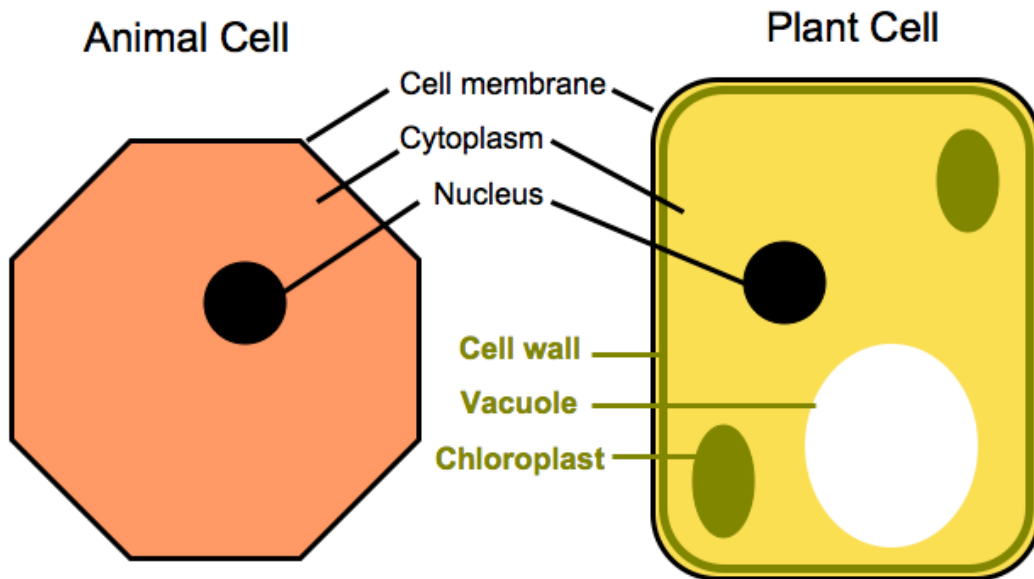


Figure 3.1: Basic Cell Structure; animal and plant cells, the features marked in green are unique to plant cell structure.

Proteins also need to be removed from samples to properly purify DNA. Some proteins like peripheral proteins (weakly bound to cell membranes) can be dissolved relatively easily with salt solutions or buffers, while integral proteins (bound to the cell

membrane surface) require detergents or other agents to interfere with membrane structure to remove the proteins.

The cellular structure and content of samples is important when isolating DNA, particularly because plants vary widely physiologically and isolating DNA is more efficient when we know which compounds are present. Maize, like other major cereals, are economically important plants because of their high starch and protein content in the kernel (Figure 3.2). *Zea mays L.* kernels contain 75% – 80% starch in dry matter at maturity, and 12-15% (mostly zeins) storage proteins (Manicacci et al. 2009).

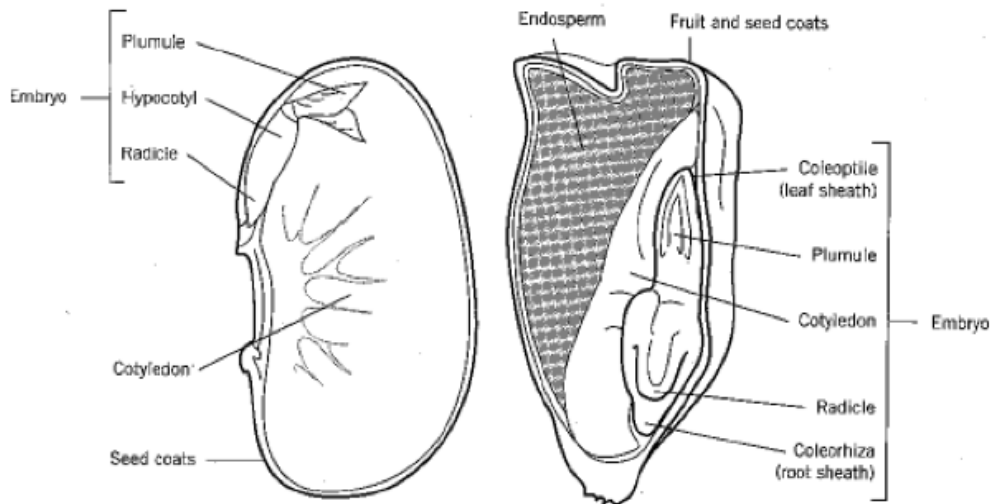


Figure 3.2: Maize kernel structure from Hopkins and Hüner 2004

Kernel quality traits found in modern forms of *Zea mays L.* from European, tropical and North American origins have a positive correlation: 1) between embryo size

and saturated fatty acid content and; 2) between kernel and endosperm weight. The same study showed three PCA axes that explain 70% of kernel phenotypic variation of which 39% accounts for protein-versus starch balance (Manicacci et al. 2009). Using the same collection of 375 maize inbred lines, Camus-Kulandaivelu et al. (2006) constituted five genotypic groups: Tropical, Northern Flint, European Flint, Corn Belt Dent, and Stiff Stalk. These groups were categorized based on character traits of contrasting kernel phenotypes (Table 3.1). Of particular interest is the nominal difference between starch/protein content and grain sizes. This could be explained by the diverse climates maize was introduced to, as well as cultural variations in usage or farming practices (e.g., horticulture versus agriculture).

Genotype	Geography	Vitreousness	Grains	Endosperm	Protein/Lipid Content	Starch Content
Tropical Origins and Varieties	Tropics	Low	Heavy	Large	non-specific	non-specific
Eastern North American and European	Early Northern and European Flint	High*	Very Small	Small	High	Low
Corn Belt Dent and Stiff Stalk	Eastern and Central North America	Low	Very Large	Large	Low	High

Table 3.1: Contrastive Kernel Phenotypes of 5 maize Genotypes
 Corn Belt and Stiff Stalk maize from the large modern corn producing regions in North America. (data from Manicacci et al 2005) *Flint means vitreous.

European Flint is a variety introduced to Europe in the early 16th century *from* Northern Flint (Rebourg et al. 2003). The large starch content is indicative of intense

selection by farmers in the 20th century to increase yield and sugar content (Duvick and Cassman 1999; Manicacci et al. 2009). The types of kernels found at the Bingo archaeological site and studied for this project are reviewed in Chapter 4.

3.2.2 Polyploidy

Polyploids are species with three or more complete chromosomes in their nuclei. Mammals typically only have two sets – for example humans have two sets of twenty-six from each parent – whereas up to 80% of angiosperms (maize included), and most major crops (wheat, sugar cane, potato, coffee and cotton) are polyploids (Leitch and Bennett 1997). This has significant implications for understanding domestication and life histories because polyploid species can have multiple origins. Maize for example, is a tetraploid, $2n = 4x = 20$, where n is the gametic chromosome number. Specifically, DNA evidence has suggested a segmental allotetraploid origin for maize (from diploid lineage), meaning more than one genetic origin. Gaut and Doebley (1997) tested four models for the evolution of the maize genome (autotetraploidy; genomic allotetraploidy, multiple segmental duplications, and segmental allotetraploidy) by examining patterns of sequence divergence of 14 pairs of duplicated genes. Blanc and Wolfe (2004) confirmed Gaut and Doeley's results and later noted that maize diverged from segmental allotetraploid events 10-20% based on expressed sequence tags (ESTs).

Many grass species have an evolutionary history that is complicated by the divergence from diploid progenitors (20.5 million years ago in the case of maize) to tetraploids. Analysis of this gene duplication and deep time evolutionary processes, although complex and seemingly uninvolved in bioanthropological research, has

implications for research on the domestication and phenotypic expression of archaeological maize. For example, Emrich et al. (2007) identified that roughly 1% of maize genes have a NIP (nearly identical paralogs: paralogous genes with >98% identity) of which both pairs are expressed and therefore potentially functional. Because gene duplication function is not well known, NIP's were used in combination with expression patterns to look at selective advantages during domestication and 'genetic improvement' of maize by early farmers (Emrich et al. 2007).

Grasses are highly adaptable and have been domesticated independently by a variety of ancient groups including rice in China (Khush 1997), wheat in the Levant (Nesbitt 1998), millet in Africa (de Wet and Harlan 1997) and maize in Mesoamerica (Piperno and Flannery 2001). Polyploidy or gene duplication has an obvious and advantageous utility for humans essentially because the number of genes are doubled and therefore farmers have more 'variety' to favor and cultivate. This is likely one of the main reasons grasses are a primary domesticate in most agricultural societies. Furthermore, this can help us to understand why a tropical plant like maize was able to thrive in a variety of climatic conditions, including regions of southwestern Ontario with fewer frost-free days than more tropical settings. Indeed, genetic diversity provided by NIPs has led Emrich et al. (2007) to explain the environmental stability of maize and its ability to grow in diverse kinds of ecological niches. NIPs are also believed to act as reservoirs of genetic variability so that multiple copies in a given sequence can allow for the recovery of higher quality mutations. The evolutionary potential of being polyploid is

outlined in Leitch and Bennett (1997) as an advantageous adaptation made by many species compared to diploid organisms.

There are a number of contemporary manipulative mechanisms that have allowed genes to coalesce into regions of the nucleus resulting in new interactions and patterns of development. This is a branch of genetics that has grown extensively in the last couple of decades and will continue to grow as hybridizing and genetic modification of plants persists in agro-business industries. Bioanthropology will benefit from growth in this area, allowing us to utilize techniques and new methodologies to pursue anthropological and ethnobiological questions. For example, the use of ploidy identification helped in understanding wheat diversity at a Neolithic site in Europe (Schlumbaum et al. 1998). These types of studies also help substantiate the need for more work on ancient plant DNA, in contrast to the narrow focus on mammal aDNA analyses where ploidy is universal. Furthermore polyploidy may increase the likelihood of recovering targeted sequences.

There are a number of methods for identifying and studying polyploids. Restriction fragment length polymorphism (RFLP) analysis showed that maize, previously thought to be a diploid, is actually tetraploid. Genomic *in situ* hybridization (GISH) works to discriminate between chromatin of parental origins. The use of random amplification of polymorphic DNA (RAPD) and competition among priming sites has shown to have no effect with the ploidy number of a specimen (Weising et al. 2005).

However there has been a marked correlation between ploidy level and scored amplified fragment length polymorphism (AFLP) bands in Solanaceae taxa (Weising et al. 2005).

Although polyploidy allows for long-term evolutionary flexibility, polyploidy also changes the organization and function of a genome at genetic and epigenetic levels, perhaps even leading to epigenetic remodeling and suppression of gene expression (Comai 2006). In addition, polyploid mitosis and meiosis has been seen to produce aneuploid (cancerous) cells in some yeast and mammalian organisms (Borel et al. 2002), although the plasticity of plant development may slow the process (Comai 2006). Therefore some of the disadvantages of polyploidy might not fully affect genome development in plants or affect this particular study. Studying the nature of polyploidy and synteny (the conservation of linkage groups between species, or in the case of maize, variety) is important in two ways: first, for the geneticist and second, for the archaeologist. Polyploid formation and genetic history offers a model for studying molecular mechanisms and processes involved in genome evolution (Leitch and Bennett 1997). Furthermore, the high frequency with which polyploids are formed means determining horizontal relationships between plants and humans (how many times a plant is domesticated), and resolving issues regarding the origin and spread of agriculture (Brown 1999). This is important for investigating the potential differences between maize types at Western Basin and Iroquoian sites where different gene expressions (polymorphisms) recombination or heterosis may have occurred based on hypothesized contrasts between farming practices.

3.2.3 Cellular Organelles

Before extracting DNA, the researcher must choose which part of the cell they will target for DNA isolation and amplification. All plant cells contain a number of organelles, or discrete areas of DNA assemblage, each with different structures and functions. Most higher order plants have a rather large vacuole (Figure 3.1) containing enzymes, sugars and pigments, surrounded by DNA membranes: the nucleus, chloroplasts and mitochondrion. Knowing what these membranes are and how they function is of particular interest to paleo-geneticists seeking to answer questions of anthropological significance, since each part of the cell will carry different kinds of DNA with different copy numbers and information about a particular organism such as its life history, community, population or species. This is one reason for collaboration between the archaeologists and geneticists. It is important to know that if, for example, we want to learn about domestication and have relatively well-preserved samples, isolating nuclear DNA will be more useful than mitochondrial DNA. As there are different protocols for isolating different kinds of DNA, I will provide a brief overview of the various forms of plant DNA while specifically focusing on DNA found in the chloroplast region of the cell which was targeted for this research.

A literary review of plant ancient DNA articles published in the last thirty years revealed that the main objective for studies included identification, phylogenetic assignment and intraspecific diversity (Gugerli et al. 2005). The same research concluded that the choice of molecular markers were primarily based on research questions. For

example, for species identification chloroplast markers were used, while inter-species diversity and phylogenetic relationships relied on nuclear markers (Figure 3.3).

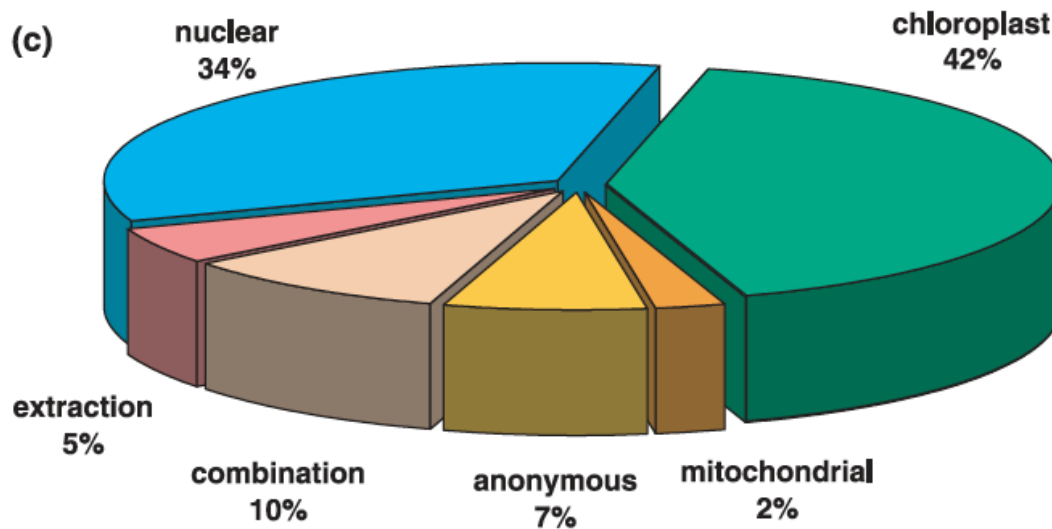


Figure 3.3: Molecular Approaches and types of markers used in ancient plant DNA analysis (Gugerli et al. 2005)

When choosing a DNA marker there will be a variety of properties that need to be ascertained. First, based on the preservation and identification status of certain materials, a specific or universal primer can be used. Universal plant primers are subject to more contamination but are best for unidentified specimens. Second, markers have to be designed for highly degraded samples, meaning they cannot be too large (or they will not anneal during PCR) and they cannot be too small (there will not be enough information or be specific enough to amplify). For this reason primers are typically between 80 and 300 bp, (Schlumbaum et al. 2008). Finally, DNA markers from different organelles can be used for different purposes. As a general rule, chloroplast and nuclear ribosomal DNA have high copy numbers and therefore are more likely to preserve while nuclear genes

have more inter-species specific information because of high mutation rates (best, for example, when examining domestication events).

3.2.3.1 Nuclear DNA

Some proteins are formed when DNA transcribed into messenger ribonucleic acid (mRNA) migrates from the nucleus into the cytoplasm where it attaches to ribosomes and forms polypeptide chains (and after several steps multiple polypeptide chains assemble together to form compounds). Specifically, nuclear ribosomal DNA contains the information that are the most popular markers in this group – internal transcribed spacer regions 1 and 2 (*ITS1* and *ITS2*), which contain hundreds of units resulting in a higher chance of preservation. The threat of ‘jumping PCR’ is increased however, as several copy numbers within polyploids like maize can result in primer dimers (i.e., primers amplifying on themselves), and other amplification problems (see Schlumbaum et al. [2008] for a range of studies that have utilized these markers). Nuclear DNA (nDNA) carries important information regarding domestication events and other economically important traits. Unlike chloroplast and mitochondrial DNA, which have uniparental inheritance, nuclear DNA is transmitted biparentally (Weising et al. 2005), therefore nDNA contributes more information about species history and evolution than the other organelles.

For example, HMW glutenin genes found in nuclear DNA have been used extensively in the literature (Allaby et al. 1999; Schlumbaum et al. 1998; Blatter et al. 2001). Because of the higher mutation rates and gene association with functional traits,

nDNA is especially used in population genetics. Microsatellites are the most popular and particularly useful types of nDNA because of their highly polymorphic singly-copy loci spread throughout the nuclear genome. Genetic variation is analyzed in a hierarchical structure for example, within a single individual, between individuals in a population, between populations within a region of origin, or between all populations from all regions (Wiesing et al. 2005). Polymorphism and high mutations rates are therefore critical when analyzing these structural groups. Microsatellites are shorter, easier to amplify and more abundant than other target regions, and they also have a large number of alleles and high variability among related organisms (Wiesing et al. [2005] reviews the use of microsatellites and other nuclear markers).

3.2.3.2 Mitochondrial DNA

Unlike their mammalian counterparts, few studies involve the detection and amplification of mitochondrial DNA (mtDNA) in plants. One of the main reasons is that mtDNA sequences evolve slowly (Weising et al. 2005). The type and quantity of mutations represents the amount and quality of information available from genetic data. Although mtDNA have very high copy numbers, they are not easily defined or identified. For example nDNA evolves twice as fast as chloroplast DNA, while mtDNA has a substitution rate of one-third cpDNA (Wolfe et al. 1989). As with mammals, mtDNA is transmitted through the female plant parent, although the value of the information varies depending on the organism. Plant and animal mtDNA are actually very dissimilar, and plant mtDNA mutates 100 times slower than animal mtDNA. For example, the D-loop region most effective in studying the evolution of vertebrate species is not specific

enough for plants (Larizza et al. 2002). Plant mtDNA has so far rarely been employed in plant aDNA analyses (Schlumbaum et al. 2008).

3.2.3.3 Chloroplast DNA

Chloroplast organelles are unique to plant cells. Like mitochondria, chloroplasts are the primary energy-transducing organelles (for photosynthesis), with four main compartments: thylakoids, lumen, the stroma (background matrix), and the envelope that surrounds all features (Figure 3.4). The stroma is home to all the DNA and RNA and enzymes responsible for reducing carbon during photosynthesis, including the target of DNA used for this project: ribulose-1,5-biphosphate carboxylase (*rbcL*). Because there are 1,000-10,000 copies of chloroplast DNA (cpDNA) per cell they are more likely to preserve and therefore useful for highly degraded aDNA analyses. However there are many chloroplast markers better suited to some plants versus others. For example, *rbcL* genes within angiosperms (like maize) have been more extensively sequenced and used for plant systematics of closely related genera (Gielly and Taberlet 1994). Generally cpDNA are best for identification and for some studies of population variation because of their lower mutation rates (compared to nDNA). However some non-coding sequences like *trn* introns and spacers evolve faster and can be more variable than other cpDNA, and therefore useful for higher resolution phylogenetic research.

The most popular spacers between transfer RNA coding segments are *trnL-trnF* and *trnD-trnT*, however in higher plants many of these spacers are from 300-800 bp, which are too large for highly fragmented aDNA (Schlumbaum et al. 2008). Although they are not exceptionally variable, the *rbcL* markers used for this project were chosen because of

their high copy number and successful extraction in previous maize analyses (Moore 2012). It is important to note here that the *rbcL* gene used in this research has been used extensively in aDNA plant research (Banerjee and Brown 2002; Blatter et al. 2002; Fernández et al. 2013; Manen et al. 2003; Willerslev et al. 2003).

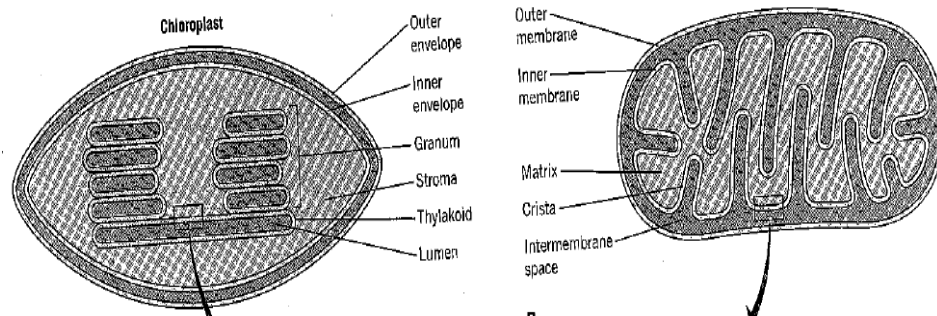


Figure 3.4: Chloroplast (left) and mitochondria (right) cellular structure (Hopkins and Hüner 2004).

The question of which marker to use in the laboratory is related to questions formulated in the field. If research can begin and end with identification of degraded samples then relatively short, high copy-number DNA should be targeted. Higher resolution questions should be framed realistically on 1) the preservation of plant material; 2) sequence data available (e.g. on GENBANK and other open-source databases to aid in interpreting data); and 3) the amount of available information from archaeological contexts and related research. For example, one way of choosing a marker may be based on the time-scale of a research question:

“Given that population genetics can be studied at a wide range of scales with different questions...the choice of marker system is

important...markers based on slowly evolving DNA sequences are adequate for the analysis of historical events on longer time scales, whereas markers derived from fast-evolving sequences are more suitable for analyzing recently diverged populations.”

(Weising et al. [2005:249]).

For the purpose of addressing archaeological questions, the time-scale referred to above is concerned with ‘recently diverged populations.’ Although maize genetic diversity has changed immensely within the last 1,000 years, in geological or deep time, our branch of study is accurately labeled as ‘recent.’ This kind of example should underscore the importance of continuous collaboration and understanding of the prospects and limitations involved in laboratory and field research. The following section looks at recent archaeological aDNA analyses of plants, which can help in understanding the potential for aDNA analyses, and in particular, the feasibility of the types of contexts and characteristics of plant specimens used for aDNA research.

3.3 Value of Plant DNA Analyses

Ancient plant DNA provides important semantides for archaeologically-oriented research questions as a result of the genetic information that is stored in specimens from controlled temporal and spatial settings. Once we have satisfactorily met the standards and procedures for working in a clean lab, and overcome the challenges of sample preservation, storage and choosing purification protocols and primers, it is possible to analyze and compare sequences of plant aDNA in an anthropologically meaningful way.

The DNA retrieved from a specific locus at a specific site gives a real time context for questions we have about ancient plant and people relationships. The following section looks at the value of ancient DNA analyses and how they have been applied specifically within archaeological research.

Morphological analyses of macroremains (seeds, nuts, shells, wood, rinds) have dramatically increased our knowledge of subsistence strategies, plant domestication, environmental interpretations, stratigraphic analyses and culture change (Hastorf and Popper 1988; Pearsall 2008). But the information potentially preserved in those remains (molecular information) are of further interest to archaeologists because of the potential for more accurate and precise plant identifications, evaluating hypotheses modeled by modern DNA, understanding long-term changes not visible morphologically, assessing various adaptations made by local communities, and investigating domestication events which are still poorly understood (Zeder 2006).

Most plant materials recovered archaeologically are charred and can be difficult to identify. A groundbreaking paper by Jacomet et al. (1989) showed that there existed large morphological variation of charred wheat grains within taxa from a particular site. Similarly, Dezendorf's (2013) experiential study on maize morphology evolved out of the difficulty of distinguishing maize varieties and processing techniques based on macro morphology alone. Although starch, phytolith and pollen can help to identify maize remains, carbonized samples can be difficult to distinguish.

Ancient DNA research can, for example, work with morphological analyses by providing another line of evidence for substantiating identifications. Indeed, Schlumbaum et al. (1998) show this with their investigation of the variety of wheat remains from a storage feature at a site in Switzerland (dated to 3906 B.C.). The rachis remains recovered from the site were compared with more contemporary finds and were identified morphologically as tetraploid naked wheat. This was complemented with aDNA analyses of charred wheat grains from the same feature. Sequencing of the high-molecular-weight subunit genes of glutenin also identified charred remains as hexaploid *Triticum aestivum*, which resulted in a clearer picture of what wheat proliferation looked like in Neolithic Europe. Pollmann et al. (2005) initially identified a portion of waterlogged *Prunus* fruit stones to species level based on morphological and metric data. Those that were unidentifiable were subjected to aDNA analyses, specifically using chloroplast *trnL-trnF* and nuclear ITS1 markers confirmed phylogenetically.

Identifications based on morphological traits are not faulty in of themselves, it is simply the frequency of variability and the subjectivity of taxonomic classification and systematics that limits our ability to precisely designate samples to a group. In the future, nomenclatural types based on particular genetic information may increase the accuracy and precision of less precise morphologically-based designations. For example, designating genetic syntypes and correlating them with morphological traits may be a helpful key that surpasses the need for destructive analyses. Linda Scott Cummings (personal communication 2012), working at the Paleoresearch Institute Inc. in Colorado, has relayed the need for genetic information to help confirm the identification traits of

particular phytolith keys, especially maize. Although the amount of time and resources needed to produce keys for phytolith and macrobotanical identifications would be enormous, the long-term benefits including reducing the destruction of ancient samples and laboratory costs are well worth exploring.

If floral analyses are meant to go beyond inventory lists of specimens identified by context and support more anthropologically significant research questions, then destructive analysis is unfortunately a by-product of our science. However, the knowledge gained from sequencing ancient samples is both dramatic and beneficial. For example, it is possible to identify genes selected for during domestication including the detection and selection of dietary and medicinally important genes for metabolic pathways or perceived economically advantageous traits (Schlumbaum et al. 2008). Blatter et al. (2001) studied a partial promoter region of the high-molecular-weight glutenin gene in *Triticum spelta* L. (AD 1700) and *Triticum aestivum* L. (AD 1750), of which specific alleles were compared. It was concluded that bread wheat in Europe had a polyphyletic origin (homoplasies or convergent evolution). Phylogenies and hypotheses regarding developmental genetic changes to improve plants agriculturally modeled on modern DNA can be confirmed or rejected by aDNA. For example Jaenick-Després et al. (2003) identified allelic diversity of plant architecture and starch characteristics in early maize domesticates. Anthropological studies of aDNA however, are not endless. An organism's DNA is not independent of cultural impacts and manipulations, Brown (1999) notes, so that while we may re-construct the trajectory of a domesticate, it is not possible to identify how it moved (migration of populations versus diffusion). Genetic research

therefore has its limitations but is nevertheless a valuable tool with many applications to understanding the spectrum of human-plant relationships through time.

Chapter 4

4 Materials and Methods

This section outlines the materials and methods used for modern and ancient maize DNA analysis. Modern maize was used in order to determine what the optimal parameters (temperature and cycling during the polymerase chain reaction [PCR]) should be to achieve ancient amplification, and to act as modern control or spike during ancient amplifications. The quantitative polymerase chain reaction (qPCR) was used for optimizing parameters so that a quantitative evaluation of positive results could be confirmed. Positive controls are samples with modern DNA only, to ensure PCR actually occurred, while internal positive controls or spikes are for samples with both ancient and modern DNA. The ladder control is to test whether or not DNA was amplified. If DNA was not amplified, then the ancient sample was too inhibited (allowing not even modern amplification to occur) or, if there was a reaction, both ancient and modern DNA was co-amplified, or modern DNA was amplified but there was simply no ancient DNA to be amplified (sample was too degraded). The first round of ancient amplifications with qPCR produced entirely negative results. As such, changes to PCR enzymes and the purification process made for the bulk of experimental research. Trial and error is recorded in this section and a summary of the results is presented below.

4.1 Modern Optimization

Parameters for the ancient qPCR were calculated using modern samples of maize DNA. Modern sample preparation, extraction and amplification were completed in separate laboratory facilities. Three modern kernels were each cut into four pieces with sterilized

blades. Half the kernel was further disrupted using a mortar and pestle and purified using the Qiagen DNeasy® extraction kit (Appendix A). To ensure DNA was quantifiable and could provide for precise measurements, a Qubit® Fluorometer was used by mixing 5 µL of DNA template with working solution; 1:200 Quant-it reagent (dye) in Quant-it buffer (Appendix B). Each sample was measured three times and averaged out and read DNA in ng/mL (see Table 4.1).

Sample	1 st read (ng/mL)	2 nd read (ng/mL)	3 rd read (ng/mL)	Avg. (ng/mL)
Modern 1 (M1)	0.0858	0.091	0.0862	0.088
Modern 2 (M2)	0.92	0.92	0.934	0.924
Modern 3 (M3)	1.32	1.35	1.37	1.347

Table 4.1: Qubit® Fluorometer results calculating DNA in modern samples. For amplification samples M1 and M2 were pooled to increase total amount of DNA.

A plate configuration following the TaqMan® Universal Master Mix guidelines was used to verify at which concentration primers and probes had the most successful reaction. The objective was to determine the minimum concentrations needed to obtain the maximum normalized reporter (R_n) and the minimum threshold cycle (C_T). This results in a three-staged detection (Figure 4.1 is an example of normalized real-time quantitative curves, with a the log scale curve is similar to a normal amplification of PCR product). The first stage reflects when R_n appears as a flat line, indicating there is no fluorescent signal detected. The next stage shows detection as the florescence increases relative to the products of the PCR. Finally the plateau, or third stage is when the ratio of Amplitaq polymerase to PCR product decreases (at about 10^{-7} M).

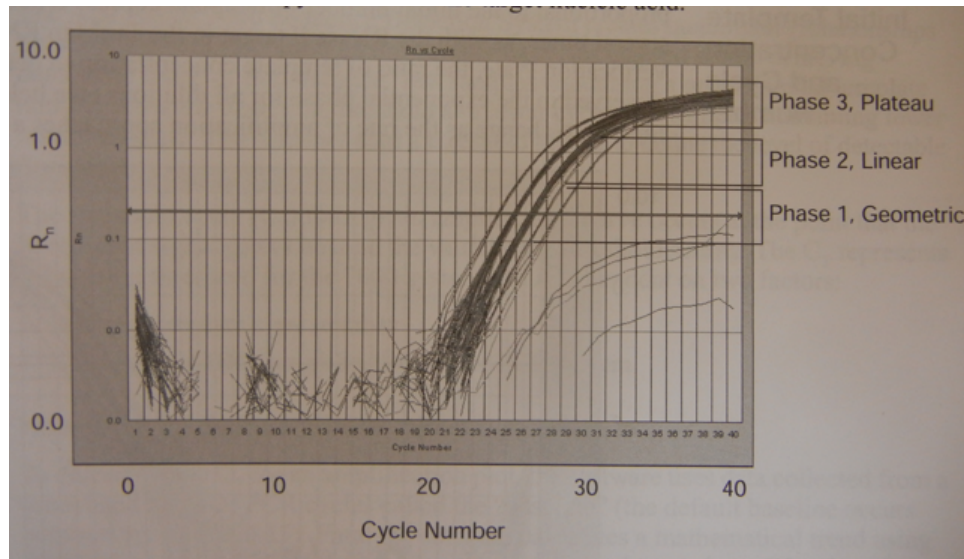


Figure 4.1: Normalized Curves. From the ABI Prism® 7000 Sequence Detection System

The final volume of each sample was 50 μL and contained the following: 25 μL of TaqMan® Universal PCR Master Mix (AmpliTaq Gold® DNA Polymerase, UNG, dNTPs with dUTP, and optimized buffer), varying combinations (0.5-9 μL) of the forward and reverse primers designed for *Zea mays* (Table 4.3), 1-5 μL of the TaqMan® probe, and 5 μL of modern DNA template and adjusted volumes of deionized water based on primer and probe concentrations (Table 4.2). Four samples for each trial were used to average final results. Reaction parameters were chosen according TaqMan® protocol with an initial denaturation at 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Primer Optimization:

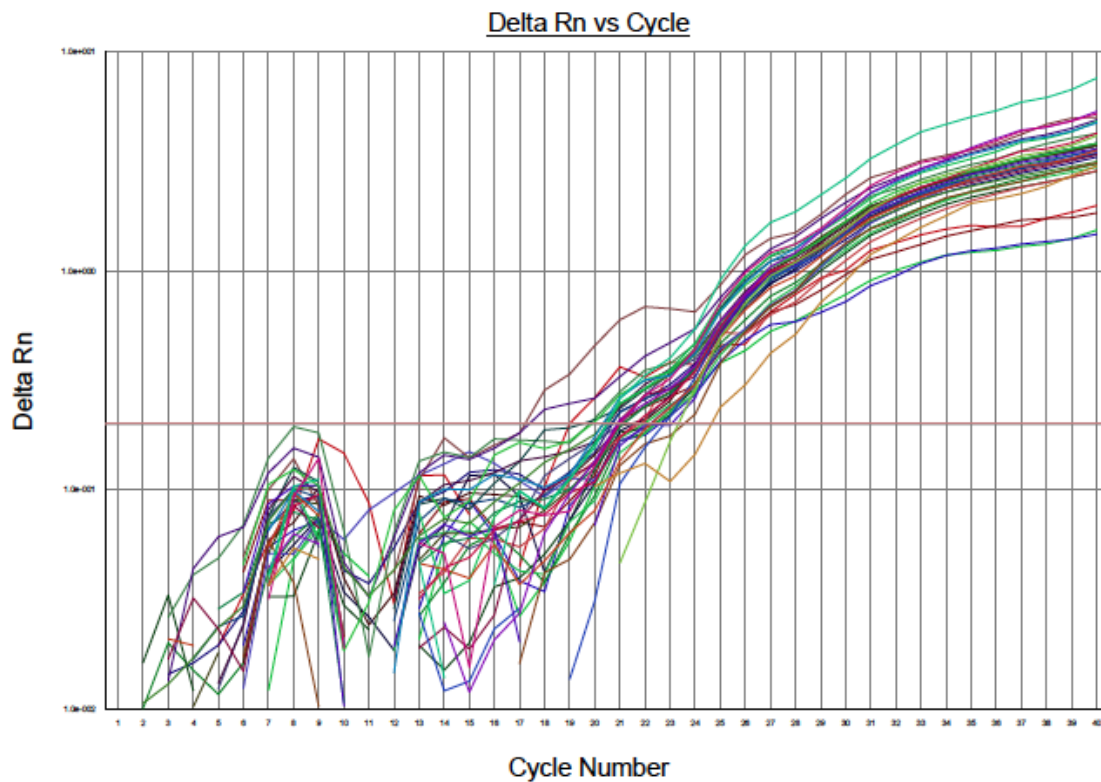
Wells	Universal PCR Mastix mix 2X (uL)	10-uM FAM Probe (uL)	FAM Template Target (uL)	20-uM Forward Primer (uL)	20-uM Reverse Primer (uL)	Deionized Water	Total Volume Per Well (uL)
A1-A4	100	5	20	0.5	0.5	74	200
A5-A8	100	5	20	0.5	3	71.5	200
A9-A12	100	5	20	0.5	9	65.5	200
B1-B4	100	5	20	3	0.5	71.5	200
B5-B8	100	5	20	3	3	69	200
B9-B12	100	5	20	3	9	63	200
C1-C4	100	5	20	9	0.5	65.5	200
C5-C8	100	5	20	9	3	63	200
C9-C12	100	5	20	9	9	57	200

Probe Optimization:

Wells	Universal PCR Mastix mix 2X (uL)	10-uM FAM Probe (uL)	FAM Template Target (uL)	20-uM Forward Primer (uL)	20-uM Reverse Primer (uL)	Deionized Water	Total Volume Per Well (uL)
A1-A4	100	1 (50uM)	20	0.5	0.5	78	200
A5-A8	100	2 (100uM)	20	0.5	0.5	77	200
A9-A12	100	3 (150uM)	20	0.5	0.5	76	200
B1-B4	100	4 (200uM)	20	0.5	0.5	75	200
B5-B8	100	5 (250uM)	20	0.5	0.5	74	200

Table 4.2: The first table shows varying quantities of primer solution in different combinations between the forward and reverse primers. Each combination was tested with four samples (eg. A1-A4), to better assess the outcome of each reaction mix. The most successful reaction according to real-time results was the first combination with the lowest amount of primer solution.

A total of 36 samples with varying parameters were amplified using the Applied Biosystems® 7000 Real-Time PCR System. Results indicated that the optimal quantity was a total volume of 0.5 μ L primer solution and 0.25 μ L probe solution per sample as evidenced by the R_n C_T values (Figure 4.2). These results provided me with standardized PCR parameters to apply for the remainder of ancient DNA amplifications for this project.



Selected Detector: All
Well(s): A1-C12

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Figure 4.2: Results from modern real-time optimization

4.2 Ancient DNA Sample Preparation, Extraction and Purification

Before securing my research position at the Paleo-DNA lab in Thunder Bay, I had to apply for visiting research status, which included a description of academic support, a proposed research plan and a confirmation of financial support. While SSHRC and OGS provided some of the funding, the majority of costs were covered by my personal funds. McMaster is the only other ancient DNA facility in Ontario but it was more difficult to

secure a research position (communication with lab managers and faculty was challenging and estimation of costs were never able to be determined). For that reason, and because a previous mentor at the Simon Fraser University, ancient DNA Laboratory endorsed Lakehead's lab, I was given research status a few months before beginning work.

The duration of actual *in-lab* time for this research was over 250 hours over the course of four months. Stephen Fratpietro (Technical Manager) provided the hands-on training and supervision for the entire project, however, research status requires previous knowledge of the extraction and amplification processes, I had to undertake all of the research myself design and I was responsible for all experiments. All experiments were conducted at the Lakehead University Paleo-DNA Laboratory in Thunder Bay, Ontario, Canada. The laboratory is accredited under the Standards Council of Canada general requirements for the competence of testing and calibration laboratories and the guidelines for the accreditation of forensic testing laboratories. The laboratory also follows strict contamination controls and protocols using sterilized materials, clean suites and double gloves, reverse air ventilation and independent rooms with strict access (floor plan layout in Appendix D).

Bulk samples were chosen from eight different features recovered from the Bingo Late Woodland site in southwestern Ontario. One to two full kernels were chosen for each sample based on positive IDs, available context information and a general visual assessment of morphological preservation (full kernels preferred to fragments).

Identification and selection of samples was conducted at the Museum of Ontario Archaeology. Counts, weights and fluorometer results are provided in Appendix C. At the Lakehead University Paleo-DNA Laboratory samples were then cleaned with ethanol and bleach, and dried and ground with a Qiagen® TissueLyser. The kernels were processed into a fine powder by adding a stainless steel bead (5mm mean diameter) to the sample and centrifuging for 2 minutes at 50 Hz. Samples were transferred via a pass through to the clean lab and treated with a lysis buffer. A number of extraction and purification methods were attempted in order to attain a successful PCR reaction. This included the modified Silica Spin method (Yang et al. 1998; Moore 2011), Ethanol Precipitation, Micro Bio-Spin Chromatography column purification, and Silica bead purification.

4.2.1 Modified Silica Spin Extraction and Purification

A lysis buffer (EDTA 0.5M, pH 8.0; 0.25% SDS concentration of 10%; 0.5 mg/mL Proteinase K enzyme) of 3 μ L was added to each sample. The lysis mix allows for binding of DNA to a silica membrane and removes carbohydrate, polyphenolics and other plant metabolites (Moore 2011; Yang et al. 1998; Kistler 2012). When this buffer was added to samples, the sample tubes were vortexed and centrifuged, then placed in a rotating incubator for 50°C overnight. Another 50 μ L of Proteinase K was added to each sample the following morning and rotated for another 3 hours in the incubator at the same temperature. Following incubation, samples were centrifuged first at 5,000 rpm for 10 minutes and any samples that were not transparent were centrifuged for a second time at the same parameters. Any samples there were still opaque were transferred to 2 ml tubes

and centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully cleaved from the resin at the bottom of the tube. The solution was transferred to Amicon® tubes (Amicon® Ultra-4 Centrifugal Filter Devices) to further concentrate particles. Amicon® samples were centrifuged at 5,000 rpm until the liquid in the column was below 100 µL. 200 µL of PB Buffer was added to Amicon tubes to facilitate transfer of the solution to new tubes for nucleotide removal. Final extraction was conducted using the Qiagen® Qiaquick Nucleotide Removal Kit (Appendix E).

4.2.2 Micro Bio-Spin P-30 Chromatography Column Purification

This purification method was used with the products obtained from the Silica-spin column. P-30 columns were placed in a collection tube and seals broken before centrifuging at 3,400 rpm for 2 minutes. Collection tubes with packaging buffer were discarded and extraction solution was added in the top of the column. Samples were centrifuged for 2 minutes at 3,400 rpm.

4.2.3 Ethanol Precipitation Purification

Ten percent (2.5 µL) of 3M sodium acetate was added to the entire volume of DNA (45 µL) after the silica spin and mixed with 2.5 times the volume (123.75 µL) of cold 100% ethanol. After placing solutions on ice for 30 minutes, they were centrifuged for 5 minutes at 13,000 rpm. The liquid was discarded without disturbing the pellet and 500 µL of cold 95% ethanol was added, mixed (vortex) and centrifuged for 1 minute at 13,000

rpm. The supernatant was once again removed without disturbing the pellet and dried for 30 minutes. Samples were re-suspended in TE Buffer at 37°C for 15 minutes.

4.2.4 Silica Bead Purification

Silica beads are used to bind with DNA while other compounds in the extract are broken down. The beads are suspended in pure water to cleave DNA, resulting in a purer extract. For purification in a 1.5 mL tube, 1 mL of Guanadinium Thiocyanate (GuScN) and 7-15 µL of silica beads (pH of 1 or 2) was added to each sample. After mixing (vortex) for 30 seconds, tubes were placed on ice for 1 hour (some overnight). Samples were subsequently centrifuged for 8 seconds at 10,000 rpm and the supernatant was carefully discarded. 1000 µL of wash buffer (similar to ethanol) was re-suspended in the silica beads by mixing (vortex) for 1 minute. This step was repeated 2-3 times depending on the color of samples. Typically, if liquid was dark we added more wash buffer. Following the wash 200-250 µL of ethanol (100%) was added and mixed with beads for 1 minute then spun down for easy removal of the supernatant. This step was once again repeated if the liquid was dark. Pellets were air dried in a speed-vac for 5 minutes. Water was added (50-250 µL) and samples were incubated at 56°C in a thermomixer for 1 hour to cleave DNA from beads. Liquid was removed and silica beads discarded.

4.3 Assessment of DNA Recovery

Primers were chosen based on Moore's (2011) successful DNA extractions from artificially degraded *Zea mays*. The purpose for using chloroplast *rbcL* markers is

discussed at length in Chapter 3. Moore designed universal primers based on *rbcL* reference sequences from *Zea mays* (NC001666.2, Z11973.1), *Pisum sativum* (NC014057.1, X03853.1), and *Cucurbita pepo* (AF206756.1, L219358.1) found on Genbank. These primers (Table 4.3) were used throughout the project for standard and real-time PCR as well as for sequencing.

Primer/Probe	Position	Sequence (5' to 3')	Region	Amplicon Length
F17 (universal)*	56917- 56942	GCTGGTGTTAARGATTATAAATTGAC	<i>rbcL</i>	166 bp
R183 (universal)*	57063- 57083	CAMACAGTTGTCCATGTACCA	<i>rbcL</i>	
<i>rbcL</i> _P2 Probe	56973- 56999	TACTGATATCTTGGCAGCATTCCGAG	<i>rbcL</i>	

Table 4.3: Primers and probe for amplification modified from Moore 2011:42.

4.3.1 PCR and Gel Visualization

Quantitative PCR works on the premise that the fewer cycles it takes to reach a detectable level of fluorescence the greater the initial copy number of the target nucleic acid.

Fluorescent levels are displayed in Chapter 5. PCR parameters were based on modern optimization, which called for the following 200 μ L concentrations: 100 μ L of Universal PCR Master Mix 2X, 5 μ L of probe (10- μ M FAM), 0.5 μ L of F17 (forward primer 20- μ M), 0.5 μ L of R183 (reverse primer 20- μ M) and adjusted amounts of deionized water (when using blanks and internal controls). The universal mix consisted of the following (per reaction): 5 μ L of PCR buffer, 1 μ L of dNTP mix (10mM), 2 μ L of magnesium, 0.2 μ L of DNA polymerase, and for standard PCR (see below) 2.5 μ L of bovine serum albumin (BSA). Over the course of this research varying combinations of AmpliTaq

Gold® and Platinum® Taq were used and produced very different results (Chapter 5). Every reaction contained blanks and positive controls. Standard PCR was used to visualize potentially positive results as indicated by the qPCR. Amplification parameters remain the same as real-time save for BSA additive where only Platinum® Taq was used. Products were visualized using polyacrylamide gel electrophoresis with a gel staining concentration of 1xTBE Buffer 3µL of 5x loading dye and 5µL of sample.

4.4 Sequencing, Alignment and Reproducibility

4.4.1 Sequencing

Potentially positive samples and those that produced a band on the polyacrylamide gel were sequenced at the Paleo-DNA laboratory. PCR products were first cleaned with ExoSAP-IT® before sequencing to get rid of unincorporated nucleotides and unused primers. The enzymatic solution consisted of 2 µL of ExoSAP-IT® for every 5 µL of post-PCR reaction product. The solution was incubated at 37°C for 15 minutes and then 80°C for 15 minutes. For sequencing PCR product consisted of 7 µL ExoSAP-IT and DNA product and 5 µL of deionized water. Reaction parameters in the C1000 Thermal Cycler included 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes for 45 cycles. In order to remove any unincorporated dye terminators that may affect the sequencing read a Qiagen® DyeEx™ spin column kit was used. Samples were cleaned according to the protocol (Appendix F) and prepared for sequencing in the ABI 3130x1 Genetic Analyzer.

4.4.2 Alignment

The ABI™ (Applied Biosystems) output was identified using the Standard Nucleotide BLAST® and then uploaded into the Molecular Evolutionary Genetics Analysis 4 (MEGA 5.1) Software. Sample nucleotides were aligned using the ClustalW pairwise and multiple alignment with a 15 base adjustment. Sequences were aligned with other species that showed a high maximum identity including: *Zea mays B73*, *Zea mays 6*, *Zea perennis*, *Zea diploens*, *Zea parviglumis*, *Sorghum bicolor*, *Triticum aestivum*, *Vetiveria zizanioides*, *Panicum amarum*, *Coix lacryma* and *Setaria italica*. Homoplasy was evaluated based on 150 bp alignment (from universal primers F17 and R183), *Cucurbita pepo* and *Pisum sativum* sequences from Genbank were used to identify outgroups. All positive samples in this thesis were reproduced according to protocol at the Paleo-DNA Laboratory.

Chapter 5

5 Results

This chapter reports on the results of the failed and successful amplifications from both modern optimization and ancient samples. Modern optimization refers to the modern maize extraction and quantitative PCR (qPCR) amplification, which was conducted in order to find, first, the best PCR parameters; how many μL of probe/primer to use in the ancient PCR amplification and second, to use as a control during the ancient experiments. qPCR was also used for ancient DNA amplification in order to authenticate results and help visualize DNA copy numbers. The rate of success for modern experiments are based on the curves obtained and detected from the qPCR output and results are displayed below. Troubleshooting negative ancient qPCR results is what allowed for the testing of a number of extraction methods. Once a sufficient quantified amplification was measured, results were replicated with standard PCR, visualized with gel electrophoresis, and sequenced. The results are reviewed below, as well as the cutting, alignment and tree-building of positive sequences.

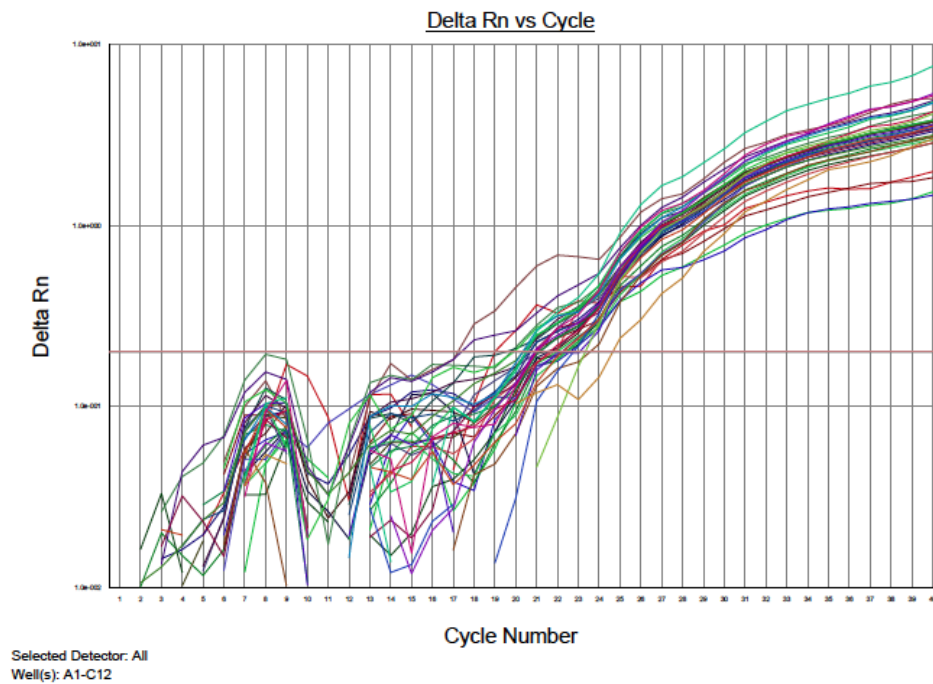
5.1 DNA Recovery

5.1.1 Optimization Results

As outlined in Chapter 4, the optimization of PCR parameters was evaluated using modern maize DNA. In order to find optimal parameters, various concentrations of maize, probe, primer and water were used during amplification to test which combinations produced the best results. Standard PCR will detect positive results by presence/absence, whereby qPCR detects how many times the DNA copies – this is

translated into C_T values that are explained in Chapter 4. According to Figure 5.1 and Table 5.1 the most successful reaction for primer/probe concentrations were from samples A1-A4. The results indicated that minimum concentrations for probe, primer and water yielded the maximum normalized reporter (R_n) and the minimum threshold cycle (C_T), In other words, samples A1-A4 returned the highest C_T values for the primer test and the most consistent results for the probe test. Although the results for the probe test were more variable we decided qualitatively to use the same concentrations as the primers to maintain more consistent volumes. For the remainder of this research (on all ancient samples) these parameters were used (0.5 μ L of forward and reverse primer and 1 μ L (50 μ M) of probe).

A)



B)

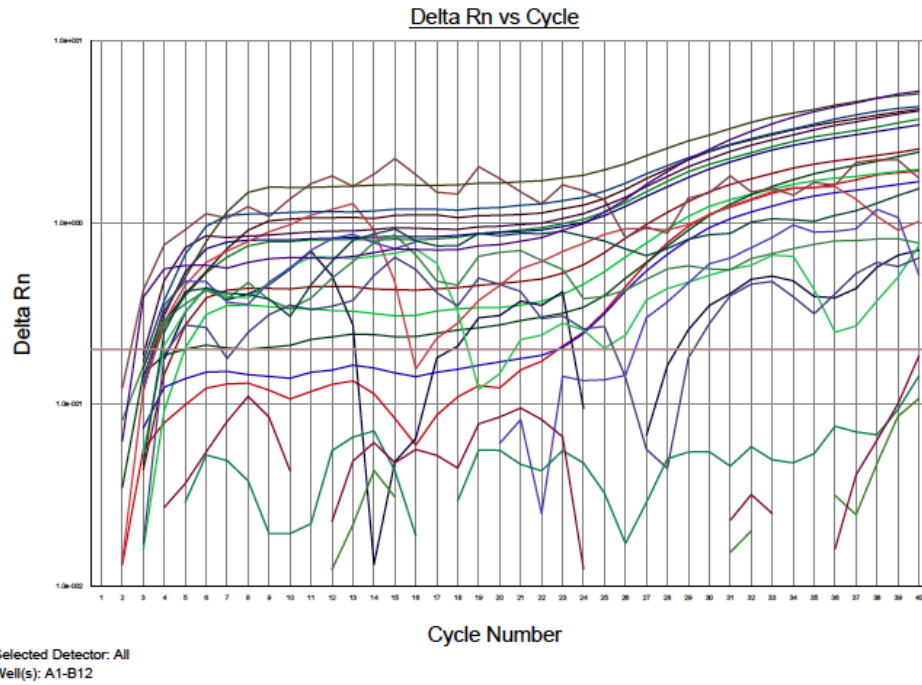


Figure 5.1: Absolute quantification of modern maize A) primer and B) probe optimization.

A)

Well	Sample Name	Detector	Task	Ct
A1		C Armstrong	Unknown	24.32
A2		C Armstrong	Unknown	24.54
A3		C Armstrong	Unknown	24.27
A4		C Armstrong	Unknown	24.22
A5		C Armstrong	Unknown	23.06
A6		C Armstrong	Unknown	23.82
A7		C Armstrong	Unknown	23.86
A8		C Armstrong	Unknown	23.01
A9		C Armstrong	Unknown	23.84
A10		C Armstrong	Unknown	22.53
A11		C Armstrong	Unknown	24.19
A12		C Armstrong	Unknown	25.43
B1		C Armstrong	Unknown	19.49
B2		C Armstrong	Unknown	23.15
B3		C Armstrong	Unknown	21.72
B4		C Armstrong	Unknown	22.67
B5		C Armstrong	Unknown	22.77
B6		C Armstrong	Unknown	22.52
B7		C Armstrong	Unknown	23.43
B8		C Armstrong	Unknown	21.51
B9		C Armstrong	Unknown	22.77
B10		C Armstrong	Unknown	22.31
B11		C Armstrong	Unknown	20.91
B12		C Armstrong	Unknown	21.51
C1		C Armstrong	Unknown	23.05
C2		C Armstrong	Unknown	24.34
C3		C Armstrong	Unknown	23.30
C4		C Armstrong	Unknown	21.42
C5		C Armstrong	Unknown	23.25
C6		C Armstrong	Unknown	22.87
C7		C Armstrong	Unknown	23.11
C8		C Armstrong	Unknown	20.98
C9		C Armstrong	Unknown	22.15
C10		C Armstrong	Unknown	20.25
C11		C Armstrong	Unknown	22.46
C12		C Armstrong	Unknown	23.56

B)

Well	Sample Name	Detector	Task	Ct
A1		C Armstrong	Unknown	25.23
A2		C Armstrong	Unknown	27.30
A3		C Armstrong	Unknown	25.89
A4		C Armstrong	Unknown	27.34
A5		C Armstrong	Unknown	28.96
A6		C Armstrong	Unknown	29.36
A7		C Armstrong	Unknown	31.97
A8		C Armstrong	Unknown	26.20
A9		C Armstrong	Unknown	38.29
A10		C Armstrong	Unknown	10.60
A11		C Armstrong	Unknown	Undet.
A12		C Armstrong	Unknown	Undet.
B1		C Armstrong	Unknown	Undet.
B2		C Armstrong	Unknown	Undet.
B3		C Armstrong	Unknown	Undet.
B4		C Armstrong	Unknown	Undet.
B5		C Armstrong	Unknown	27.95
B6		C Armstrong	Unknown	6.97
B7		C Armstrong	Unknown	30.91
B8		C Armstrong	Unknown	24.62
B9		C Armstrong	Unknown	Undet.
B10		C Armstrong	Unknown	Undet.
B11		C Armstrong	Unknown	Undet.
B12		C Armstrong	Unknown	Undet.

Table 5.1: Absolute quantification of modern maize **A)** primer and **B)** probe optimization, C_T detections (see Chapter 4, Figure 4.2 for plate configurations that correspond to table.)

5.1.2 Inhibition Testing

Quantification of the starting copy number is necessary for ancient DNA research in order to authenticate results. If there is an abnormally large starting copy number of DNA in a sample it can be assumed that contamination of modern DNA is present. In total, 11 qPCR's were run throughout this research. False results (no detection) meant that 1) DNA was too degraded and undetectable, or 2) there was too much inhibition, meaning samples were perhaps too dark (not purified enough) and blocked the potential for the reaction necessary for amplification. In other words, the polymerase chain reaction relies on thermal conductivity that allows DNA strands to open and close. While DNA strands are open (think of the double helix unraveling), the Taq will help bind primers to the selected strand of DNA. However if there are too many inhibitors (compounds that did

not take to purification) primers will not bind to DNA and therefore will not amplify. Because DNA, primers and other desired compounds in a purified solution are clear, any colour in the samples mean that it may be inhibited – in other words the sample is too dirty.

False results in the first scenario (if DNA is too degraded) are not uncommon with ancient DNA, so when the first amplification of ancient DNA produced false results (Figure 5.2), a sub-set of ancient samples were spiked with modern samples (in-vitro positive control). The null hypothesis for the second amplification (Figure 5.3) – H_0 = no modern DNA will be detected – was then tested. That meant: H_1 = if modern DNA is detected, there is no inhibition in the ancient sample (the ancient sample may be clean) and therefore there is simply no DNA in the ancient samples (at which point our research would be over), or: H_2 = modern DNA is not detected therefore ancient samples inhibited modern DNA.

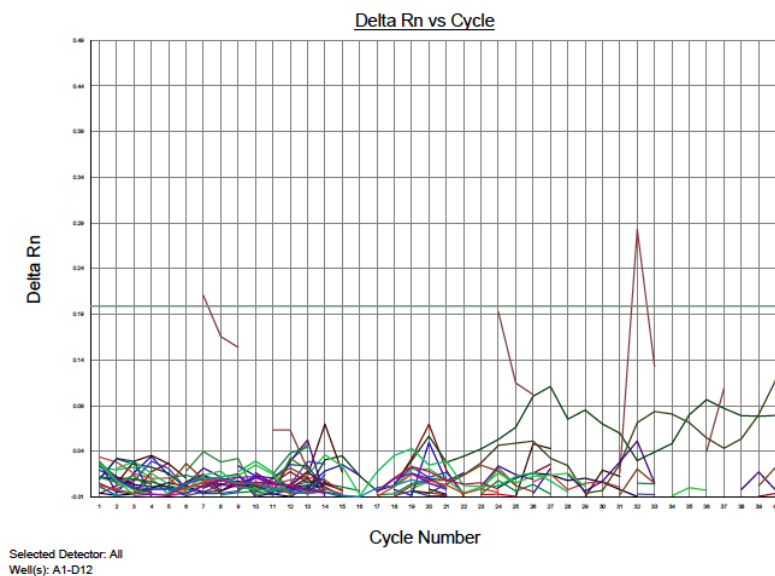


Figure 5.2: Results from first amplification of ancient charred maize. Note: samples do not even reach minimum Delta R_n (horizontal green line).

Figure 5.3 shows that the first scenario (H1) can be rejected because no modern DNA was detected. This result means the initial extraction and purification were not sufficient in isolating ancient DNA. This is not surprising given the extremely dark solution that was left after the first round of extractions (Figure 5.4). As a result of our findings from the second amplification, then, troubleshooting during the purification process was qualitatively steered by a need to reduce the dark colour from samples, in other words, purifying the sample to remove inhibitor compounds.

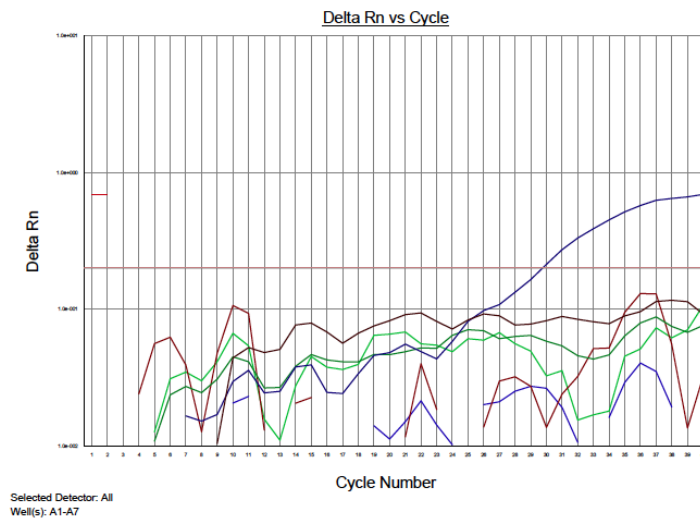


Figure 5.3: Second ancient amplification showing no detections (lines should look like the blue normalized curve – in this Figure the blue line is a positive control (not a spike).

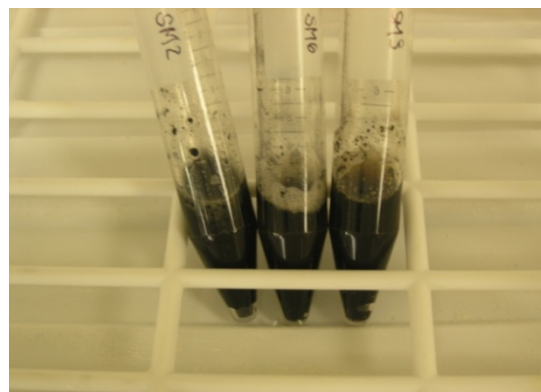


Figure 5.4: Example of samples before they were placed in Amicon tubes during the modified silica spin extraction

For the first two rounds of amplification a total of 30 samples were run. All samples were purified using the Modified Silica Spin Extraction and Purification process (Yang et al 1998). Unfortunately samples remained very dark even after additional centrifuging in both the 2 mL and Amicon® tubes. I then decided to further purify samples by using an ethanol-based precipitation (as reviewed in Chapter 4), which proved effective at eliminating some of the darker colour (potential inhibition) from the samples. Although additional noise was detected in the results, the output produced negative results (Figure 5.5). Moving on from an ethanol-based precipitation, I then used the Platinum® Taq DNA Polymerase and bovine serum albumin (BSA), which is meant to stabilize enzymes during DNA digestion (previously we were using AmpliTaq Gold™ that had been called for in Moore's [2011] qPCR amplifications). Using the Platinum® Taq produced positive results. While the potential for false-positives remained, for the first time in the amplification sequences ancient samples assembled in a normalized curve (Figure 5.6).

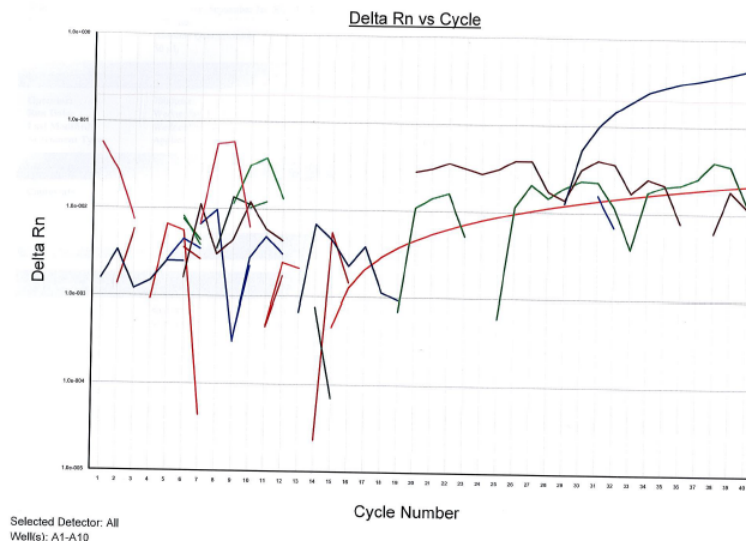


Figure 5.5: Results from third amplification using modified silica spin and ethanol precipitation.

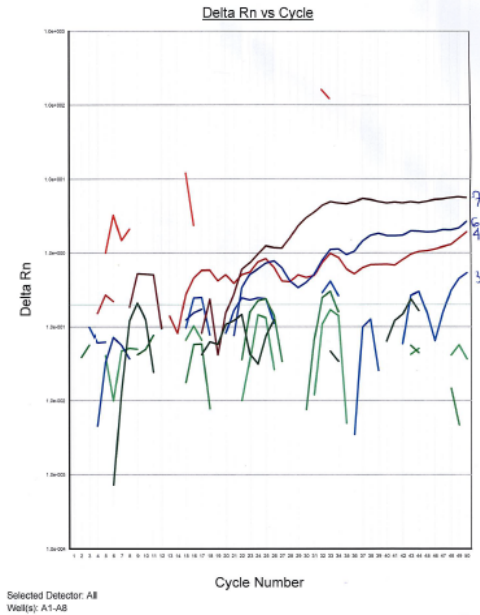


Figure 5.6: Results of fourth amplification using modified silica spin and ethanol precipitation purification and substituting AmpliTaq Gold™ with Platinum® Taq DNA and BSA.

Subsequently I experimented with and adjusted a number of purification methods, including silica bead purification, Micro Bio-Spin P-30 Chromatography Column Purification, as well as modifying the ethanol precipitation and modified silica spin methods. I focused modifications towards improving qualitative perceptions of colour. If I thought a sample was too dark I would add an extra step. For example during silica bead purification, I would add 500 μL of cold 95% ethanol to samples, mix (vortex) and centrifuge them for 1 minute at 13,000 rpm. Before and after removing the liquid, if I noticed the colour was still too dark I would repeat the step again. Figure 5.7 shows the kind of colour variation each sample produced. This process was also mitigated by the potential for *too much* purification, wherein breaking down impurities in samples can simultaneously breakdown any DNA also present.

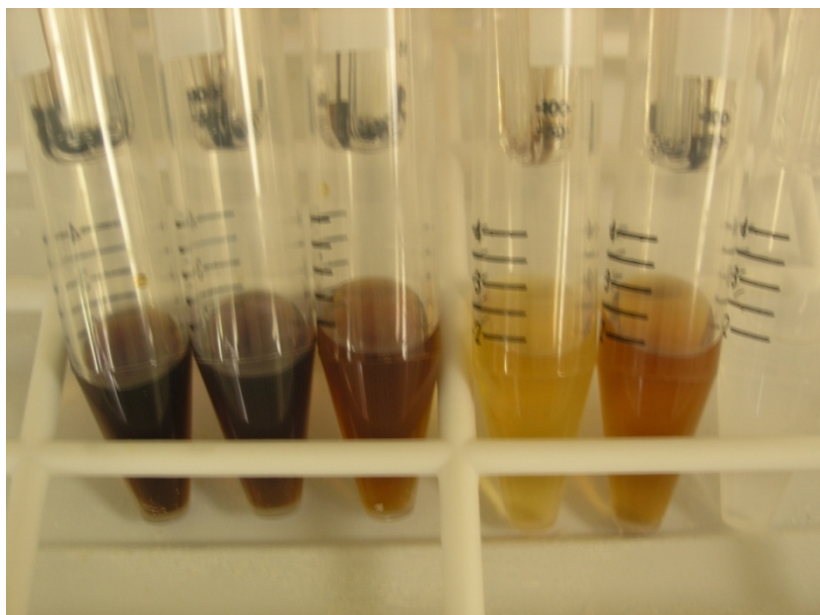


Figure 5.7: Variation of colour during purification process (intuitively, the darker the colour the less likely a positive result would be obtained).

The uncertainty of inhibition versus over-purifying resulted in an abundant number of ‘trials and errors’ (i.e. running a qPCR almost every day for weeks at a time). The best results are displayed in Figure 5.8. These results used the following (and most successful) extraction and purification protocol which was essentially a combination of the modified silica-spin and ethanol precipitation (see Chapter 4), according to Yang et al. (1998), and a lysis buffer (EDTA 0.5M, pH 8.0; 0.25% SDS concentration of 10%; 0.5 mg/mL Proteinase K enzyme) of 3 μ L was added to each sample. Tubes were vortexed and centrifuged then placed in a rotating incubator for 50°C overnight. Another 50 μ L of Proteinase K was added to each sample the following morning and rotated for another 3 hours in the incubator at the same temperature. Following incubation, samples were centrifuged first at 5,000 rpm for 10 minutes and any samples that were not transparent were centrifuged for a second time at the same parameters. Any samples that were still

opaque were transferred to 2 ml tubes and centrifuged at 14,000 rpm for 10 minutes. The supernatant was carefully cleaved from the resin at the bottom of the tube. Ten percent (2.5 μ L) of 3M of sodium acetate was added to the entire volume of solution (45 μ L) and mixed with 2.5 times the volume (123.75 μ L) of cold 100% ethanol. After placing solutions on ice for 30 minutes, they were centrifuged for 5 minutes at 13,000 rpm. The liquid was discarded without disturbing the pellet and 500 μ L of cold 95% ethanol was added, mixed (vortex) and centrifuged for 1 minute at 13,000 rpm. The supernatant was once again removed without disturbing the pellet and dried for 30 minutes. Samples were re-suspended in TE Buffer at 37°C for 15 minutes.

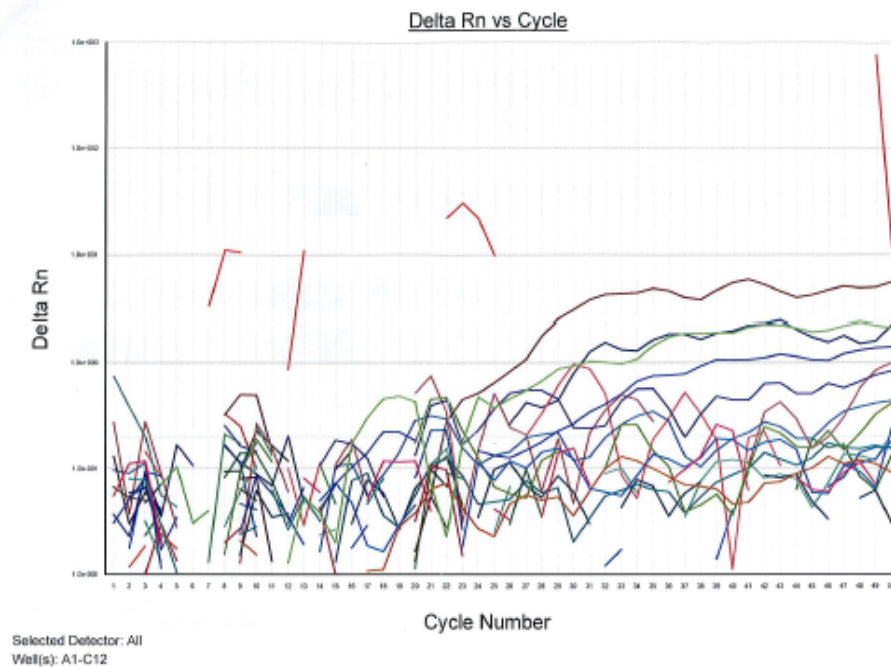


Figure 5.8. Results of a combination of the modified silica spin (without Amicon tubes) and ethanol precipitation.

5.1.3. Standard PCR and Gel Visualization

A standard PCR was conducted for the 13 samples that showed some potential during qPCR. The parameters are described in Chapter 4. Figure 5.9 shows the gel visualization which indicated that in total, four samples produced a band, demonstrating presence of DNA. In order to confirm that these samples were yielding DNA related to ancient maize, the sample bands were subjected to sequencing to understand their relationship to various plant DNA profiles (protocol for sequencing is also reviewed in Chapter 4).

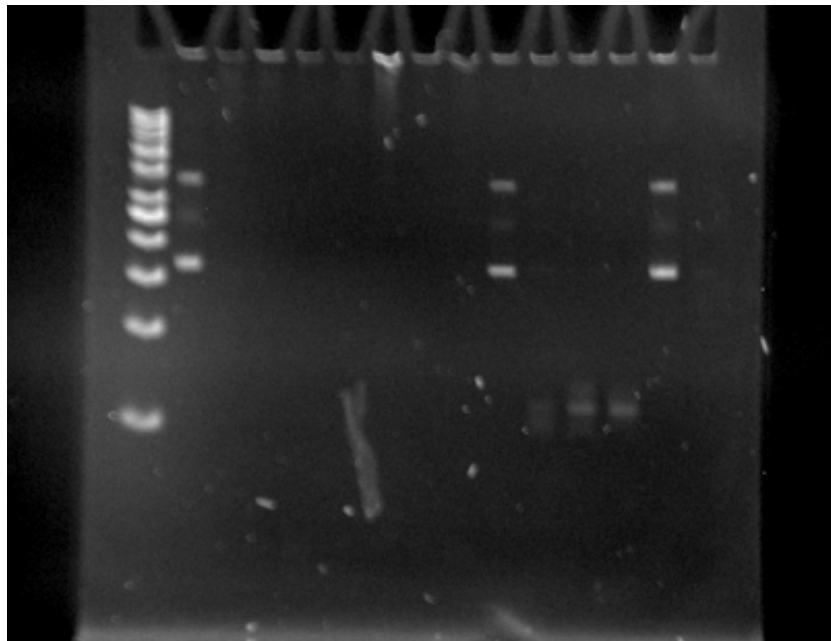
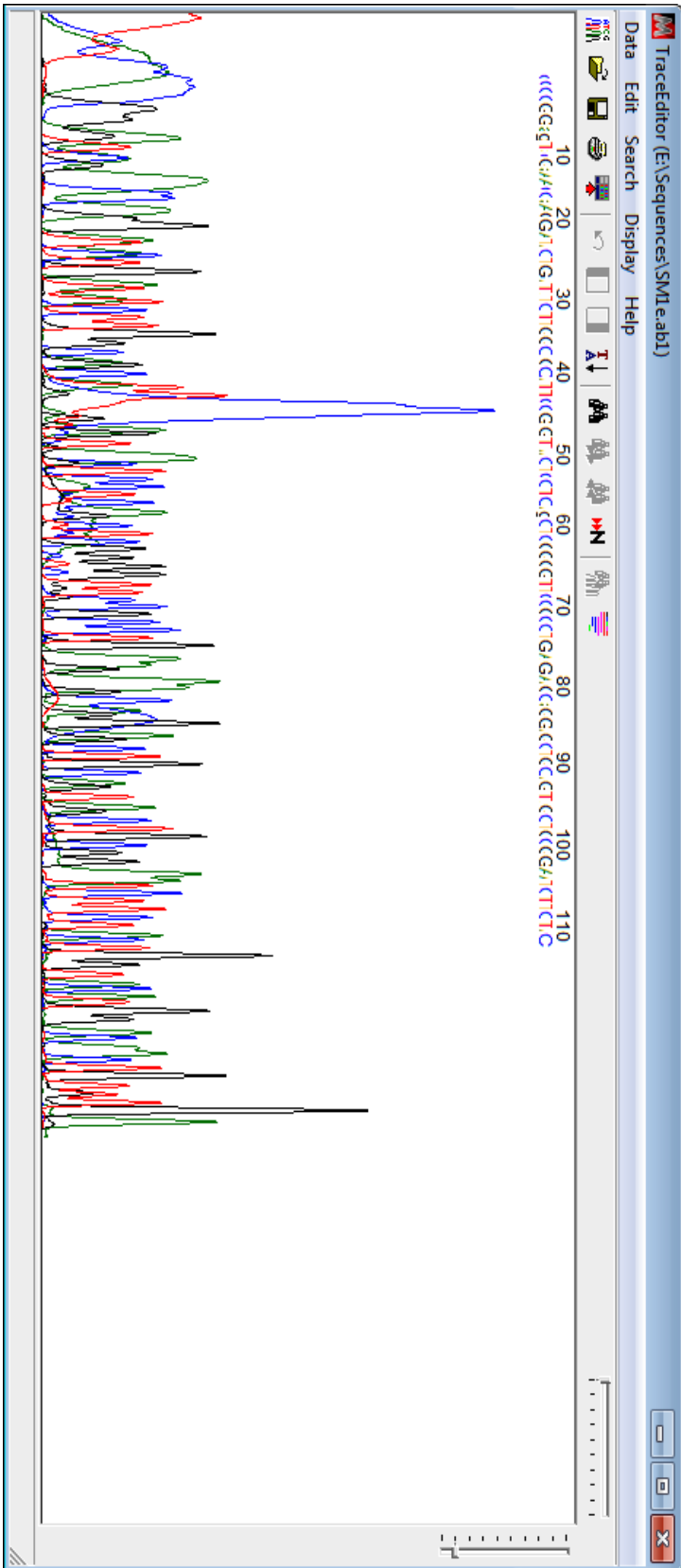


Figure 5.9: Standard PCR results visualized on a Gel with O'GeneRuler 50bp ladder (50bp, 100bp, 150bp, etc). Samples with clear and potential bands were sequenced.

5.2 DNA Alignment and Phylogenetic Reconstruction

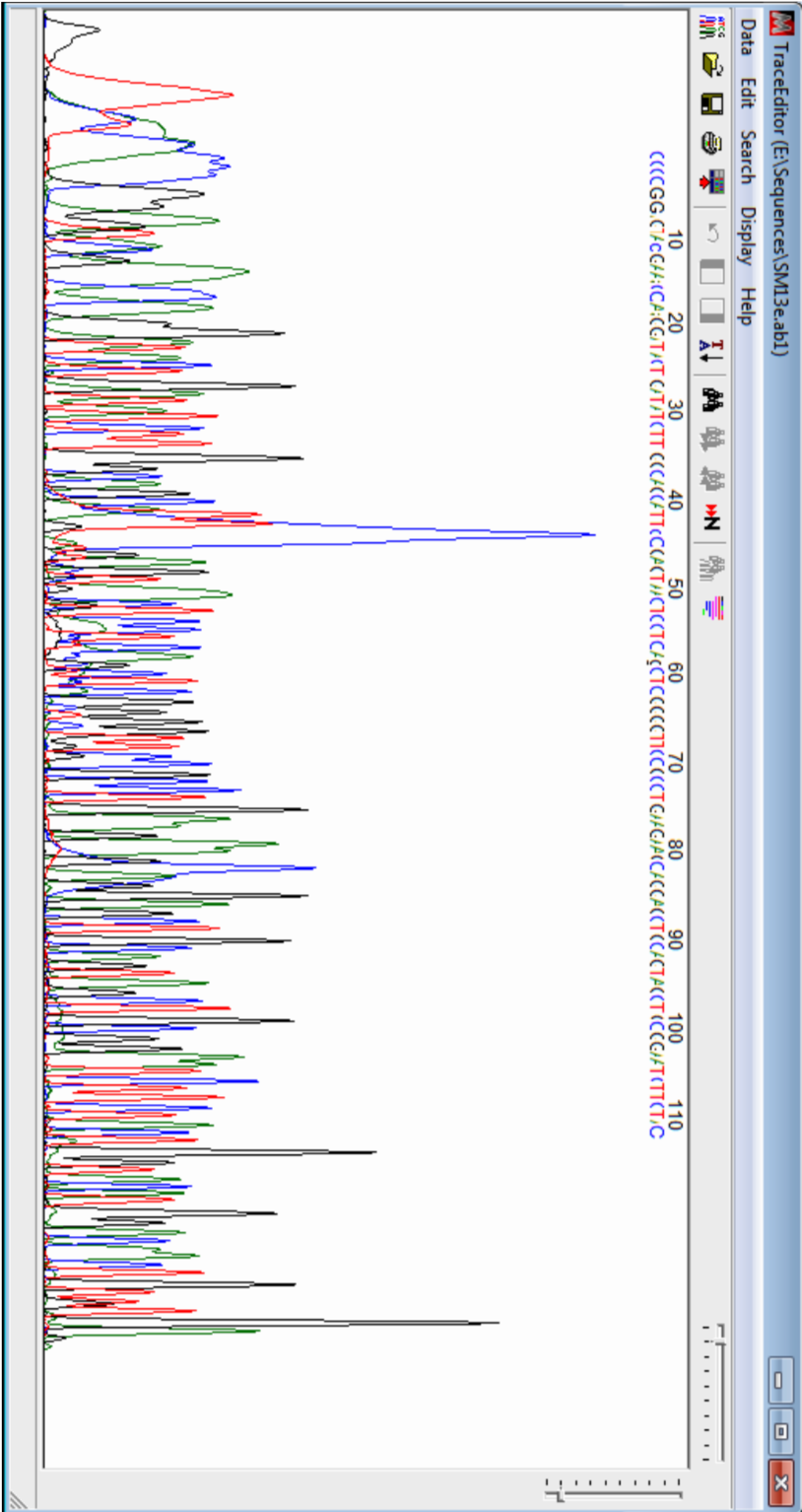
In total 4 samples were sequenced, and a Basic Local Alignment Search Tool BLAST® (NCBI) assembled (compared) the sequences that I generated in the lab, to millions of

sequences on Genbank (internet database). This process allows us to identify from which family, species, genus or variety our samples are most closely related. The BLAST® search showed that 3 of our samples returned at least 98% identity with dozens of species from the *Poaceae* (Grasses) family, specifically domesticated versions of *Zea*. I exported those closely related sequences (random selection of the 98% identity grasses) from Genbank to the MEGA 5.1 alignment program to test for homology. Sequence alignment allows the user to play with sequences, compare them with Genbank sequences, clean them up (edit ambiguous nucleotides and shave primer endings) and construct trees and other visual representations. To compare my samples with other species, I chose sequences from Genbank by using BLAST® searches with options for “teosinte”, “maize” or “corn” and these were imported into MEGA with my other samples. Sequences were cut and trimmed by deleting primers and deciphering nucleotide ambiguity based on the *Zea mays* genome sequence and the original electropherograms from the sequencing output (Figure 5.10). A Muscle Sequence Alignment (multiple sequence comparison by log-expectation) was used with default parameters (parameters are most parsimonious framework) . After trimming we were able to identify 112 nucleotide positions excluding primers.



(A)

(B)



Phylogenies were constructed to show relational placement of samples, for example how close ancient maize samples aligned with other grasses genes on Genbank. The primers used for this research were *universal to plants* so phylogenies are not about diversity or species reconstruction but rather, they are a visual representation of sequences with out groups. Therefore the only thing these trees show is that sequences I generated from charred ancient maize are grasses closely aligned to maize. All phylogenies were constructed by myself and Dr. Marc-André Lachance from the Biology department at the University of Western Ontario. The evolutionary history (again, where grasses split from other species) was inferred and visualized in three ways: 1) using a maximum parsimony analysis of taxa method tree; 2) a neighbor-joining tree and; 3) a maximum likelihood tree. These three trees are statistical methods most commonly applied when looking at related species and homologies of sequences in MEGA. This first tree (Figure 5.11) is constructed out of the 10 most parsimonious trees (length= 21). The scale bar is the number of substitution sequences it takes to get to *C. pepo* and *P. vulgaris* or how any positions in the sequence it takes for squash and bean to diverge from grasses. In other words, how many nucleotides need to be changed to get from a grass to squash/bean (grasses are more similar to each other than to bean or squash). *Curcubita* and *Phaseolus* were used because they are also new world domesticates and had readily available *rbcL* genes on Genbank. Similarly, the scale for the neighbor-joining tree (Figure 5.12), a popular algorithmic version of the minimum evolution parsimony principle, shows the same output. Lastly, the maximum likelihood tree (Figure 5.13), which reconstructs character branches and branch lengths on complex models, takes into account the probability of various nucleotide changes and confirms the 2%

shift that matches the two previous trees. None of the trees show that *Zea parviglumis* had some variation in the alignment but these are likely to be polymorphisms within the wild taxa. Phylogenies confirm that DNA isolated, amplified and sequenced from ancient maize in the Paleo-DNA laboratory is related most closely to modern specimens in the Poaceae (Grass) family. As BLAST results show, they are most closely related to domesticated maize varieties.

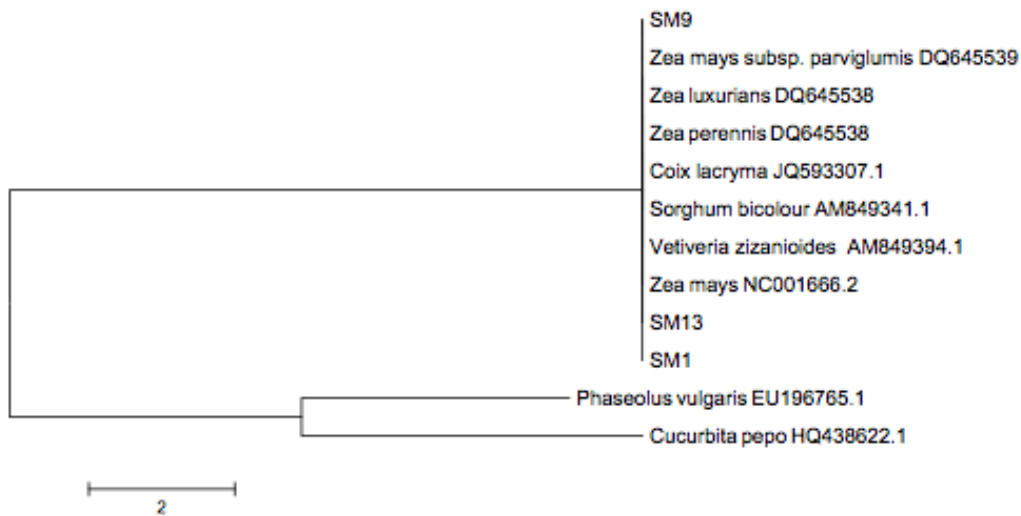


Figure 5.11: Maximum parsimony tree of Bingo village samples, relatives and outgroup.

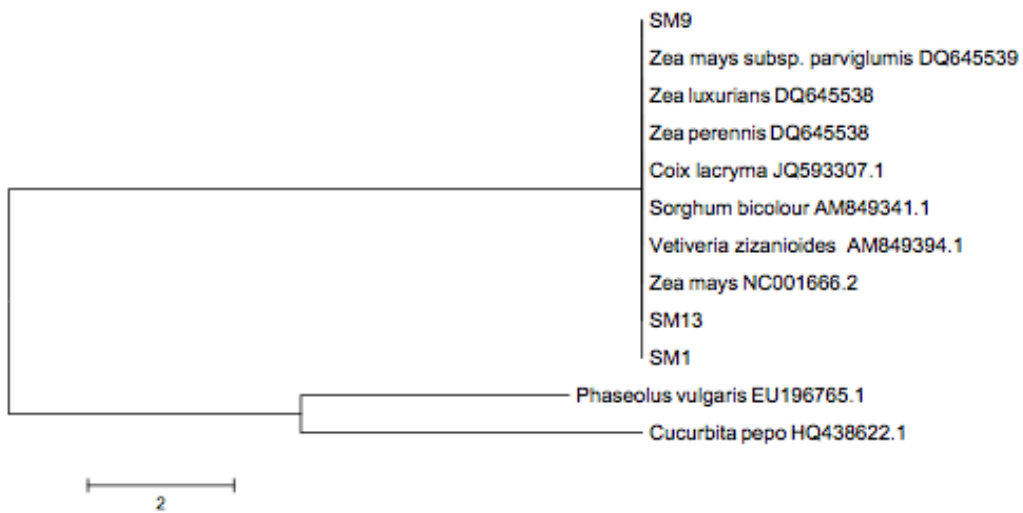


Figure 5.12: Neighbor joining tree of Bingo village samples, relatives and outgroup.

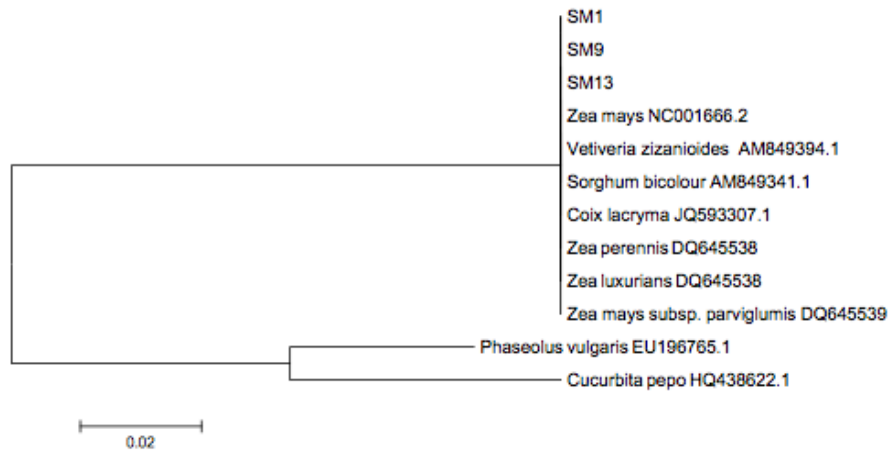


Figure 5.13: Maximum likelihood tree of Bingo village samples, relatives and outgroup.

5.3. Conclusion

Despite clear researcher expectations that this project would not be successful, and despite a number of challenges that had to be worked out in the lab in order to achieve success, in the end three samples (SM13 a modern control) out of twenty-one samples of charred maize kernels from subterranean pits in Bingo Village returned positive DNA results. Although not all samples could be purified, the two samples (SM1 and SM9) with positive results had almost transparent liquid. One hypothesis is that samples without clear liquid will not return results because there are too many compounds inhibiting amplification (we were not able to purify them enough). The most effective method of purification in this particular research was a variation of the modified silica-spin column with an ethanol precipitation. The caveat is that not all samples will take to purification in the same capacity and that multiple washes and repeated steps may be required for optimal results. That positive DNA was recovered from this very limited study and

sample clearly underscores the significance and importance of paleoethnobotanical remains for research, beyond simple macro-identifications. The implications of this important discovery are reviewed in the final chapter of this thesis.

Chapter 6

6 Discussion

*“Nature speaks in many tongues and they are all alien.
What a scientist tries to do is decipher the many dialects.”*
— Slightly modified from Dudley Herschbach (Harvard),
quoted in Pierotti (2011:65).

Over the course of this research I consulted with other Paleoethnobotanists who assured me it would be impossible to garner DNA from charred specimens. Most geneticists I talked to – and including Stephen Fratpietro at the Paleo-DNA lab- were more optimistic but assured me it would take a lot of work and time. Despite large assumptions that it was not possible, and despite the often-unrefined CRM practices of recovery and storage, I have demonstrated that with careful troubleshooting and qualitative analysis of material – aDNA can be recovered from archaeologically charred maize. Although samples in this research were identified to the species level, it is possible to imagine a world where archaeologists enter the field with the intent to recover plant materials for aDNA analysis. In this scenario research questions could lend themselves to larger anthropological thought and materials could be recovered and stored in a way that prevents further deterioration of important molecular information. I now want to situate this research in the context of contemporary debates in the social and hard sciences, mainly how and why certain kinds of knowledge are valued. By generating ‘more’ information from burnt kernels than was previously thought possible, I am naturally inclined to think about what this research actually means. The broader implications for this study are discussed below.

6.1 Contributions of Ancient DNA

6.1.1 *Combining Biological and Archaeological Approaches*

Here I consider the usefulness of converging disciplines, particularly combining the ‘hard’ sciences with archaeology. Archaeology is inherently multi-disciplinary because it is essentially, the history of *everything*. It should not be surprising, based on other areas where bio-chemical and archaeological approaches have converged (C14 dating, Isotope analysis), that the benefits of a collaboration between archaeologists and microbiologists are many. Smith (2001) summarizes the very basic intercession of genetics and archaeology in addressing questions of when, where and from what progenitor populations of domesticates appeared (Smith 2001). Schlumbaum et al. (2008) and Brown (1999) provide extensive overviews on the kinds of prospects and limitations that ensue when applying scientific methodologies to archaeological data.

To many, better research means combining and collaborating horizontally. For example, Jaenicke-Després and Smith (2006:84) have outlined the “4 cells” or sets of approaches to studying the history of maize, which includes: morphology-modern; morphology-ancient; genetics-modern; and genetics-ancient. Non-genetic studies of maize can consider the size and morphology of starch granules, phytoliths and pollen (not only kernels and cobs), while modern genetics can identify genes artificially selected and ancient genetics can test hypotheses posed by modern genetics and give more regional contexts for a species change over time. Archaeological contexts and records are useful for holistic interpretations of a settlement or cultural lifeways. For example, Hard et al.

(1996) correlated the stable isotope ratios and macrobotanical evidence of maize production with the growth in *mano* size to an increase in maize production in six different regions of the American Southwest. A number of studies in Staller et al.'s (2006) extensive edition on the multidisciplinary history of maize are an excellent resource for exploring the importance of combining methodological approaches. Below are brief examples of how some methodologies and collaborations between disciplines have serviced archaeology and biochemistry, agriculture and resource management and Indigenous Research to help answer the question "why bother with aDNA".

6.1.2 *Identifications*

One of the very obvious benefits to molecular bioarchaeological analyses is the identification of plants remains, which have undergone enough morphological changes to render the plant otherwise unidentifiable. The techniques used in modern plant identification are quite different than those used in the paleo-genetic approach. Some of these techniques, like hybridization-based methods such as restriction fragment length polymorphism (RFLP), have been extensively studied in chloroplast DNA. This method was further developed into marker-building techniques like cleaved amplified polymorphic sequences or CAPS which defines a DNA sequence with sequence specific primer, the product is digested with restriction enzyme which may or may not show polymorphisms on gels. These methods are useful but testing archaeological specimens can be challenging because of the highly degraded nature of DNA or maybe the questionable contexts in which plants were found.

Paleoethnobotanical remains are retrieved in a variety of conditions that have been affected by both environmental and cultural factors. Chapter 3 reviewed the many states in which we find archaeobotanical materials; below I look into the use of aDNA analyses to examine those materials, particularly when trying to identify specimens. In the past, paleoethnobotanical analyses relied entirely on visually identified morphological analyses, however identifications of this fashion can have its limitations (Schlumbaum et al. 2008). Local varieties of plants or environmental changes to plants over time may have meant that archaeological samples may not correlate to reference collections. Some herbariums have ‘untouched’ seeds – ones that have not been artificially charred and therefore may not resemble charred plants found archaeologically. The archetypical figure for some seeds will almost never have a one-to-one correlation with samples retrieved from sites. While seasoned paleoethnobotanists are certainly able to identify morphological variations in many botanical remains, identification of wild plants, especially grasses or carbonized remains, can be difficult to score. As Dezendorf has shown through alkali processing, cultural practices have an extreme effect on changing kernel size. Her experimental work observed an increase in kernel thickness from dried to alkali-processed-carbonization, ranging from 21.64% growth in the Anasazi flour variety and 71.38% growth after treatment for Hickory King varieties. This renders identification of processed remains challenging and ambiguous. Other cultural processes can alter plants to versions that we would never be able to identify using typical keys or references. Baskets, tools, cooking implements, clothing and ritual or cosmetic items may have gone through transformations that render identification impossible. Nonetheless these items still have the potential to be identified to the species level (sometimes variety)

through ancient DNA analyses. Morphological identifications are not rendered useless in this light however; by combining both morphological and ancient DNA analyses Pollman et al. (2005) looked at the increase in diversity of cultivated fruits in Roman Europe. Both chloroplast and nuclear DNA were used to construct an almost complete domestication history for varying waterlogged *Prunus* fruit stones. Ancient DNA should not replace current paleoethnobotanical methodologies, but enhance them.

6.1.3 Development of New Plant Varieties

Plant breeding is essential for the maintenance of world food supply (Henry 1997:101). Typical gene traits observed in phenotypic variation such as endosperm content in lipids, proteins and starch quality (many of the traits selected for by early farmers) are more often studied, with little attention paid to the alleles associated with *natural* variation among cultivated maize (Manicacci et al. 2009). Certainly ancient maize genes (not only those selected by early farmers) can play a role in maintaining food crop diversity and disturbance resistance (naturally acquired genes being more sustainable than artificially selected ones). Genetically modified crops (GMO's) are a hot topic in popular media and government legislation and subsidies. Companies like Monsanto are engineering crops, patenting variety, and the result is causing a number of humanitarian and environmental conflicts. One of the very obvious places for ancient genetics to contribute here is to provide open access databanks where ancient variety sequences are localized and open to the public. Ancient DNA can also help in the revitalization and maintenance of bio-diverse cropping for contemporary farmers. For example, applied anthropology studies

have looked at early archaeological sites where food management and early farming was based on more sustainable practices (highly variable crop rotation, diverse crop maturation times and fallow periods which increased nutrient cycling, local cover cropping), and translated traditional practices to contemporary subsistence level farming practices (e.g., Erikson 1998), or even assist the distressed agri-industry of North America, currently trying to mitigate an oncoming food crisis (Nabhan 2013). As well, ancient DNA can identify exactly what varieties and how many varieties were grown by ancient farmers and localize the practice for modern fields. Localizing or contextualizing crops to their most native ecological niche has endless benefits for the sustainability and health of the environment and ensures more successful yields for farmers. (Holmgren 2002). Ethnobiologists have been successfully employing indigenous food management systems to contemporary environmental management and conservation (e.g. Anderson et al. 2011) – ancient DNA lends itself to this niche by identifying plants at a higher and more localized resolution and potentially identifying particular genes selected for during early domestication.

6.2 Constructing Relations: Phylogenies, Western Science and Beyond

The organization of biological organisms has roots in every culture. Many ethnobiologists have recognized the cross-cultural tendency of humans to classify the natural world (Atran 1990; Berlin 1992), and that each of these systems ('folk' or scientific) are purely cultural or social constructions and not 'natural' categories (Anderson 2011a). Unfortunately many in the Western Science tradition have (and in

some cases continue to) maintain that their ‘way of knowing’ is superior to other forms of traditional or folk science. In this section I wish to explore how genetics, particularly ancient DNA, can play a role in breaking down the misrepresentations of, and bridging both Indigenous and Western Science. No one-classification system is perfect, nor is one better than the other, but modern and paleo-genetics can help connect and bring out the best of both.

There are a number of ways to build and construct relationships among taxonomic categories. Some systems taxonomists have and continue to use a variety of systems to construct relationships, such as: ontological, typological, evolutionary, genospecies, chronospecies, phylogenetics, biological, genic, cohesion, and differential fitness. However, the variation and disagreement among biologists underscore the fact that defining terms that are seemingly basic such as “species” are in actuality rather complex and, in some case, arbitrary. Species are often defined by descriptive morphological characteristics. During the age of antiquarianism and natural classification of plants and animals based on morphological characteristics, the Linnaean *Systema Naturea* (kingdom, phylum, class, order, family, genus, species) allowed for the effective organization of the world and helped define the strange creatures discovered in European colonies (Alexander 1995; Dawkins 2004). Indeed, these types of classification systems based on the exclusion/inclusion of basic entities are still in use today and are the backbone of Western Science. However, despite these formal, structural approaches to classification, genetics allows us to significantly refine and re-define species and related organisms at a higher resolution. Notably, modern genetics has shown that traditional

indigenous taxonomies can be more accurate than European science (Anderson 2011a; Hunn and Brown 2011). For example, according to Hunn and French (1981), Columbia Plateau indigenous elders distinguish *mamin* from *sasamit'a*, both of which were classified under a single Linnaean plant species in guidebooks and espoused in University botany programs (Hitchcock and Cronquist 1973). But indigenous elders distinguished *mamin*, a food plant, from *sasamit'a*, which was only of interest to groundhogs. Genetic analysis later verified these were indeed different species (Hunn and French 1981; Schlessman 198). The importance of this study and others like them (e.g. Anderson et al. 2011) is that genetics demonstrates that no one system is perfect and that multiple models of classification will serve to better understand the relationship between plants and, in our case, the human hands that cultivated them.

One reoccurring theme I have come across in my studies is the importance of varietal and sub-species designations. Morphological analyses will rarely break down specimens into varieties, but it is at this level of identification where much anthropological knowledge can be extracted (such as looking at crop history, trade relationships, growth patterns, etc.). For example, Tuxill et al. (2010) have shown that analyzing maize at the level of *variety* has important implications for studying Maya ecology, culture and society (ancient and contemporary). Modern genetics has allowed scientists to further break down the diversity that occurs within a single species but it has also validated some of the indigenous classifications already constructed at this level.

While studying the classification of maize ‘races’ in the Yucatan, Arias et al. (2000) found that Yucatecan farmers had a sophisticated way for describing maize varieties that paralleled taxonomies defined by Wellhausen et al. (1952). Both classificatory systems established three principal landraces, but as they differentially varied in maturation time or kernel colour, the number of varieties increased to 16. Additional varieties grown intensively and long enough exclusively in Yaxcaba are now locally adapted or “creolized” varieties (Tuxill et al. 2010). This information is not always available archaeologically but genetics allows us to look at maize development and diversification in local contexts when no visible phenotypic signatures are available (morphologically). Some of these traits unrecognizable in the archaeological record are: traits effecting plant architecture, starch properties, kernel colour and growing habits (Jaenicke-Despres and Smith 2006).

While my research focused mainly on recovering ancient DNA to assist in species identification, these studies show the potential when archaeologists go into the field with the foresight of recovering botanical remains for genetic analyses; in effect we can begin to look at maize variety and crop history in specific regions, like the Great Lakes.

If Lowenthal (1985) is right, and we cannot “know that past” or that two pasts exist (the “actual past,” which is gone forever, and the “perceived past” that is paraded through memory, at archaeological sites, and differently remembered within various socio-political milieus), then how does the value of a kernel of maize play out in this “perceived past” (as reflected by our current socio-politico milieus)? In other words, what

kind of past should archaeology be contributing to? Bridging the Indigenous world-view and the realm of Western science is perhaps one of the most powerful ways of answering this question. As noted in Pierotti (2011:67), “Indigenous perspectives are most effective in observing and understanding wholes rather than parts, because they operate at the level of human perception and concentrate on functional relationships and coevolutionary processes rather than internal structure” (See also Barsh 2000). Research like aDNA analyses conventionally tend to contribute directly to Western science ways of knowing. My inclination is to explore how genetics, specifically ancient plant DNA analyses, can serve to combine ways of knowing in a holistic way. This is demonstrated in the examples above, but also can be explored more now that we know ancient charred maize, which is so often asserted in a general sense are a critical instigator of cultural change and emerging social complexity in the Americas, is also a meaningful and important source of genetic value from which to explore both questions of science and Indigenous ways of knowing the past.

6.3 The Value and Implications of *Zea* in Southwestern Ontario

I am going to finish off this chapter with a more contextual and localized discussion on what this research could mean for the archaeology of Southwestern Ontario as a case study for our understanding of ‘agriculture’. It has been hypothesized that the people of the Western Basin Tradition were mobile farmers. Because the maize used in this research came from their stores it begs the promise for more genetic analyses at a higher resolution to help answer larger anthropological questions about farming and sedentism.

The genetic diversity of a farmed species is critical to its survival and the survival of the humans who rely on that species for food. Large-scale famines, drought, pests and all other kinds of disturbances harm agriculturalists more than hunter-gatherers, but there are ways of farming that can minimize risk (Anderson 2011b). One way to farm more securely is to increase the varieties of a single crop such as: varieties that grow at different times of year or varieties that grow in different climates. Mobile farmers, such as those of the Western Basin Tradition, could have mediated risk with any number of mechanisms. We need to start thinking of some of the ways they could have done this, perhaps utilizing high-diversity maize, perhaps making use of diverse grain stores, or perhaps interacting with multiple trading partners (for seed). These hypotheses are now considered in light of the discovery that genetic material is present in charred maize kernels. Additional studies will help better explain the Bingo Village site, particularly how maize was grown and stored, what and how many varieties inhabitants were growing and add another component to the very long and complex history of maize.

As a geneticist I am inclined to question what genetic signatures set off a change in plant varieties, and as an anthropologist I am curious about the shifts in food production and the experiences or relational shifts between humans and plants. Adding ancient DNA analyses to any debates surrounding agricultural production such as: the inevitability of agriculture, the correlation between agriculture and sedentism or the difference between horticulture and agriculture, is something we might better understand when exploring a regional context like the Western Basin Late Woodland in Southwestern Ontario. Many studies (e.g. Bean and Saubel 1972; Laird 1976; Lee 1978;

Nabhan 1997; Shipek 1989; Turner 2005) have shown agricultural (food management) variation that exists across space, and we can now start thinking of those variations across time. The prevailing notions of Western imposed concepts of progressive agriculture and First Nations landscape management can be challenged or absorbed by genetics, bridging more anthropological thinking with the world of molecular biology.

6.4 Conclusion

Although some precedents exist for the isolation and amplification of ancient DNA from charred plant remains, this is the first, to my knowledge, of its kind for maize in the new world and one of only a few such studies accomplished in recent years since the amelioration of aDNA technologies. The initial ambivalence and difficulty obtaining positive results was overcome with patient troubleshooting and endless inhibition testing. Despite the fact that these plant remains were recovered with only limited, macro-identification needs the conventional end result of recovery, and the rather sketchy recovery and storing of maize remains without consideration of potential or consideration of genetic analyses – all of typical of CRM standards and practices – ancient DNA was successfully retrieved from charred maize remains recovered archaeologically from a 13th century Western Basin Late Woodland settlement. The repercussions of this research and these findings are many. Ancient geneticists and archaeologists need to work closely together for more collaborative analyses of plant materials. Archaeologists, particularly in Cultural Resource Management, need to become aware of the highly sensitive and valuable information that we now know is recoverable from charred plants.

Finally, collaborations should be based on the long-term research trajectories representative of ‘good’ science; studying a few kernels will not answer the kinds of questions we want to ask. Rather, we need to follow up on previous genetic and archaeological research for holistic interpretations and advancement of our knowledge, taking into account anthropological, science-based, and Indigenous ways of knowing the past and understanding human-plant relationships and landscapes. Aware of the destructive nature of aDNA analyses, stakeholders and scientists need to frame research questions that consider the current socio-political milieu. What are the varieties and species of plants that are the descendants of these kernels, and what was the long term genetic history of these? How are scientists shaping, and how are they shaped by, this research? The methodologies and materials in this thesis is open to the public, not only in an attempt to encourage open access of scientific knowledge, but for the hope of facilitating a more interactive and engaging community, and broader discussion about the paleoethnobotanical history of southern Ontario, for all those interested.

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Appendices

APPENDIX A: Protocol for modern maize extractions (from Qiagen® DNeasy Plant Mini Kit)

Protocol for Isolation of DNA from Plant Tissue with the DNeasy Plant Mini Kit

Important notes before starting

- If using DNeasy Plant Mini Kits for the first time please read "Technical Information" (page 11).
- Buffers AP1 and AP3/E concentrate may form precipitates upon storage. If necessary, warm to 65°C to redissolve (before adding ethanol to Buffer AP3/E). Do not heat Buffer AP3/E after ethanol has been added.
- Buffer AP1 may develop a yellow color upon storage. This does not affect the procedure.
- Buffers AW and AP3/E are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a water bath or heating block to 65°C.
- Preheat Buffer AE to 65°C.
- All centrifugation steps are carried out at room temperature in a microcentrifuge.

1. Grind plant or fungal tissue under liquid nitrogen to a fine powder using a mortar and pestle. Transfer the tissue powder and liquid nitrogen to an appropriately sized tube and allow the liquid nitrogen to evaporate. Do not allow the sample to thaw. Continue immediately with step 2.

Note: See "Disruption of plant material" (page 11).

2. Add 400 µl of Buffer AP1 and 4 µl of RNase A stock solution (100 mg/ml) to a maximum of 100 mg of ground (wet weight) or 20 mg (dried) plant or fungal tissue and vortex vigorously.

No tissue clumps should be visible. Vortex or pipet further to remove any clumps. Clumped tissue will not lyse properly and will therefore result in a lower yield of DNA. In the rare case where clumps cannot be removed by pipetting and vortexing, a disposable micropestle may be used.

Note: Do not mix Buffer AP1 and RNase A prior to use.

3. Incubate the mixture for 10 min at 65°C. Mix 2–3 times during incubation by inverting tube.

This step lyses the cells.

4. **Add 130 μ l of Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.**

This step precipitates detergent, proteins, and polysaccharides.

(Optional) Centrifuge the lysate for 5 min at full speed.

Some plant materials can generate very viscous lysates and large amounts of precipitates during this step resulting in shearing of the DNA in the next step (see "Lysate filtration with QIAshredder", page 12). In this case optimal results are obtained if the majority of these precipitates are removed by centrifugation for 5 min at maximum speed. After centrifugation, apply supernatant to QIAshredder spin column and continue with step 5.

5. **Apply the lysate to the QIAshredder spin column (filac) sitting in a 2 ml collection tube and centrifuge for 2 min at maximum speed.**

It may be necessary to cut the end off the pipette tip to apply the lysate to the QIAshredder column. QIAshredder removes most precipitates and cell debris, but a small amount will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet in step 6.

6. **Transfer flow-through fraction from step 5 to a new tube (not supplied) without disturbing the cell-debris pellet.**

Typically 450 μ l of lysate is recovered. For some plant species less lysate is recovered. In this case determine volume for the next step.

7. **Add 1.5 volumes of Buffer AP3/E to the cleared lysate and mix by pipetting.**

Example: To 450 μ l lysate add 675 μ l Buffer AP3/E. Reduce the amount of Buffer AP3/E accordingly if less lysate is recovered. A precipitate may form after the addition of ethanol but this will not affect the DNeasy procedure.

Note: Ensure ethanol has been added to Buffer AP3/E (see "Important notes before starting").

Note: It is important to pipet Buffer AP3/E directly onto the cleared lysate and to mix immediately.

8. **Apply 650 μ l of the mixture from step 7, including any precipitate which may have formed, to the DNeasy mini spin column sitting in a 2 ml collection tube (supplied). Centrifuge for 1 min at $\geq 6000 \times g$ (corresponds to ≥ 8000 rpm for most microcentrifuges) and discard flow-through.***

Reuse the collection tube in step 9.

9. **Repeat step 8 with remaining sample. Discard flow-through* and collection tube.**

10. **Place DNeasy column in a new 2 ml collection tube (supplied), add 500 μ l Buffer AW to the DNeasy column and centrifuge for 1 min at $\geq 6000 \times g$ (≥ 8000 rpm). Discard flow-through and reuse the collection tube in step 11.**

Note: Ensure ethanol is added to Buffer AW (see page 15).

* Flowthrough fractions contain Buffer AP3/E, and are therefore not compatible with bleach.

11. **Add 500 μ l Buffer AW to the DNeasy column and centrifuge for 2 min at maximum speed to dry the membrane.**

It is important to dry the membrane of the DNeasy column since residual ethanol may interfere with subsequent reactions. This spin ensures that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.

After washing with Buffer AW, the DNeasy mini spin column membrane is usually only slightly colored. In the rare case that the membrane remains significantly colored after washing with Buffer AW, refer to "Darkly colored membrane" in the Troubleshooting Guide on page 21.

Note: Following the spin, remove the DNeasy column from the collection tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.

12. **Transfer the DNeasy column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied) and pipet 100 μ l of preheated (65°C) Buffer AE directly onto the DNeasy membrane. Incubate for 5 min at room temperature and then centrifuge for 1 min at $\geq 6000 \times g$ (≥ 8000 rpm) to elute.**

Elution with 50 μ l (instead of 100 μ l) increases the final DNA concentration in the eluate significantly, but also reduces overall DNA yield. If larger amounts of DNA (>20 μ g) are loaded, eluting with 200 μ l (instead of 100 μ l) increases yield. See "Elution" on page 12.

13. **Repeat elution (step 12) once as described.**

A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, the microcentrifuge tube can be reused for the second elution step to combine the eluates.

Note: More than 200 μ l should not be eluted into a 1.5 ml microcentrifuge tube because the DNeasy column will contact the eluate.

APPENDIX B: Qubit® Fluorometer Quantification protocol

Qubit Fluorometer Quantification

Assays Protocol

1. Set up your tubes: 2 (or 3) for the standards and one for each sample. Label only the tube lids.
2. Make the Quant-it Working Solution by diluting the Quant-it reagent 1:200 in Quant-it buffer. 200µL of Working Solution is required for each sample and standard in a plastic container, do not use glass.
3. Prepare the assay tubes according to the table below:

	Standards	Samples
Working Solution	190 µL	180-199 µL
Standard	10 µL	
Sample		1-20 µL
Total Volume per tube	200 µL	200 µL

4. Vortex all tubes for 2-3 seconds
5. Incubate tubes for 2 minutes (15 minutes for protein assay) at room temperature
6. Turn the Qubit on by pressing any key. Press **HOME** and use the **▲** and **▼** keys to highlight the correct assay type and press **GO** to initiate the assay
7. On the calibration screen, highlight either **Run new calibration** or **Use last calibration** and press **GO** (follow section on calibrations below for details)
8. Insert and read the samples in the Qubit. The measurement will take approximately 5 seconds. Upon the completion of the measurement, the result will be displayed on the screen. The number displayed is the concentration of the nucleic acid or protein in the assay tube.
9. Calculate the concentration of your original sample by following the calculation below or choose the **Calculate sample concentration** to have the Qubit fluorometer perform this calculation for you.
10. Record the reading
11. Remove the sample from the instrument, insert the next sample and press **GO**, repeat for all samples

Notes: Keep 0.5 mL reaction tubes at room temperature (do not hold them for an extended period of time or leave them in the Qubit longer than required for a reading)
0.5 mL tubes must be clean and dry
Ensure there are no bubbles in the reactions
Protect Quant-it stock solution from light as much as possible

Calibrating the Qubit fluorometer

For each assay, you have the choice to run a new calibration or to use the values from the previous calibration. Highlight either **Run new calibration** or **Use last calibration** and press **GO**.

Running a New Calibration

Calibration of the Qubit fluorometer requires the preparation of the appropriate standard solutions. Prepare the appropriate standard solutions for the assay of choice.

1. Insert Standard #1 and press **GO**. Ensure you use the standard appropriate for each assay
2. The reading will take approximately 5 seconds
3. Repeat 1 for each standard
4. The Quant-iT PROTEIN Assay requires a three point calibration, so if you are performing this assay, a prompt to insert Standard #3 will appear on the screen.
5. The calibration is complete after Standard #2 or #3 is read. If you get a "Standards incorrect" message, you have made an error. Checking the calibration values may help you determine the source of your error.

Using the Last Calibration

You can choose to apply the previous calibration to your sample readings by pressing **GO** when **Use last calibration** is highlighted on the calibration selection screen. You are then directed to insert the assay tube containing your sample.

Calculating the Concentration of Your Sample

The Qubit fluorometer gives values in either $\mu\text{g/mL}$ or ng/mL . This value corresponds to the concentration after your sample was diluted into the assay tube. To calculate the concentration of your sample, use the following equation:

$$\text{Concentration of your sample} = \text{QF value} \times (200/x)$$

Where: QF value = the value given by the Qubit fluorometer

x = the number of microliters of sample you added to the assay tube

Or press **Calculate sample concentration** and select the number of microliters of sample you added to the assay tube.

Quant-iT dsDNA HS Assay Kits

0.2-100 ng, two standards

Quant-iT dsDNA BR Assay Kits

2-1000 ng, two standards

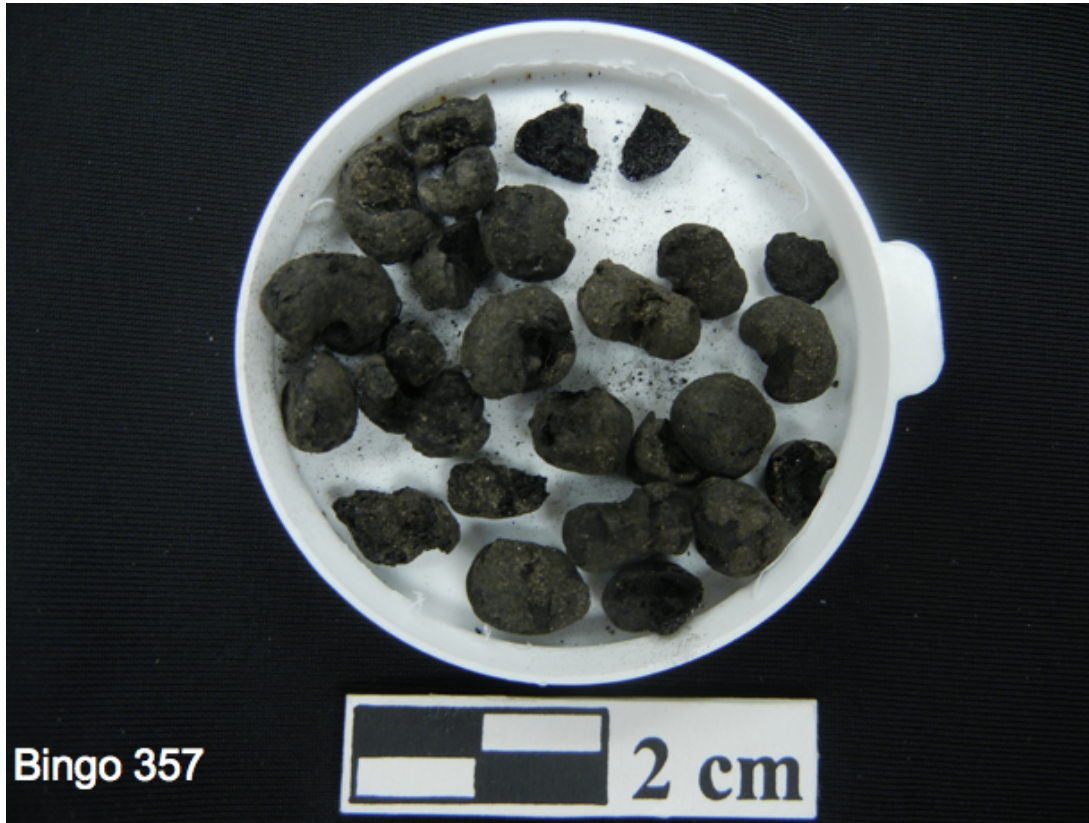
Quant-iT RNA Assay Kits

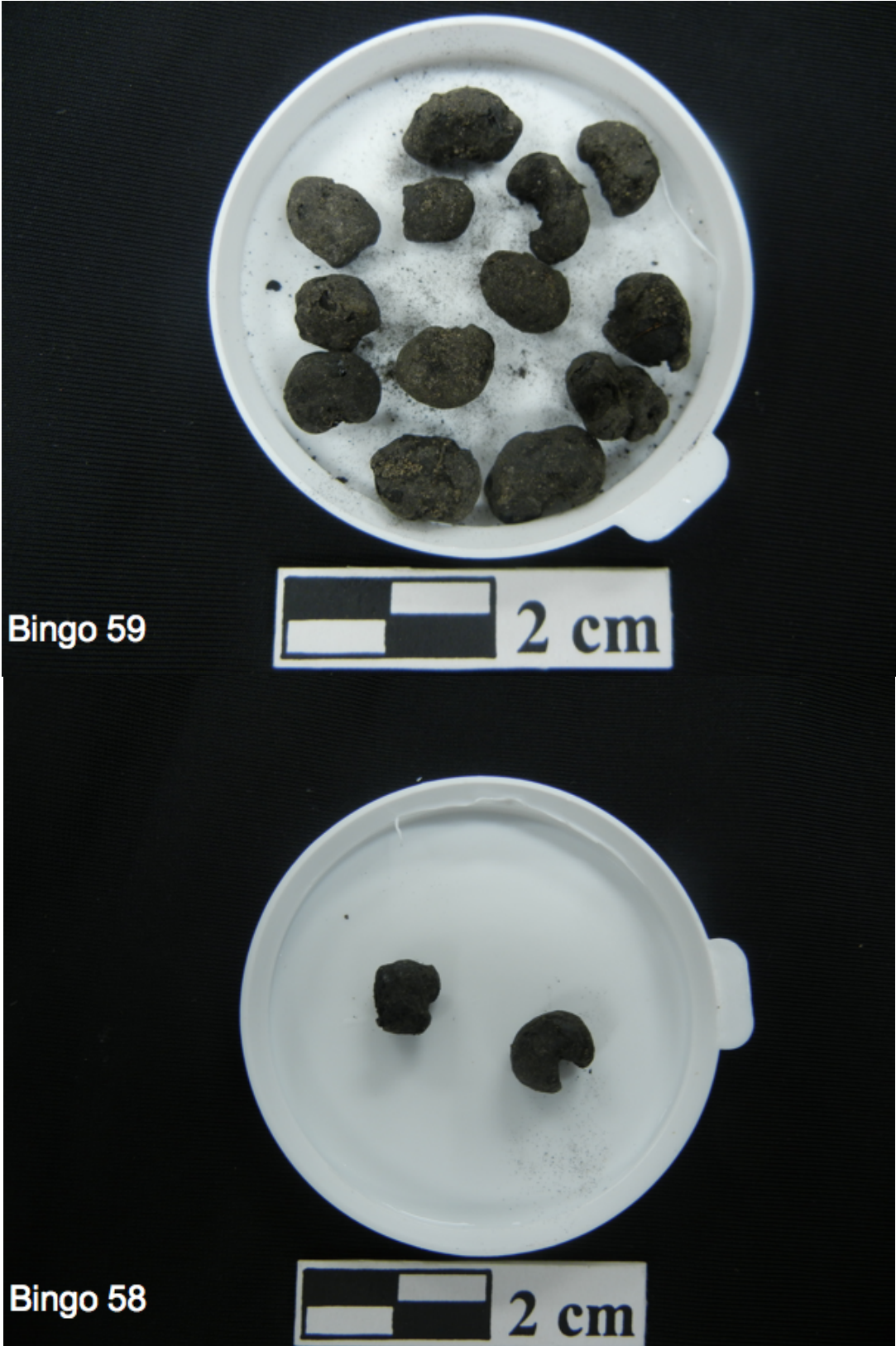
5-100 ng, two standards

Quant-iT Protein Assay Kits

0.25-5 μg , three standards

APPENDIX C: Ancient maize pictures, kernel counts and volumes





Bingo 59

2 cm

Bingo 58

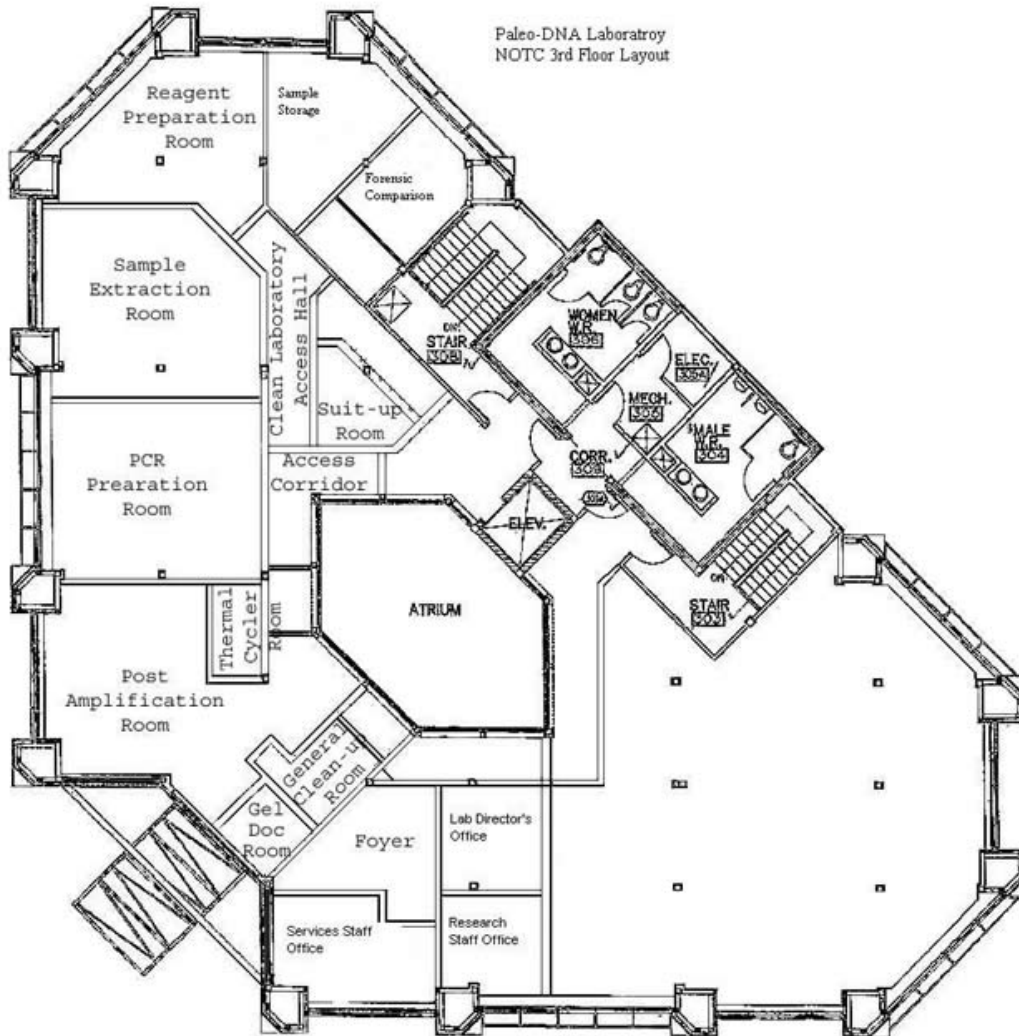
2 cm





Sample	Feature	Fluorometer (ng/mL)*	Number of Kernels	Ground Volume (mL)
SM1	Bingo 420	0.24	1	0.25
SM2	Bingo 420	0.116	1	0.3
SM3	Bingo 420	Too low	2	0.3
SM4	Bingo 420	0.306	1	0.25
SM5	Bingo 420	0.238	1	0.25
SM6	Bingo 420	0.371	2	0.3
SM7	Bingo 357	0.444	1	0.25
SM8	Bingo 357	0.265	2	0.5
SM9	Bingo 357	0.211	1	0.4
SM10	Bingo 420	0.224	1	0.4
SM11	Bingo 238	0.18	1	0.5
SM12	Bingo 588	0.12	1	0.35
SM13	Bingo 59	0.279	2	0.3
SM14	Bingo 238	Too low	2	0.25
SM15	Bingo 588	0.343	1	0.45
SM16	Bingo 58	0.096	1	0.25
†NF1	Westpit 417	0.266	1	0.5
NF2	Bingo 357	0.3	2	0.35
NF3	Bingo 301	0.215	2	0.3
NF4	Bingo 357	0.261	1	0.45
NF5	Westpit 417	0.093	1	0.25
NF6	Bingo 301	0.397	1	0.2
NF7	Westpit 417	0.349	2	0.3

Appendix D: Paleo-DNA Laboratory at Lakehead University, Thunder Bay, Ontario, Canada



QIAquick Nucleotide Removal Kit Protocol

using a microcentrifuge

This protocol is designed for cleanup of radioactive-, biotin-, or DIG-labeled DNA fragments and oligonucleotides ≥ 17 nucleotides from enzymatic reactions (see page 8). The protocol ensures removal of primers < 10 bases, enzymes, salts, and unincorporated nucleotides. It is possible to use this kit with a vacuum manifold as well as with a microcentrifuge, and a protocol for vacuum processing is available on request from QIAGEN Technical Services or your local distributor. However, we do not recommend processing radioactive samples with a vacuum manifold.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are in a conventional tabletop microcentrifuge at room temperature.

Procedure

1. **Add 10 volumes of Buffer PN to 1 volume of the reaction sample and mix.**
For example, add 500 μ l Buffer PN to a 50 μ l reaction sample. For DNA fragments ≥ 100 bp, only 5 volumes of Buffer PN are required.
2. **Place a QIAquick spin column in a provided 2 ml collection tube.**
3. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 min at 6000 rpm.**
4. **For radioactive samples:**
Place the QIAquick column into a clean 2 ml collection tube and discard the tube containing the radioactive flow-through appropriately.
For non-radioactive samples:
Discard the flow-through and place QIAquick column back into the same tube.
Collection tubes are reused to reduce plastic waste.
5. **For radioactive samples:**
To wash QIAquick column, add 500 μ l of Buffer PE and centrifuge for 1 min at 6000 rpm. Discard the flow-through appropriately and repeat wash with another 500 μ l of Buffer PE.
For non-radioactive samples:
To wash QIAquick column, add 750 μ l of Buffer PE and centrifuge for 1 min at 6000 rpm.

6. Discard the flow-through and place the QIAquick column back in the same tube, which should be empty. Centrifuge for an additional 1 min at 13,000 rpm (17,900 x g).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifuge.

7. Place the QIAquick column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 100–200 μ l of Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min at 13,000 rpm (17,900 x g). Alternatively, for increased DNA concentration, add 30–50 μ l elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

9. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Appendix F: Qiagen® DyeEx™ spin column

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn.
This is necessary to avoid a vacuum inside the spin column.
3. Snap off the bottom closure of the spin column (Figure 1), and place the spin column in a 2 ml collection tube (provided).
4. Centrifuge for 3 min at the calculated speed.
5. Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the sequencing reaction (10–20 μ l) to the gel bed (Figure 2).
Notes:
 - Pipet the sequencing reaction directly onto the center of the slanted gel-bed surface (Figure 2). Do not allow the reaction mixture or the pipet tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipet tip.
 - This protocol is suitable for sequencing reactions with volumes of 10–20 μ l. For easier handling, more reproducible pipetting, and reduced error with sample volumes <10 μ l, we recommend adjusting the volume to 20 μ l using distilled water, before application to the gel-bed.
 - It is not necessary to remove mineral oil or kerosene prior to cleanup of dye-terminator sequencing reactions.
 - It is not necessary to replace the lid on the column.
6. Centrifuge for 3 min at the calculated speed.
7. Remove the spin column from the microcentrifuge tube.
The eluate contains the purified DNA.
Optional: If using the ABI PRISM 3700 with a water loading protocol, it is possible to load the eluate directly onto the sequencer without drying down the sample.
8. Dry the sample in a vacuum centrifuge and proceed according to the instructions provided with the DNA sequencer.

CHELSEY G. D. ARMSTRONG

PROFESSIONAL SUMMARY: Master's candidate in Ecological Archaeology at the University of Western Ontario (expected end date August 2013). Interests include: ethnobiology, ancient DNA, paleoethnobotany, archaeology, modern and ancient environmental resource management

FIELD EXPERIENCE:

2012 (May-September)

**Archaeological Field Technician, Golder Associates:
London, Ontario**

- Archaeobotanical analyses (designed sampling strategies and performed excavations, flotation, identification and analyses)
- Identified and catalogued thousands of historic and pre-historic artifacts
- Completed stages 1-4 under the Standards and Guidelines of the Ontario Ministry of Tourism and Culture including:
 - Assessed and mitigated development impacts
 - Performed background studies and property inspections
 - Property assessments, pedestrian surveys, test pit surveys, property formation analysis
 - Controlled surface pick-ups, unit excavations, heritage value assessments, artifact analysis
 - Test pit, screening and excavation

2011 (May-Sept)

**Soil Quality Research Assistant, Environment Canada
and Pacific Agriculture Research Center: Agassiz,
British Columbia**

- Collected field samples for lab analysis (wet aggregate distribution and stability)
- Ran *Guelph Permeameters* and water infiltrometers
- Collected cores for water retention analysis
- Collected earthworm samples for analysis
- Weeded, harvested and applied lime and insecticides (*all work in corn, raspberries and blueberries plots)

2010 (June – July)

University of Arkansas Field School: Tell Qarqur, Syria

- Completed basic archaeological excavation, methods and theory
- Performed flotation, recovery and sorting and analyses of archaeobotanical remains
- Completed GIS survey and total station mapping on and around the site

2010 (May)

Volunteer, Tribal Heritage Preservation Office: Fort Apache, Arizona

- Surveyed and controlled surface pick up over vast and rugged terrain
- Performed large-scale screening
- Processed historical artifacts (basic documentation and data input)
- Organized and catalogued historical maps, legal papers, deeds, survey documents and other historical records

2007 (June-Sept)

Naturalist and Heritage Educator: Killarney Provincial Park, Ontario

- Studied geology, flora and fauna of Killarney Provincial Park and was tested weekly to verify knowledge
- Lead overnight canoe trips and hikes for park guests
- Collected and managed data to present in an engaging and interactive way to Park guests
- Worked with Environment Canada to conduct Butterfly and Loon counts to document local ecological conditions

2007 (May-June)

SAHARA Organic Farming Project: Himachal Pradesh, India

- Worked on organic farms in Kulu/Manali, India
- Performed interviews and collected ethnographic data on local and ancient organic farming techniques
- Plowed, sowed, maintained and harvested crops with locals on a number of farmsteads
- Compiled research and presented data to IPCC
- Worked with grassroots NGO's like SAHARA

LAB EXPERIENCE

2012 (Sept-Nov)

Visiting Research Scholar, Paleo-DNA Laboratory: Lakehead University, Thunder Bay Ontario

- Performed sample preparation, extraction, purification of over a hundred ancient *Zea mays L.* samples
- Set up standard and real-time PCR's, visualization gels, sequencing
- Worked with a range of chemicals and reagents in closed and confined areas with strict contamination protocols
- Analyzed results with various computer software programs (D.Imager, Sequencher, Bioedit, BLAST)

- All research compiled and used for Masters thesis

2011 (Jan-Sept)

Contract Archaeobotanist, Sources Archaeological and Heritage Research Inc: Vancouver, British Columbia

- Floated, recovered and analyzed all soil samples from 2010 excavation of Hopetown, near Vancouver Island
- Studied wet site archaeobotany, recovery strategies and Vancouver Island botany
- Provided weekly updates to Sources team and compiled a final lab report of all findings

2010-2011 (Sept-May)

Garry Oak Ancient DNA Extraction and Analyses, Honors Research: Vancouver, British Columbia

- Supervised by Dr. Dongya Yang
- Developed primers and used multiple extraction methods for PCR set up and aDNA analysis
- Trained on PCR and Electrophoresis machines
- Trained to utilize *GenBank*, *Bioedit*, *Chromas*, *Mega4* and *Net Primer*

2010-2011 (Aug-May)

Volunteer Archaeobotanist for Dr. Cathy D'Andrea: Vancouver, British Columbia

- Volunteered in Dr. D'Andrea's archaeobotany lab
- Helped sieve, sort, identify and fill out sample sheets of remains
- Sorted for Dr. D'Andrea's Masters candidate Pam Wadge (samples from Ethiopia)
- Sorted for Dr. D'Andrea's Masters candidate Molly Capper (samples from Turkey)

OTHER RELATED EXPERIENCE

2011 (Sept) – Current

Teaching Assistant at the University of Western Ontario: London, Ontario

- Assisted faculty with instructional responsibilities
- Tutored, held office hours, proctored exams, lead labs and discussion
- Gave lectures in "North American Archaeology"
- Classes taught: ANTH 1025F Sociocultural Anthropology; ANTH 1003 Introduction to Biological Anthropology; ANTH 2225 North American Archaeology; ANTH 1025b Linguistic Anthropology

2011 (September-Present)

Co-Editor for Totem: Student Journal of Anthropology

- Organized work flow schedule for 22nd edition of journal

- Created and ran training session for student peer-reviewers (how to provide effective feedback and conduct reviews professionally)
- Sorted submissions for review, comments to authors and editors
- Trained on Totem website in order to update and coordinate software for ‘blind’ review process
- Negotiated contract with EBSCOhost™
- Continuing with process until Journal is published and uploaded to online server

2011 (Jan-Present)

Social Media Volunteer Contributor for the *Society of Ethnobiology*

- Administrator for the Society of Ethnobiology’s *Facebook, Twitter, Tumblr* and Blog accounts.
- Add updated news and events to the Society’s website (See Ethnobiology.org)
- Participating in working group for podcasting the 2013 conference in Texas

2012 (November)

Presented Thesis Research Results at University of Western Ontario Graduate Seminar: London, Ontario

2012 (August)

Wrote Best Practices policy for Archaeobotanical analyses of Stage 4 excavations for Golder Associates

2012 (May)

Presented Working Masters thesis at Society of Ethnobiology Conference: Denver, Colorado

2011 (April)

Presented Complete Honours Thesis at the Simon Fraser University Graduate Seminar Series: Burnaby, British Columbia

2011 (May)

Presented Poster of Honors Thesis at the 2011 Society of Ethnobiology Conference: Columbus, Ohio

2011 (Feb-June)

Developed Index for Ethnobiology Textbook

- Employed by Gene Anderson from University California Riverside to write the Index for textbook “Ethnobiology”
- Completed a twenty-two-chapter overview of key definitions, concepts, authorities, theories and organized data into an extensive and properly formatted index

2010-2011 (Sept-May)

Undergraduate Faculty Liaison

- Elected by archaeology undergraduate majors to represent student interests to the department
- As part of the SFU Archaeology Department's move into the Faculty of Environmental Sciences I was part of the committee to re-write the undergraduate curriculum with Dr. David Burley, Chris Pappianni