SAVE ME A DRUMSTICK:
MOLECULAR TAPHONOMY, DIFFERENTIAL PRESERVATION AND
ANCIENT DNA FROM THE KINGDOM OF TONGA

by
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Abstract

This project was focused on the extraction and amplification of DNA from ancient samples of chicken (*Gallus gallus*) and sea turtle recovered from the Kingdom of Tonga. Results of this project demonstrate that small amounts of highly degraded ancient DNA template material is preserved in archaeological chicken remains. Two of the twelve samples tested yielded positively amplified DNA. The chicken D-loop sequences recovered are from the Plainware Phase of prehistory dating from approximately 2650 BP. However, the sporadic nature of the results suggest it is at the borderline of what can be done with contemporary techniques. Analysis of sequenced domesticated chicken remains from Tonga display an affinity with Chinese stocks identified as ‘isolates’ and may lend support to theories of a Southeast Asian origin.

This preliminary study provides evidence that chicken from Oceanic ‘open air’ sites may provide a parallel line of evidence to studies of other domesticates and human mtDNA to reconstruct migration and interaction in prehistoric Oceania. Further refinement of these methods, such as optimization of PCR protocols and quantification of PCR products make this a potential source of secondary evidence for future study.

In order to further understand preservational differences between sea turtle and chicken bone samples a secondary measure to assess preservation
was sought. Collagen was selected because research suggested a similar structure and vulnerability to processes of degradation as DNA. The measurement of “collagen” yields indicates that this protein may also preferentially preserve in bird remains of some antiquity, but does not preserve in sea turtle or rat.

Several preliminary attempts, by other authors, to amplify aDNA from 'marginal' environments has suggested that DNA and collagen might not preserve well in hot and humid locales, particularly the tropics. It appears, however, that the unique structure of avian bones makes them more resistant to processes of diagenesis and enhances the preservation potential of aDNA and collagen. In Tonga (and the rest of Oceania) the association of these remains with midden deposits on the sea shore, also creates an environment in which DNA and collagen may bind to the minerals in sea sand conferring higher preservation potential.
For Ronald Storey April 1943 – March 2002
"The whole of science is nothing more than a refinement of everyday thinking."
– Albert Einstein 1936

"In theory, theory and practice are the same. In practice, they are not."
– Anon
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Chapter 1: Introduction

As an undergraduate student in archaeology at Simon Fraser University I became interested in the application of genetic data to the understanding of human migration and interaction. With the development of a laboratory for ancient DNA (aDNA) analysis in the department, it became possible to pursue these interests as a MA research project. Initially I hoped to examine the prevalence of the ‘Polynesian Motif’ in ancient human remains from Tonga and compare them with studies undertaken by Hagelberg et al. (1989) and Hagelberg and Clegg (1993). Problems of contamination, analysis and other issues, led to a less ambitious but equally interesting project eventually being pursued.

Pioneering work by Matisoo-Smith (1994) examining the DNA of modern Polynesian rats (Rattus exulans) has shown great potential as a proxy for tracing human migrations in the past, in much the same way other researchers use modern human data (examples include; Hertzberg, et al. 1989; Hill, et al. 1985; Hill and Serjeantson 1989; Lum, et al. 1998; Lum, et al. 1994; Oppenheimer and Richards 2001). More recently Matisoo-Smith et al. (1997) extended their original research to include ancient rat DNA for comparison with the modern rat data and also with populations of rat from different islands through their occupation history. Ancient DNA studies in Oceania have also been extended to include two domestic animals, dogs (J.S. Allen, et al. 1996) and pigs (M.S. Allen, et al. 2001) with similar intention. Chicken (Gallus gallus), a third and critical domesticate for Oceanic economies as well as colonising settlement in
prehistory, has yet to be examined. Consequently, the research presented here aims to examine the possibilities for extraction, analysis and interpretation of ancient DNA from chicken in the prehistory of tropical Oceania. Samples of chicken bone from recently excavated sites in southern and central Tonga were obtained. These samples were recovered from faunal assemblages associated with the initial colonizing populations during the Lapita phase (2950-2650 BP) and subsequent Polynesian Plainware or ancestral Polynesian phase (2650 - 1400 BP) in Tongan prehistory (Burley 1998).

During a previous attempt by Matisoo-Smith's ancient DNA team (Robins, et al. 2001) to extract and amplify DNA from Tongan archaeological rat bone samples, aDNA amplification was unsuccessful. While this result was initially discouraging for the intended project on chicken, it previously has been suggested that bird bone degrades differently than mammal bone (Davis 1997). If this held true for preservational differences between rats and chickens then DNA might be differently preserved as well. This thesis, then, is also an experiment to discern if aDNA from open air sites in the South Pacific exhibit traits of differential preservation between taxa. Initial results for the first set of ancient chicken DNA samples suggest this is indeed the case. Subsequent tests were less rewarding, but the two samples which produced sequences which were those of chicken.

Ancient DNA is a field in which claims for success must be authenticated due to the large potential for spurious results from contamination (Cooper and Poinar 2000). The Ancient DNA Laboratory at SFU is a new facility, and the chicken samples being run were among the first samples processed in this lab.
Laboratory contamination risk for modern chicken DNA was very low. The absence of amplifiable aDNA in the rat samples nonetheless potentially raises questions for the authenticity of chicken results. Thus a third faunal type, sea turtle, was tested for the preservation of aDNA. Like rats, this proved unsuccessful despite repeated attempts at varying PCR conditions and in some cases modification of laboratory protocols.

Gotherstrom et al. (2002) suggest that DNA and collagen degrade in similar ways and, therefore, the existence and quantity of collagen might be directly used to serve as a proxy for the existence of DNA. Significantly Steadman et al. (2002b) were able to extract collagen from chicken bone to obtain AMS radiocarbon dates for Tongoleleka site samples, one of the sites incorporated in the present study. If collagen is a proxy for DNA preservation, then these results support the likelihood for positive DNA results for chicken. It must be further hypothesized that collagen preservation in sea turtle and Polynesian rat would be limited. To test this hypothesis two of the turtle samples previously tested for DNA were submitted to the Stable Isotope Laboratory at SFU for collagen extraction and were found to have very little to no high quality collagen present. Four rat bones from three of the sites previously examined by Robins et al. (2001) as well as three chicken samples previously tested for ancient DNA were also submitted for “collagen” extraction. The results parallel those found in the DNA analysis, with “collagen” deficiency in rats and sea turtles, but differently preserved in chicken. These “collagen” results do not authenticate the DNA study per se, nor conclusively prove the use of “collagen”

1 After DeNiro and Weiner (1988), collagen which has been subject to degradation is denoted with quotation marks to identify it as degraded
Chapter 1: Introduction

as a proxy for DNA preservation in tropical open air sites. Yet they do serve as coincidental data with implications for both.

Progression of the Thesis

This introduction has presented an overview for the history of this research project, and the different directions and stages though which it has proceeded. This is important as the research project evolved considerably from its initial conception through to the thesis writeup. Chapter 2 provides the context for this research, with brief examinations of Tongan geography and prehistory as well as the use of faunal data for the documentation of prehistoric settlement in the Pacific. It concludes with a presentation of background data for chicken and sea turtle, the two other faunas from which attempts were made to extract aDNA. Chapter 3 provides an account of the samples, methods and results of the aDNA study. Discussion in this chapter focusses upon an interpretation of these results in the broader concern of Oceanic prehistory. Chapter 4 explores the potential parallels in “collagen” and aDNA preservation, and the use of the former as a possible proxy in the Tongan samples. It also presents the results of “collagen” extraction in the chicken, rat and turtle samples from Tongan sites. Chapter 5 provides discussion of the proceeding research and its implications not only for biomolecular studies but also Oceanic prehistory. Chapter 5 concludes with a brief review of the thesis and suggested directions for future research.
Chapter 2: Site and Sample Contexts

The following chapter will provide a brief background to Tongan geography, past archaeological investigations and cultural chronologies. This is followed by a discussion of how DNA techniques, both ancient and modern, have been applied to questions of Oceanic migration, settlement and interaction with emphasis on the use of faunal models. In order to understand the choice of samples used for this project a brief introduction to both chickens and sea turtles is included, outlining their geographic spread, place in Oceanic society and relevant information with regards to their mitochondrial DNA. This chapter will orient the reader to the features of chickens and sea turtles which make them of interest to studies both of aDNA and Oceanic archaeology.

The Kingdom of Tonga

Tonga, given the name Friendly Island by Captain James Cook, is located at the western edge of the Polynesian Triangle, as is shown in Figure 1. The contemporary kingdom, covering an area of 738 km², includes over 160 islands, forty of which are currently inhabited. While most are raised coral limestone covered with a fertile blanket of volcanic ash, there is a chain of active volcanic islands to the west. The modern Tongan population live almost exclusively on the coral-limestone islands, as was likely the case in prehistory. These islands are surrounded by resource rich fringing reefs which provide abundant food.
resources and also serve to protect the islands from the intense surf which pounds the shores of unprotected volcanic islands (Burley 1998).

The contemporary kingdom consists of three major island groups; Tongatapu, Ha'apai and Vava'u. Tonga is mainly subtropical with a humid and a dry season. Temperature, rain and humidity increase in intensity from south to north. The average annual rainfall in the Ha'apai and Tongatapu groups is 1,500 to 1,800 mm (Poulsen 1977: 5). Throughout most of its history the major site of occupation has been on beaches with easy access to reef resources. In the central islands of Ha'apai these are evidenced by single midden sites facing the sea, while Tongatapu settlements (also dominated by middens) are focussed along Fanga 'Uta Lagoon and the northern leeward coastline (Burley 1998).
The modern history of Tonga was of great interest to a variety of early explorers and ethnographers, such as Cook (1777), Mariner (Martin 1991), and Beaglehole and Beaglehole (1941), to name a few. It has also been the subject of intensive archaeological study since the early work of McKern (1929) in the 1920's. McKern's study was part of the Bay and Domineck expedition of the B. P Bishop Museum directed by Gifford (1923; 1924; 1929). Since then archaeological studies have been undertaken by Golson (1957), Poulsen's (1987) and Davidson's work on Tongatapu (Davidson 1969), Groube's (1971) work throughout the Kingdom, Kaeppler (1973) in Ha'apai, Davidson's (1971) investigations in Vava'u, work in Niuatoputapu by both Rogers (1974) and Kirch (1988), survey and test excavations on 'Ata by Anderson (1978), work by Dye (1996), and a large research project by Spenneman (1989). Since 1989 archaeological investigations have only been undertaken by Burley and his team of researchers (see Burley 1994, 1998; Burley and Dickinson 2001a, 2001b; Burley, et al. 2001; Burley, et al. 1999; Burley, et al. 2002). The current understanding of this great quantity of research has led to the formulation of the following chronology for settlement of the islands and the cultural sequences therein (Burley 1998).

The earliest known settlement of Tonga was at the site of Nukuleka on the island of Tongatapu around 2950 BP by Lapita peoples (Burley and Dickinson 2001b). After the settlement of Tongatapu, the remainder of the Kingdom was settled quickly, first in the central Ha'apai group (Burley, et al. 1999), next in the islands of Vava'u (Davidson 1971) and finally to the northern outlying island of Niuatoputapu (Kirch 1988). The Lapita period, identified archaeologically
through the proliferation of dentate stamp decorated ceramics lasted from approximately 2950 to 2650 BP (Burley and Dickinson 2001b). This was followed by a Plainware period, characterized by ceramics which lack not only the elaborate decoration but also several of the vessel forms from the preceding Lapita period, persisting from 2650 to 1550 BP (Burley, et al. 1999). The abrupt end of ceramic bearing cultural layers at 1550 BP signifies another shift in Tongan material culture and is designated by several names, the A-ceramic period, the Formative period or the Tongan Dark Age which ends at approximately 750 BP with the advent of the Classical Tongan Cheifdom (Burley 1998; Burley, et al. 1999).

**Lapita, Molecular Research and the Settlement of Oceania**

The colonization of the islands of Oceania has been of interest to Europeans since Captain Cook first reported on the widespread dispersal of Polynesian peoples in the 1770's. Archaeological investigations in the twentieth century led to the discovery of a distinctive type of dentate stamp pottery known as 'Lapita' and this has helped to trace the expansion of these ancient peoples east across Oceania. Their ultimate origin is still debated and lines of evidence to trace their migrations across the sea have ranged from comparison of physical traits to sourcing of artifacts such as pottery and lithics to detailed investigations of linguistic similarities and differences (Kirch 1997; Kirch and Green 2001).

Techniques from population genetics have oft been employed to examine a myriad of traits in Pacific island populations. At the outset so many interesting
traits were available that despite a great volume of work no unifying trait was tested uniformly. For example, some groups examined the prevalence of α-thalassaemia in nuclear genes (Hertzberg, et al. 1988), while others surveyed the mitochondrial genome for the 9 base pair gene deletion and associated mutations which constitute the ‘Polynesian motif’ (Melton, et al. 1995; Yao, et al. 2000). Many other groups studied other traits, including Y-chromosome information (Su, et al. 2000) and blood group polymorphisms (Livingstone 1984). However, the data was not drawn from the same island populations and therefore conclusions did not always point to the same location for dispersal of particular traits. For example in studies of Gamma globin (Gm) frequencies Clark and Kelly (1993) proposed an Asian Near Oceanic or East Asian homeland, Serjeantson et al. (1982) proposed Japan based on Human Leukocyte Antigens and from the 9 base pair deletion three teams (Melton, et al. 1995; Merriwether, et al. 1999; Redd, et al. 1995) identified three possible places of origin; Indonesia, an Austronesian area of Southeast Asia and/ or Taiwan.

In the early 1990's Hagelberg and Clegg (1993) were able to acquire archaeological skeletal remains and set about extracting ancient DNA. The results were highly successful with 21 of 38 specimens producing sequences. The tests suggested that the oldest Polynesian samples did not have the ‘Polynesian motif’ and called into question the direct ancestry of Polynesians from Lapita peoples. The results were difficult to authenticate and were identified as ‘preliminary’ by the authors (Hagelberg and Clegg 1993). It became clear that ancient DNA from human populations would not resolve debates relating to the ultimate origins of the Lapita and/ or Polynesian peoples.
Domesticate and commensal species purposefully introduced to the islands of the Pacific by early colonists could also be used to track human settlement patterns by examining patterns of mitochondrial (mtDNA). Their presence and abundance in archaeological sites created an alternative option for tracing ancient migration using genetic data (J.S. Allen 1996, M. Allen 2001, Matisoo-Smith 1994, Matisoo-Smith et al. 1997, Matisoo-Smith et al. 1998a, Matisoo-Smith et al. 1998b, Matisoo-Smith et al. 1999, Matisoo-Smith and J.S. Allen 2001, Matisoo-Smith et al. 2001, Matisoo-Smith 2002 and Matisoo-Smith and Robins 2002). This has provided an independent means to assess not only models developed from DNA analyses but also from linguistic and archaeological investigations.

Faunal models of Migration and Interaction

First detailed in 1994 (Matisoo-Smith 1994), live Polynesian rats (*Rattus exulans*) were trapped and their DNA analysed to look for patterns relating to specific island populations. Rats were on board ancient exploratory and colonizing voyages, as is evidenced by their remains in early archaeological sites (Matisoo-Smith 1994). Therefore, it was reasonable to assume that genetic signatures of rats could be traced as a proxy for people, and could be used to provide information on “degrees of interaction between the various archipelagos within the Polynesian triangle” (Matisoo-Smith, *et al.* 1998b: 15146). In 1996, the project was expanded by another team of researchers who began to undertake work with ancient DNA of both Polynesian rats and Maori dogs (*Canis familiaris*) (J.S. Allen, *et al.* 1996). The team insisted on using only ancient
faunal remains with reliable associated radiocarbon dates, an unprecedented level of chronological control for aDNA investigations (J.S. Allen, et al. 1996).

By 1997 Matisoo-Smith had refined her techniques from modern samples to extract DNA from small ancient bones, such as femurs and mandibles using a silica based extraction protocol (Höss and Paabo 1993). This resulted in a great deal of data and the discovery that *Rattus exulans* often expressed "point mutations unique to specific island populations" (Matisoo-Smith, et al. 1997: 1534). Based on data from a plethora of ancient Polynesian island rat populations, her team worked out an 'East Polynesian Homeland' in the Society and Southern Cook Islands as well as Samoa [data from Tonga is unavailable; see Robins et al. (2001)] (Matisoo-Smith, et al. 1998b: 15146). They also found that "multiple contact, to a greater or lesser degree, rather than isolation was the general pattern in Polynesian prehistory" (Matisoo-Smith, et al. 1998b: 15149). This was particularly evident on the Kermadec Islands where genetic diversity revealed multiple introductions of rat from regions with populations of high genetic variability (Matisoo-Smith, et al. 1999). This contrasted with evidence for isolation in the Chatham Islands, where a relatively homogeneous population of Pacific rats had existed from early settlement to the present (Matisoo-Smith, et al. 1999). However, this population still had a unique genetic signature among other populations of *R. exulans* with an A to G transversion at position 106 (Matisoo-Smith et al. 1999). Work on Norfolk Island showed an important link between that island and New Zealand, although genetic tests alone could not predict the direction of contact (Matisoo-Smith, et al. 2001). The results in Norfolk, the Kermadecs and Chatham Islands were consistent with other lines of
evidence, including archaeology and linguistics, demonstrating the potential of applying faunal DNA to the reconstruction of human migrations.

In 2001 Matisoo-Smith expanded her exploration of ancient rat DNA into Melanesia and island southeast Asia. The western Pacific presented unique problems in that *Rattus exulans* was not the only species of rat present in prehistory and morphological traits were not always useful in distinguishing one species from another (Matisoo-Smith and Allen 2001). Addition of these Pacific rat samples increased the tally of ancient rat remains tested to over 400 individuals from the Pacific and Asia. As in Polynesia, the team discovered regional rat markers in the western Pacific (Matisoo-Smith and Allen 2001).

Ancient DNA work with pigs was undertaken to "provide a history of human migration and interaction in the Pacific independent from, but complementary to that of the Pacific Rat" (M.S. Allen, *et al.* 2001: 5). While pigs are of great interest due to their distribution and importance in western Pacific sites and overall diversity in Asia, they are scarce or absent in Lapita age sites in Tonga (Shutler Jr., *et al.* 1994) and Samoa (Nagaoka 1993). They are also "patchy" in distribution in later Polynesian sites (M.S. Allen, *et al.* 2001: 7). While they can be used to investigate primary waves of colonisation from island southeast Asia through the western Pacific, their utility in Polynesia is restricted by their unavailability in the archaeological record.

Most recently Matisoo-Smith reported new applications and results from her continuing research with the Polynesian rat. One of the most important was the ability to compare ancient and modern DNA data sets in rat populations and the implications of this comparison to interpretation of modern human DNA data
as they relate to ancient populations (Matisoo-Smith 2002). She concluded that by sampling contemporary populations we are likely to be missing significant information about the past populations. This means that we need to be very careful when interpreting patterns of genetic variation in these regions today and making statements about population origins and prehistoric mobility (Matisoo-Smith 2002: 495).

While these warnings had come before from a myriad of sources, this empirical evidence gave it a new set of teeth. The second major discovery was the result of her work with samples from the Philippines and island southeast Asia (Matisoo-Smith and Robins 2002). Matisoo-Smith and Robins (2002) found that there were likely two introductions of rat into Near Oceania and the point of distribution as evidenced by their phylogenetic reconstructions was Halmahera. This is supported by evidence found by Bellwood (1997) for similarities in material culture and settlement pattern in this area of southeast Asia.

Matisoo-Smith's decade of work with Polynesian rats has led to technical innovation and complex reconstructions of prehistoric migration and interaction. However, they have not been without problems. Results are "only inclusive and not exclusive" (Matisoo-Smith, et al. 1998a: 271). This is because "any mtDNA phylogeny present represents a descendant of a founding female, yet there may be, and most likely are, some founding lineages not represented. This can be due to both lineage extinction and sampling error" (Matisoo-Smith, et al. 1998a: 271). The other major problem is difficulty with the successful extraction and amplification of DNA sequences from the oldest faunal remains recovered from open air contexts (Robins, et al. 2001). Samples from two areas tested by Matisoo-Smith and her team have failed to yield DNA results for comparison with other Oceanic sites, these are Tonga and the Reef Santa Cruz islands (Robins,
et al. 2001). The project reported here will be the first study to use ancient chicken remains from the South Pacific for ancient DNA analyses and will explore the possibility of using older open air site samples.

The Chicken in Oceanic Prehistory

The chicken was among the suite of domesticated plants and animals which were imported to Pacific islands by the Lapita people (Delacour 1977; Green 1979, 2001; Kirch 1997; Spriggs 1997; Steadman, et al. 2002b). There is both archaeological (Kirch 1997, 2000; Kirch and Green 2001) and linguistic evidence (Green 2001; Kirch 2000; Kirch and Green 2001; Smith 1993) to support this claim. Ball (1933: 80) believed that “the reason for the resemblance of the fowls first seen wild in Tahiti by Europeans to the typical wild Gallus gallus was not a reversion from different domesticated birds, but direct descent from the original fowls transported from Malaysia by the migrating ancestors of the Polynesians.” Green (2001: 73) concurred that “there was only one breed in Polynesia, the red jungle fowl (Gallus gallus).” However, the fowl had to be domesticated for transport. Modern wild jungle fowl will not allow themselves to be confined and will kill themselves in an effort to escape confinement in cages (personal communication, I. L. Brisbin 2003).

Crawford (1984) cited linguistic similarities in names for chicken and terms which related to chicken as well as morphological similarities between chicken breeds to show Oceanic fowl was introduced from both India and China. This was supported by Carter (1971) who believed that linguistic evidence supports a dual introduction of chicken to the Marquesas from these same mainland
sources. Ball (1933: 7) asserted that irregardless of where they originated early Polynesian jungle fowl likely possessed considerable variation. He reported that in the main, phenotypic variation in Polynesia was not population specific.

**The Red Jungle Fowl (Gallus gallus)**

The first major scientific study of jungle fowl and their relationship to domestic chickens was undertaken by Darwin (1899). He believed that the red jungle fowl (or *Gallus bankiva*) was the sole ancestor to all extant breeds of domestic chicken. Delacour (1977: 119) similarly reported that “the genus *Gallus* occupies an isolated position among Pheasants and it is not closely related to any of the others.” He lists four “well-defined” species of junglefowls (Delacour 1977: 121); *Gallus gallus* (red junglefowl), *Gallus lafayetti* (La Fayette’s junglefowl), *Gallus sonerati* (Sonerat’s or gray junglefowl), and *Gallus varius* (green junglefowl). Within the red jungle fowl family he further lists five subspecies; *Gallus gallus gallus* (Cochin-Chinese Red Junglefowl), *Gallus gallus spadiceus* (Burmese Red Junglefowl), *Gallus gallus jabouillei* (Tonkinese Red Junglefowl), *Gallus gallus murghi* (Indian Red Junglefowl) and *Gallus gallus bankiva* (Javan Red Junglefowl).

The present range of wild jungle fowl extends “from northwestern India to southern China and Hainan, and south to Sumatra and Java” (Crawford 1984: 299). The original domestication of the fowl is still disputed and may have occurred in China (Ho 1977; Wood-Gush 1959), India (Crawford 1984; Davis 1997; Stevens 1991; Wood-Gush 1959), or even Indonesia (Ball 1933; Fumihito, et al. 1996; Niu, et al. 2002; West and Zhou 1989). Irregardless of the site of
initial domestication it is likely that chicken spread with people practising shifting cultivation agriculture as red jungle fowl tend to prefer these recently cleared habitats (Collias and Saichuae 1967). The Lapita peoples, as described earlier, did practise a form of swidden horticulture as part of their ‘transported landscape’ (Kirch 2000). This type of landscape is an ideal environment for red jungle fowl, perhaps partially explaining their prodigiousness in Oceanic archaeological sites and successful transport across the Pacific.

It has been postulated that the original purpose of red jungle fowl domestication was not for food but for cock-fighting (Ball 1933; Carter 1971; Collias and Saichuae 1967; Crawford 1984; Delacour 1977; Giese 1996; Stevens 1991; Wood-Gush 1959). Some evidence that their feathers were important in ritualized personal adornment is also available (Carter 1971). Crawford (1984: 300) believed that “chickens were first used for cock-fighting, later they assumed religious significance, and only much later were they utilized as a source of eggs and meat.” He further asserts that “this progression appears to have been followed in all early civilizations which possessed chickens.”

Several authors discuss the ritual/spiritual importance of chicken (Brothwell 1997; Carter 1971; Wood-Gush 1959). In Oceania archaeological evidence shows that “aside from the butchery, cooking, and consumption, other sources of bird bone modification in Polynesia related to the bones being used for tool production (needles), ornamentation (beads), and musical instruments such as flutes and whistles” (Steadman, et al. 2002a). While chickens assumed cultural significance in the form of wealth and status symbols in Melanesia, it does not appear that this same attribution was continued in Polynesian societies.
The Chicken in Oceanic Prehistory

DNA and Chicken

The principle goal of this study was to use ancient chicken remains to study human migration and interaction. Therefore it is important to understand the available information relating to chicken DNA. Studies with a focus on ancient avian DNA are numerous. These include studies of geese (Barnes, *et al.* 1998; Barnes, *et al.* 2000), New Zealand wrens (Cooper 1994), moas (Cooper, *et al.* 2001), ratites (Haddrath and Baker 2001), dodos (Shapiro, *et al.* 2002) and moa-nalos (Sorenson, *et al.* 1999). Cooper (1997: 345) asserts that “ancient DNA techniques are particularly suited to the study of avian evolution since birds make up a disproportionate number of the world’s recently extinct and currently threatened taxa.”

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is the most common target for ancient DNA studies, as the mitochondrial genome is much more abundant in the cells of animals and is small and relatively well documented. Baker and Marshall (1997: 52) found that “applications of control region sequence data to population
structure and systematics of birds are few, but have mostly been instructive in revealing the increased resolution afforded by faster mutating sequences.” Since mtDNA does not recombine and the control region has a high mutation rate it can offer much insight into population history and the evolution of maternal lines.


Shen et al. (2002) believe that the cytochrome b gene can be useful in differentiating chicken from other pheasants; it was not useful to differentiate individuals as “no variations were observed among individuals within each species” (Shen, et al. 1999a: 291). D-loop, however, is more promising. Niu et al. (2002: 166) report that among the species of the genus Gallus, the tandem duplication of one 60-base unit containing a nearly invariant tetradecameter, AACTATGAATGGTT, is found... and the copy number of the unit varies in different species. For G. varius, two copies were found in two individuals, whereas three or four copies were found in the remaining three. All specimens of G. lafayettei and G. soneratii have three copies. All subspecies of G. gallus have two copies, of particular interest is the fact that all the domestic fowl have the same copy number as G. gallus, implying that the domestic fowl likely originated from G. gallus alone.
This finding reinforced studies by Fumiho et al. (1994; Fumihito, et al. 1996; Fumihito, et al. 1995) who noted this same tetradecameter. They also noted that this region was immediately proceeded by a 50 bp region which is hypervariable in all species of the genus Gallus (Fumihito, et al. 1994; Fumihito, et al. 1996; Fumihito, et al. 1995).

**The Sea Turtle in Oceanic Prehistory**

Sea turtles and their eggs were very important in Oceanic prehistory as is evidenced by their abundance in archaeological midden deposits of Lapita and later age (Dye and Steadman 1990; Kirch 2000; Shutler Jr., et al. 1994; Weisler 1995). Ethnographically in Tonga three types are recorded as present: Green (Chelonia mydas), Hawksbill (Eretmochelys imbricata), and Loggerhead turtles (Caretta caretta) (Pritchard 1981). The Food and Agricultural Organization (FAO) guide (Marquez 1990) suggests that two others may have been occasionally present in the waters off Tonga; the Kemp’s ridley (Lepidochelys kempii), and Flatback turtle (Natator depressus) and this is also noted by Pritchard (1981) who mentions the Olive ridley (Lepidochelys olivacea) as opposed to the Kemp’s ridley.

Many "vernacular" names for turtles exist which vary from village to village, with some basic terms for turtle in the Tongan language. Hawksbill turtles are known as Fonu Koloa, Olive Ridley or Loggerhead turtles are called Tuangange, and there are several names for Green turtles which vary depending on sex, age and colouration (Pritchard 1981: 258). Green turtles, are currently most common in the Ha'apai group and while they are available year round, they
are most easily caught during the summer nesting period of November to December (Pritchard 1981). Pritchard (1981: 259) observed turtle nesting sites on only three inhabited islands in Ha’apai; Mango; ‘Uiha (liku side), and Ha’ano (Muitoa). Vava’u’s turtle population is almost identical to that of Ha’apai with nesting concentrated in the southwestern area of this group (Pritchard 1981). Tongatapu is also dominated by Green turtles with nests being reported on Malinoa Island just off of Tongatapu.

Kirch (1994) has noted the ritual importance of turtle, particularly Green turtles, to Polynesian chiefly society. The nesting of sea turtles in Futuna is in winter, at the same time as the tumeric harvest and the rising of the Pleiades, making their arrival and the opportunity to feast on them in great numbers very culturally significant. In examining ethnographic material he believed that he could reconstruct, with some degree of reliability, the importance of these to Ancestral Polynesian society based on “shared retentions.”(Kirch 1994: 285). He asserted that “archaeological evidence lends some insights to this aspect of ancient Polynesian ritual, for the earliest settlement sites on many Polynesian islands characteristically yield large quantities of turtle bone in their midden deposits” (Kirch 1994: 285). This is particularly true of Lapita sites in Tonga (Dye and Steadman 1990; Shutler Jr., et al. 1994).

**DNA and the Sea Turtle**

Like the jungle fowl no ancient DNA studies have examined sea turtles. Only one study for ancient tortoise DNA exists and was undertaken by Austin and Arnold (2001). Studies of modern sea turtle DNA are myriad and are concerned mainly with issues of conservation. The reader is referred to Cao et
al. (2000), Casale et al. (2002), Fitzsimmons et al. (1995), and Karl et al. (1992) for further information. Sequence for both D-loop and Cytochrome b are available for all species common to Tonga in GenBank.

Summary

The Kingdom of Tonga has been the subject of intense archaeological investigations since McKern (1929) in the early 1920's and has been the focus of over twenty years of research by Burley (1998). The earliest known settlement of Tonga was at the site of Nuukleka around 2950 BP and questions of the ultimate origins of these colonists and their descendants have long been of interest not only to archaeologists but also to early explorers such as Cook (1777). The advent of statistical applications of genetic data, population genetics, has led to investigations of modern DNA patterns as a means of examining these origins. Ancient DNA studies have also been undertaken (Hagelberg and Clegg 1993), but due to a dearth of ancient human skeletal remains robust reconstructions of ancient populations has proven an unrealistic goal (Kirch 1997).

Lapita settlers stocked their canoes for colonising voyages with a suite of domesticates, including dogs, pigs and chickens, as well as some commensal animals such as rats (Matisoo-Smith 1994) and skinks (Perry 1999). The abundance of these faunal remains has allowed ancient animal DNA to serve as proxy for the humans who transported them. This theory was first advanced by Matisoo-Smith et al. (1994), and was extended to examine dogs (J.S. Allen, et al. 1996) and pigs (M.S. Allen, et al. 2001). The studies of ancient rat DNA has
proven very successful, each island population having unique genetic signatures, has been in agreement with the bulk of archaeological and linguistic evidence for the colonisation of Oceania. A final critical domesticate, the chicken, has yet to be investigated for its potential to serve as proxy for human colonisation and interaction. This study was developed to examine the possibility of extracting and applying genetic data from chickens to investigate human migration in the prehistory of the tropical Pacific.
Chapter 3: Ancient DNA Analyses

As the primary goal of this project was to extract and amplify ancient DNA from Tongan chicken bones, the major portion of this project was dedicated to that purpose. From sample selection, through primer development and into laboratory techniques the goal of obtaining authentic DNA at a suitable yield was of primary concern. This chapter will outline the materials and methods used, the results of ancient DNA analyses and their implications for Oceanic Archaeology. It will also review the samples and unsuccessful attempts to extract aDNA from sea turtle.

Materials and Methods

Sample Selection: Chicken

Chicken samples for this project were selected from faunal remains recovered during the 1995 and 1997 Ha'apai Islands Lapita Project (Burley 1996, 1997) and the 1999 excavations on the island of Tongatapu (Burley and Dickinson 2001a). Primary samples of chicken were chosen by Dr. David Steadman of the University of Florida who had previously identified the bird remains. Dr. Steadman selected large elements which were relatively intact. Specimens were from distinct levels with well defined stratigraphic boundaries and represented more than one level of occupation through time. This would allow comparison with some degree of certainty that samples represented
temporally distinct populations. Twelve samples from three sites were initially selected, the sites included; Mele Havea and Tongoleleka in the Ha'apai Islands and Ha'ateiho from the main island of Tongatapu (for site locations see Figure 2, for sample details see Table 1). Photos of all samples can be found in Appendix C.

Figure 2 Locations of Sites in the Kingdom of Tonga from which chicken and sea turtle samples were drawn.
<table>
<thead>
<tr>
<th>Site</th>
<th>Unit</th>
<th>Level</th>
<th>Excavation Year</th>
<th>Element</th>
<th>SFU ID</th>
<th>University of Florida ID</th>
<th>Cultural Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>To5: Ha'atehio</td>
<td>5</td>
<td>2</td>
<td>1999</td>
<td>Tarsometatarsus</td>
<td>TA</td>
<td>UF 59519</td>
<td>Aceramic/ Disturbed</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7</td>
<td>1999</td>
<td>Femur</td>
<td>TB</td>
<td>UF 59639</td>
<td>Lapita</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9</td>
<td>1999</td>
<td>Humerus</td>
<td>TC</td>
<td>UF 59584</td>
<td>Lapita</td>
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<tr>
<td></td>
<td>9</td>
<td>5</td>
<td>1999</td>
<td>Femur</td>
<td>TD</td>
<td>UF 59748</td>
<td>Plainware</td>
</tr>
<tr>
<td>Li7: Tongoleleka</td>
<td>6</td>
<td>9</td>
<td>1995</td>
<td>Femur</td>
<td>LA</td>
<td>Li7: 54</td>
<td>Plainware</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6</td>
<td>1995</td>
<td>Tarsometatarsus</td>
<td>LB</td>
<td>Li7: 6</td>
<td>Plainware</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>Plainware</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>1995</td>
<td>Tarsometatarsus</td>
<td>Li7:38</td>
<td>Li7: 38</td>
<td>Aceramic/ Disturbed</td>
</tr>
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<td>2</td>
<td>1997</td>
<td>Tibiotarsus</td>
<td>HA</td>
<td>UF 55952</td>
<td>Aceramic/ Disturbed</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>1997</td>
<td>Femur</td>
<td>HB</td>
<td>UF 56415</td>
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</tr>
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<td></td>
<td>11</td>
<td>8</td>
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<td>Tarsometatarsus</td>
<td>HC</td>
<td>UF 56710</td>
<td>Lapita</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>13</td>
<td>1997</td>
<td>Tarsometatarsus</td>
<td>HD</td>
<td>UF 59739</td>
<td>Lapita</td>
</tr>
</tbody>
</table>

Table 1: Provenience and ID numbers for Chicken Samples Tested
For Photos of Samples Refer to Appendix C.

Cultural Associations:
Lapita 2950 to 2650 BP
Plainware 2650 to 1550 BP
Aceramic predates AD 500
Sample Selection: Sea Turtle

Using field notes and level records sea turtle remains were selected from four sites in Ha'apai as well as Ha'ateiho on Tongatapu. These specimens had previously been identified by Steadman. Samples were generally selected for their stratigraphic integrity and chronological association. Samples were also chosen based on gross morphological preservation, with minimal exposed cortical bone. To limit the possible cross-contamination of these samples each was removed using a new pair of latex gloves and then placed in an individual Ziploc bag. Table 2 provides information on samples and their provenience. Photos can be found in Appendix C. As a positive control, a sample of ancient salmon was run in tandem with all turtle samples. Unrelated tests of salmon aDNA have proven very successful (personal communication D. Yang 2003) and their inclusion provided a control of methodology.

<table>
<thead>
<tr>
<th>Site</th>
<th>Unit</th>
<th>Level</th>
<th>Excavation Year</th>
<th>SFU ID Number</th>
<th>Cultural Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi1: Mele Havea</td>
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<td>M1</td>
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<tr>
<td></td>
<td>8</td>
<td>10</td>
<td>1997</td>
<td>M2</td>
<td>Plainware</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7</td>
<td>1997</td>
<td>M3</td>
<td>Plainware/Lapita</td>
</tr>
<tr>
<td>To5: Ha'ateiho</td>
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<td>H1</td>
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<td></td>
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<td>1997</td>
<td>H2</td>
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</tr>
<tr>
<td></td>
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<td>1997</td>
<td>H3</td>
<td>Lapita</td>
</tr>
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<td>Ui4: Vaipuna</td>
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<td>5</td>
<td>1997</td>
<td>V1</td>
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<td>9</td>
<td>1997</td>
<td>V2</td>
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<td></td>
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<td>6</td>
<td>1997</td>
<td>V3</td>
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<td>T1</td>
<td>Aceramic</td>
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<tr>
<td></td>
<td>8</td>
<td>10</td>
<td>1997</td>
<td>T2</td>
<td>Lapita/Plainware</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>12</td>
<td>1997</td>
<td>T3</td>
<td>Lapita</td>
</tr>
<tr>
<td>Fo1: Faleloa</td>
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<td>7</td>
<td>1997</td>
<td>F1</td>
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<tr>
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<td>17</td>
<td>12</td>
<td>1997</td>
<td>F2</td>
<td>Lapita</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>9</td>
<td>1997</td>
<td>F3</td>
<td>Plainware/Lapita</td>
</tr>
<tr>
<td>Ha1: Pukotala</td>
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<td>1</td>
<td>1997</td>
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<td>Aceramic/disturbed</td>
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<tr>
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<td>9</td>
<td>7a</td>
<td>1997</td>
<td>P3</td>
<td>Lapita</td>
</tr>
</tbody>
</table>

Table 2: Provenience and ID number for Turtle Samples Tested.
Chapter 3: Ancient DNA Analyses
Materials and Methods

Gross Preservation of Samples

Chicken samples were in good morphological condition (Appendix C). They were of different sizes and represented a variety of elements. Sample TA is noted as being very small. Sample HA, HB and TD were noted as having adhering soil/ sand in the bone cavity. This was left mostly intact, in hopes that it might have helped to preserve aDNA and have some endogeneous aDNA bound to it. Samples HB and TB were quite friable and the shafts of these bones broke into fragments when sawed into sections a second time. Li7:17 was in an excellent state of preservation. This tibiotarsus had thickened medullary bone and no remaining cavity, indicating that it was a laying hen (D. Steadman, personal communication, 2001).

As was stated in the sample selection procedure for turtles, samples were preferentially selected to be relatively intact, all bones appeared to be solid and in a good state of preservation (see Appendix C). Samples H1, T2 and V2 had relatively exposed spongy bone and were not exposed to acid/ base decontamination. P3 was discoloured and may have been burned, it was very difficult to crush into bone powder.

Primer Development: Chicken

The D-loop of mtDNA was targeted to design primers for the chicken samples as it not only can provide species identification but also holds the potential to separate individuals/ populations from one another. The first set of chicken primers designed were used to determine whether PCR products were jungle fowl. This was facilitated by a repeat that occurs in the chicken genome
as a conserved sequence present in all species of *Gallus*. However, the frequency of the repeat varies in number between red, green, gray and ceylonese jungle fowl, with only two repeats in the red variety. Examination of modern *Gallus* sp. sequences downloaded from GenBank by the author revealed that the 'invariant tetradecameter' sequence being targeted was not fourteen but twenty-five nearly invariant base pairs which did indeed repeat as reported by Fumihito *et al.* (1994) and Niu *et al.* (2002). The repeat is then;

\[ \text{TAACTATGAATGGTTACAGGACATA} \]

with the original fourteen base-pairs identified by Fumihito *et al.* (1994) and Niu *et al.* (2002) in red. This was a crucial recognition which formed the basis of the reverse primer for the first primer set developed. PCR primers are designed to be between 18 to 25 bp in length. The 25 bp repeat is a perfect target for primers and allows for the amplification of several bands in suitably preserved DNA fragments.

A second and third set of primers were later developed to extend the sequence obtained by the first set and to confirm the authenticity of positive ancient DNA samples. The second set embeds in the first and the third set uses the reverse primer of the second set as the forward primer (see Table 3 for primer sets and sequences). It was anticipated that the degraded state of ancient DNA might prevent amplification of longer DNA fragments with two or more repeats. If this was the case, subsequent sequencing with abutting primer sets would reveal species identity and individual variations. However, the amplification of longer fragments might also indicate better DNA preservation in these remains. The first runs of any new primer set included a sample of K-562 (modern human DNA) to ensure that the primers would not bind to, and amplify.
human mitochondrial DNA. This test excludes the possibility that any human
DNA introduced to samples could be misinterpreted as authentic red jungle fowl
DNA.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position 5’</th>
<th>Sequence of Primer</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGF1</td>
<td>161</td>
<td>ACCCATTATATGTATACGGGCATTAA</td>
<td>Forward</td>
</tr>
<tr>
<td>GGR1</td>
<td>305 and 365</td>
<td>TGTCCTGYAACCATTCATAGTTAGGTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>GGF2</td>
<td>333</td>
<td>AAACAGTCACCTAATGTTAGGTTAC</td>
<td>Forward</td>
</tr>
<tr>
<td>GGR2</td>
<td>625</td>
<td>ACAGATAATCCACAGATGCCTG</td>
<td>Reverse</td>
</tr>
<tr>
<td>GGF3</td>
<td>599</td>
<td>CACGAACTCATCTGTGGATTATCTGT</td>
<td>Forward</td>
</tr>
<tr>
<td>GGR3</td>
<td>847</td>
<td>TTTGTGAGGGGAGTTATGGGG</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

**Table 3:** Primer Sets and Sequences for Amplification of the D-loop Region of *Gallus gallus*. Y = C or T. 5’ Position listed according to Desjardins and Morais (1990) reference sequence NC_001323.1

**Primer Development: Sea Turtle**

After consulting the FAO Guide on the world distribution of turtles (Marquez 1990); five species were identified as having the potential to be present in the waters surrounding the Kingdom of Tonga; *Caretta caretta* (the Loggerhead Turtle), *Chelonia mydas* (the Green Turtle), *Eretmochelys imbricata* (the Hawksbill Turtle), *Lepidochelys kempii* (Kemp’s Ridley Turtle), *Natator depressus* (the Flatback Turtle) and *Dermochelys coriacea* (the Leatherback Turtle). Sequences for D-loop and Cytochrome b were downloaded from GenBank for comparison and primer development. It was a surprising observation that the conserved regions of D-loop were better shared across species than those found in Cytochrome b. Primers which would target all or any of the five possible species were developed using sequences from GenBank and examined using BioEdit (T.A. Hall 1999) Software. See Table 4 for resulting primers.


Table 4: Primer Sets and Sequences for Amplification of D-Loop and Cytb for Sea Turtle.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position 5'</th>
<th>Sequence of Primer</th>
<th>Position 3'</th>
</tr>
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<tbody>
<tr>
<td>CYTB-ST-F47</td>
<td>47</td>
<td>CTACCAAAACCTACGAAAAAACCAACC</td>
<td>1258</td>
</tr>
<tr>
<td>CYTB-ST-R252</td>
<td>252</td>
<td>TCAKCCGTATTGATCRTCTCGGGGTGA</td>
<td>1739</td>
</tr>
<tr>
<td>DLOOP-ST-F382</td>
<td>382</td>
<td>TCACGAGAAATAAGCAACCCTTGTTAG</td>
<td>1739</td>
</tr>
<tr>
<td>DLOOP-ST-535</td>
<td>535</td>
<td>ATGTATAGAACTCATTYACARAGGC</td>
<td>1739</td>
</tr>
</tbody>
</table>

Y = C or T, R = A or G.

Sample Decontamination, Preparation and Incubation

Sub-sets of both chicken and turtle samples were chosen for initial extraction experiments. One sample from each site was selected as being the most recent (highest level) and a second was chosen based on relative size (the largest representative sample being preferentially chosen). This was done as it was thought that size and age might each have an influence on the ability to successfully extract and amplify DNA. Each element was documented and then cut in half using a clean, new hacksaw blade. Half was resealed in its original bag and placed in the freezer for repeat experiments and the other half was decontaminated. Decontamination began with surface polishing. A piece of sandpaper soaked in a 10% bleach solution (mixed with ultrapure dH₂O, pH 7) was used to polish the surface of the bone, to remove surface contaminants and adhering soils from excavation. Next the specimen was submersed in a test tube with a 10% bleach solution to dislodge any contaminants adhering inside the bone cavity or which were difficult to reach through polishing. After a short soak the bleach solution was poured off and ultra pure water was added to rinse the bone fragment (for later repeats of chicken extractions the liquid decontamination step was completely removed in hopes of increasing the yield of DNA fragments available for decontamination). The water was poured off and
approximately 3mL of 1N Hydrochloric acid (HCl) was added to further decontaminate the specimen. The HCl was quickly poured off and approximately 3mL of 1N Sodium Hydroxide (NaOH) was added and again quickly poured off. A final rinse of ultra pure water was added to the tube to wash away residues of the decontaminating chemicals which might inhibit PCR reactions later in the process. As a final measure of decontamination bone fragments were laid on labelled weighing trays and subjected to UV light (254 nm) in the UV crosslinker for thirty minutes on each side. Fragments were then left in the open crosslinker over night to dry. The second set of chicken bones was similarly prepared with one extra step. After liquid decontamination ethyl alcohol was poured over the specimens to facilitate quicker drying during exposure to UV light in the crosslinker.

Turtle bones were subjected to the same rigorous decontamination procedures. All samples except H1, T2 and V2 were subjected to decontamination with HCl and NaOH and a second rinse of ultra pure water. The aforementioned samples were not treated with strong acid and base as they were composed primarily of spongy bone and there was a concern that this decontamination procedure was too rigorous for the survival of endogenous DNA. Dried bones were ground using a nut and bolt assembly with a wrench used for torque as detailed in Thomas and Moore (1997). Each sample was processed with a new assembly and the resulting powder was placed in a labelled tube. After all samples had been processed in this way the bone powder was submersed in a lysis buffer (approximately 0.5M EDTA, pH 8.0, 0.5% Sodium Dodeyl Sulfate and 0.2 mg/mL protanase K). Approximately 3mL
of Lysis buffer was added for each half gram of bone powder. Tubes were sealed with Parafilm™, covered with Kim Wipes™ and the lids wrapped in tin foil to prevent leaks and contamination of other samples in a rotating incubation oven. Tubes were placed in larger glass incubation tubes and secured inside the incubation oven. The temperature was set at 50°C and left to incubate overnight.

The second set of chicken bones prepared were similarly run, except the nut and bolt assemblies from the prior experiment were cleaned using HCl. This resulted in some rust forming within the threads of the assemblies. A small rust coloured supernant was observed after centrifugation of the incubated samples.

**DNA Concentration and Extraction: Centricon™ Method**

The initial run of chicken samples followed the method for concentration and extraction identified as Protocol C by Yang *et al.* (1998). After incubating, the tubes were placed in a centrifuge and spun until the bone powder was conglomerated at the bottom of the tube allowing the liquid portion to be poured into a labelled 1.5mL tube. These smaller tubes were then spun again at 14,000 RPM's for 20 minutes to ensure that all bone powder was pelleted at the bottom of the tube and would not enter the Centricon™ 30 (Millipore, Billerica, MA, USA) tubes. Centricons™ were assembled and all three parts were labelled with the sample lab ID number. Alliquoits from the 1.5mL tubes were poured into the centre part of the Centricon™ assembly and all joints were sealed with Parafilm™ to prevent leaks. The assembled Centricons™ were then spun in a
fixed angle centrifuge for 90 minutes to 2 hours until approximately 100µL of liquid remained in the centre assembly. The latter part of the Centricon™ was removed and the liquid disposed of. The remaining 100µL of liquid was resealed into the Centricons™ and spun quickly at 14,000 RPM’s.

QIAquick Columns™ (QIAGEN, Mississauga, ON, Canada) were then labelled with the laboratory sample numbers and the liquid was pipetted out of the Centricons™ into the column. 500µL of PB Buffer was added to the column and spun in the centrifuge for one minute at 12,000 RPM’s. The waste product was disposed and the Column reassembled. 500µL of PE Buffer was added to the silica membrane and spun again for 1 minute at 12,000 RPM’s. The waste was emptied and the procedure repeated. The bottom portion of the assembly was discarded and replaced with a fresh new 1.5mL tube, 50µL of EB Buffer was pipetted onto the silica membrane, the tubes incubated at 50°C for ten minutes and then spun for two minutes at 14,000 RPM’s. The resulting product was pipetted out of the tube and placed into a new tube.

The second set of chicken bones prepared followed the same procedure except 50µL extra EB Buffer (total 100 µL) was added to increase the possible yield of extracted DNA.

Concentration and Extraction: Amicon™ Method

The Amicon™ (Millipore, Billerica, MA, USA) method allows for a greater volume of incubated product to be concentrated. This method was tried as part
of an experiment to increase yields of DNA products (unpublished data Yang 2003). The Amicon™ replaces the Centricon™ but works in a similar way. Amicons™ were used to process six samples of chicken (HC, HD, LA, LB, TC and TD) and all samples of turtle. For chicken samples a test was run to test differences between Centricons™ and Amicons™ resulting in the preparation and digestion of two-thirds as opposed to half of an available bone sample. In Amicon™ tests, approximately 3 to 4mL of incubated product was poured into the tubes and spun for a period of 90 minutes to 2 hours until between 50 and 100μL of sample remained above the membrane. This was removed with a pipette and the purification protocol, as detailed above, followed. During this step samples LA and TD from the Amicon™ experiments were confused, it is likely that this was remedied but cannot be proven without repeats with secondary samples. Turtle samples T1, H2 and M1 were problematic in their extraction from the Amicon™, as during centrifugation they resulted in a thick substance which did not easily flow through the filter and were difficult to remove using the pipette. These samples were subjected to only one elution with 50 μL EB Buffer with all other samples eluted twice in hopes of increasing DNA yield.

**Polymerase Chain Reaction (PCR) Setup: Chicken**

A variety of PCR conditions were used for Tongan chicken samples. While some samples responded well to an annealing temperature of 60°C others
worked well at 55°C. A PCR Master Mix was prepared using the following volumes of solution per 50µL reaction; 5µL Buffer II X 10, 2mM MgCl₂, 0.2 mM dNTP, 0.3µM Forward Primer, 0.3µM Reverse Primer, 1.5 mg/mL BSA, 1.25 U (or 2.0 U for best results) AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 5µL ancient DNA sample and Ultra Pure water to make the volume equal 50µL. PCR conditions were set with an initial denaturing of 95°C for 12 minutes followed by 60 cycles consisting of a 30 second denaturing at 95°C, a 30 second annealing cycle at 55 to 60°C depending on the sample, an extension cycle of 72°C for 30 seconds. A final extension cycle took place at 72°C for seven minutes. PCR conditions for each run of chicken can be found in Tables 5 to 7. PCR products were run on a Dark Reader™ Electrophoresis System (Clare Chemical Research, Dolores, CO, USA) on a 1% to 2% Agrose Gel (0.5g to 1g Agrose and ~49 mL 1X TBE buffer). 5 µL aliquots PCR Products were mixed with 5 µL Blue Juice and run on the gel. After electrophoresis finished gels were submerged in a SYBR Green™ (Clare Chemical Research, Dolores, CO, USA) Solution, to fluoresce the DNA fragments.
### Chapter 3: Ancient DNA Analyses

#### Materials and Methods

**Table 5:** PCR conditions for Primer Set One. Format is temperature@ number of cycles (number of units)X(extra Taq). 1 denotes a band of correct length, (S) is a sequenced sample.

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**Table 6:** PCR conditions for Primer Set Two. Format is temperature@ number of cycles (number of units)X(extra Taq). 1 denotes a band of correct length.

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**Table 7:** PCR Conditions for Primer Set Three. Format is temperature@ number of cycles (number of units)X(extra Taq).

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Polymerase Chain Reaction (PCR) Setup: Sea Turtle

PCR Master Mix and basic setups followed the same protocols as chicken samples. During the first run of the primers, as with chicken, K-562 (modern human DNA sample) was run to ensure the primers did not bind to human mtDNA. Salmon was also run with several of the tests to ensure extraction procedures were adequate. For a list of all PCR conditions tested please see Tables 8 and 9.

### Table 8: PCR Conditions for Cyt B Sea Turtle Primers

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*Table 8: PCR Conditions for Cyt B Sea Turtle Primers. Format is temperature@ number of cycles (number of units) X (extra Taq). 1 denotes a band of correct length, x signifies sample was not run. ES1 is the ID for the salmon sequence.*

### Table 9: PCR Conditions for D-loop Sea Turtle Primers

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*Table 9: PCR Conditions for D-loop Sea Turtle Primers. Format is temperature@ number of cycles (number of units) X (extra Taq). 1 denotes a band of correct length, x signifies sample was not run.*
Purification and Sequencing of PCR Products

Using QIAquick columns (QIAGEN™, Mississauga, ON, Canada) a preliminary binding of DNA is done with 200µL PB Buffer and after this is spun off, two washes of 400µL PE Buffer is executed and a final release of DNA is done with 60µL of EB Buffer incubated prior to centrifugation. Five µL of sample and five µL of diluted forward primer was sent to MOBI at McMaster University for sequencing. For some samples, an extra step was added to the purification protocol. The new protocol followed the same basic procedures but the final flow through of EB Buffer was left to evaporate in the heat block at 70°C until the liquid had completely evaporated. 5 to 10µL of EB was then added to reconstitute the sample prior to running it through electrophoresis to check whether purified products are adequate for sequencing. This step also saw the addition of a mass ladder for more accurate and observable quantification of purified DNA products.

Repeat/ Reproducibility Experiments

Five samples were repeated from initial bone preparation to amplification of PCR Products. Samples selected for repeat were HA, HB, HD, LA and TB, with other samples submitted for independent replication and 'collagen' extraction. The protocol for the primary extractions was followed with a few exceptions. Bone decontamination was limited to mechanical abrasion with 10%
bleach solution on sandpaper. It was believed that the acid/base liquid
decontamination was too strenuous for these delicately preserved remains and
that more template DNA may be available if this step was skipped. A blank
extraction was included. PCR protocols followed the same pattern as before,
with three extra units of Taq and an annealing temperature of 55°C and 60°C.

Independent Repeat: University of Auckland

Repeat experiments were undertaken at the University of Auckland on two
of the three samples sent for that purpose. Samples TB and HB were prepared
and tested by Judith Robins at the Ancient DNA Laboratory at Auckland as
follows. Sample HB was split and cleaned. A portion (0.1g) was prepared using
the phenol chloroform method used to process rat samples (Matisoo-Smith, et al.
1997), and the remainder (0.27g) was processed using a silica based method
(Yang, et al. 1998). HB was processed using only the silica based method. A
sample of Chatham Island Rattus exulans was run in parallel of all samples as a
positive control of methods. Four different PCR conditions were attempted on
extracted DNA. The first using AmpliTaq at an annealing temperature of 50°C at
45 cycles. The second using AmpliTaq Gold at 50°C with 45 cycles. The third
and fourth followed the most successful protocols for our experiments with
annealing temperatures of 55°C and 60°C at 60 cycles. The Polynesian rat
sample amplified in every experiment but no bands of expected length were observed for chicken repeats. The third sample submitted, LA, was not run due to the complete lack of positive indicators for the first two. Further destruction of sample material was deemed unnecessary by the lab.

Results

PCR Amplification and Sequencing of Chicken DNA Samples

Results from the first set of extractions of chicken remains were among the most successful. Fragments produced from amplification with primer set one generated sequences from two samples; HB and TD. The electrophoretograms for these can be found in Figures 6 and 7. Two distinct bands were visible on the electrophoretic gel, demonstrating that the primer sets did indeed bind to the 25 base-pair repeat and were likely chicken. Since all jungle fowl species have at least two repeats, and ancient DNA is often

Figure 3 Gel showing double banded pattern in sample HA (not a sequenced sample).
Chapter 3: Ancient DNA Analyses

Results

Figure 4 Gel showing a test of all three primer sets with an annealing temperature of 55°C.

... degraded, the presence of two bands upon electrophoresis demonstrates that the amplified product is a jungle fowl, but does not guarantee that it is of the red variety. The appearance of double bands was not limited to sequenced samples. An illustration of the double banded result is shown in Figure 3. As illustrated in Table 5 (page 36), banding even of the most responsive samples was sporadic. HB responded most strongly to an annealing temperature of 55°C while other samples preferred 60°C (this can be seen in figures 4 and 5). Often PCR reactions were prepared as 50μL solutions and split into two separate PCR
Chapter 3: Ancient DNA Analyses

Results

Figure 5 Gel showing test of all three primer sets at an annealing temperature of 60°C. Note the complete absence of HB for primer set one.

reactions to examine this phenomena. Figures 4 and 5 have the same constituents of master mix and same volume of extracted DNA with the only difference between them being annealing temperature. The cause of this differential amplification has yet to be determined. For comparative purposes 55 D-loop sequences from *Gallus* species, particularly red jungle fowl and domestic chicken were downloaded from GenBank, these multiple alignments appear in Appendix A. Only sequences which were geographically
provenienced within the database were used to compare with amplified sequences from ancient Tongan chicken. Unfortunately, most provenienced samples were of Southeast Asian origin and the extent to which this skews the data is unknown. Table 10 provides a complete list of GenBank samples, their codes and provenience. Figure 8 provides a simplified alignment of sequenced ancient Tongan remains with other jungle fowl. As can be seen from this alignment the two sequences are very similar and are clearly of the red jungle fowl line as opposed to gray or green. HB appears to be distinct from TD at position 219 (numbers based on the Desjardins and Morais (1990) Reference Sequence NC_001232.1), however, this cannot be viewed in absolute terms as no other sequence data for these samples have ever been produced to verify this base difference.
Chapter 3: Ancient DNA Analyses

Results

Figure 6: Electropherogram of Sample TD
Figure 7: Electrophoretogram of Sample HB
Figure 8 Multiple Alignments of ancient sequences with those of Red, Gray and Green Jungle Fowl, Domestic Chicken and the Desjardins and Morais (1990) Reference Sequence (RefSeq) NC_001323.1.
PCR Amplification of Sea Turtle DNA Samples

No band of expected length was observed upon electrophoresis of sea turtle from any sample. Neither Cytochrome b nor D-loop was amplified for any of the eighteen samples of sea turtle processed. Often the gel showed ‘Pseudo-bands’ of approximately 100 bp in length, as can be seen in Figure 9. A few of these were sent for sequencing but matched nothing on the BLAST (Altschul, et al. 1997) database and could not be aligned with modern turtle sequences from GenBank using ClustalW (Lopez and Lloyd 1997). Extracted salmon DNA produced strong bands in every run of PCR at all conditions, demonstrating that the methodology for extraction was not to blame for the absence of sea turtle PCR products. An experiment to extract ancient DNA from Indian Ocean tortoises reported similar results. Austin and Arnold (2001: 2518) reported no success with samples from “volcanic caves and sand dunes.” They were successful with a few other samples recovered from a marsh but had equal

![Figure 9](image-url)  
*Figure 9* ‘Pseudo-bands’ observed for sea turtle. B identifies a blank extraction, N a negative control.
difficulty with remains from a sandy matrix. Further experimentation is needed to discover if any level of endogenous DNA can be extracted from Tongan sea turtle samples.

Authentication of Results

The field of ancient DNA has been plagued with results which are questionable and standardized authentication procedures have been proposed in order to determine if aDNA sequences are genuine. Several papers have been written outlining standards for authenticating ancient DNA. The protocol accepted here is set out by Cooper and Poinar (2000).

Authentication standards should include a physically isolated work area, control amplification (avoiding positive controls due to possibility of contamination), small template molecules, sequences which make phylogenetic sense, reproducibility, cloning to determine the ratio of endogenous to exogenous sequences, independent replication, independent evidence such as amino acid racemization, quantification through competitive PCR and replication of sequences from associated remains. These are listed and appropriately checked off in Table 11 to show that authentication procedures have been followed. Each of these will be discussed in turn as they were applied to current experiments.

The Ancient DNA Laboratory at Simon Fraser University consists of two
laboratories in two separate buildings on campus. The first laboratory, in the Archaeology department, has three rooms (a bone preparation area, a DNA extraction area, and a PCR set-up area) which are not only physically separated but operate on a positive air flow system. The PCR laboratory is in the adjacent Robert Brown Classroom Complex and does not share the same ventilation system. Both laboratories make use of disposable plastic supplies and protective clothing such as hooded suits, 95 Particulate masks and latex gloves which are worn at all times. Modern sea turtle and chicken samples were never used as positive controls. However, as a means of scrutinizing extraction protocols a salmon vertebrae was included in the sea turtle extractions and functioned in place of a positive sea turtle control. The two sequences which did result from the experiments were indeed of small template size (approximately 150 - 200 bp) and longer fragments have yet to be successfully sequenced from either sea turtle or chicken samples.

<table>
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<th>Unsuccessfully Applied</th>
</tr>
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<td>Control Amplification</td>
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<tr>
<td>Hylogenetic Sense</td>
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<tr>
<td>Independent Repeat</td>
<td>✓</td>
<td>✓</td>
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Table 10: Authentication Procedures and Their Application
During the course of experimentation several potential bands were sent for sequencing, but only two made phylogenetic sense and were positively identified as being of the *Gallus* sp. Bands observed in subsequent tests of Primer Set One for HB and TD were purified and sent for sequencing but both produced nonsense sequences when run through BLAST (Altschul, *et al.* 1997). Sequences returned from BLAST were patchworks of DNA from several different species, including mice, bacteria and human nuclear genes. The electrophoretogram for the repeat of HB can be seen in Figure 10. This sequence cannot be used to validate the previous sequence produced for sample HB.

Two methods were applied to independently support evidence for the authenticity of chicken DNA sequences. Direct evidence through amplification of ancient DNA in another associated species, or through reproduction of acquired sequences and indirect verification through a secondary measure. Sea turtle remains were chosen to use as the associated remain to demonstrate the probability that chicken sequences were authentic. Unfortunately, the sea turtles failed to yield amplifiable fragments of ancient DNA. Five samples of chicken were used in an attempt to replicate results of the first set of experiments from bone preparation through purification within the Ancient DNA Laboratory at SFU. These remains were tested only once and while results appeared to be positive upon electrophoresis of amplified products none survived the purification
Figure 10: Electrophoretogram of Repeat for Sample HB
protocol to be sequenced. Three other samples of chicken have been sent out to the University of Auckland for independent replication; with the two tested (TB and HB) failing to positively amplify PCR products. "Collagen" yields were used to examine a parallel molecule for preservation and these tests and their results are examined in detail in Chapter 4.

There are, at this stage, three major indicators of authentication. Sequenced fragments tend to cluster with Chinese domesticates, not the European domesticates used for commercial poultry operations. Contamination controls in place are excellent and no chicken had ever been tested in the laboratory prior to these experiments. Finally the "collagen" yields show a parallel pattern of preservation in chicken, sea turtle and rat remains.

**Implications for Oceanic Archaeology**

**Sequence Analysis**

Sequences obtained for ancient Tongan chicken were compared using Clustal W (Lopez and Lloyd 1997) and MEGA2 (S. Kumar, *et al.* 2001) software. These programs involve methods of sequence alignment in which sequences are compared statistically and base pairs are lined up based on matching sections. Phylogenetic trees were constructed from aligned sequences, using statistical methods to compare them. These are expressed graphically depending on the selection of a distance method to show the fraction of sites that differ between
two sequences (B.G. Hall 2001). Bootstrapping is used to refine the data, or examine the multitude of possibilities more closely. This method examines sub-samples of sites in an alignment and creates graphical representations (or trees) based on these sub-samples, this is repeated many times (in this case 1000) and creates a statistically based estimate of the reliability of the tree (B.G. Hall 2001).

Upon analysis, the sequences from HB and TD grouped with other red jungle fowl and domestic chicken. They tended to cluster more closely with Chinese breeds of fowl. A UPGMA tree constructed using the

![Figure 11 NJ Tree Showing only the Chinese and Indonesian branches which relate to ancient Tongan samples.](image)
Figure 12 Neighbour Joining Tree, Bootstrap Consensus. Squares show a Chinese Origin, Triangles Indonesian, Diamonds India. Red for RJF, Green for GJF and Gray for GJF. Key for ID's can be found in Table 11.
Figure 13 UPGMA Tree. Squares show a Chinese Origin, Triangles Indonesian, Diamonds India. Red for RJF, Green for GJF and Gray for GJF. Key for ID's can be found in Table 11.
<table>
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<tr>
<th>Storey ID</th>
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Table 11: Sequences of *Gallus sp.* from GenBank, citing author and geographical provenience.
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Table 11: Sequences of _Gallus_ sp. from GenBank, citing author and geographical provenience.
Kimura 2-parameter, analysing both transitions and transversions and only complete deletions at 1000 Bootstraps; HB, the sample from the Mele Havea site, groups with Chinese domestic breeds of fowl while TD, the sample from the Ha'ateiho site, forms another group with mainly Chinese specimens as can be seen in Figure 11. Using the same parameters with a Neighbour Joining Tree shows a similar pattern of clustering of ancient Tongan samples with those from China (see Figure 11 for Chinese branches including Tongan samples, Figure 12 for full tree).

While the sample size of sequences from Tongan sites is much too small for significant conclusions to be drawn, it is interesting that preliminary sequences group with those from Chinese domesticates. This information supports Crawford (1984) and Carter's (1971) assertions that Pacific Island chicken may have been introduced from China and India. Crawford (1984) based his findings on morphological similarities in chicken and similarities in Pacific Island languages for words relating to chickens, while Carter (1971) cited linguistic similarities alone. Since no provenienced samples from India could be identified on GenBank there is no way to examine that aspect of introduction in this study. A Southeast Asian origin for the Lapita peoples (or at least aspects of their culture) is well supported by both archaeological and modern genetic research. Studies of modern genetic affinities for human populations certainly suggest Taiwan was an important place in the development of Proto-
Austronesian culture (Melton, et al. 1998; Melton, et al. 1995; Su, et al. 2000; Sykes, et al. 1995; Yao, et al. 2000) and linguistic evidence also points there, through connections with the Formosan culture (Kirch 1997; Spriggs 1998). Domesticated chicken, believed to be of Chinese origin has been found in ancient sites on the island of Halmareha (Bellwood 1997), which is a place of great interest for the dispersal of *Rattus exulans* (Matisoo-Smith and Robins 2002) into the South Pacific as part of the Austronesian expansion. However, it is crucial to view these results as preliminary, and while encouraging, further inferences cannot be based on these phylogenies yet. As Houghton (1996: 159) once cautioned: “A few loci do not a phylogeny make.”
Chapter 4: Collagen Analyses

Sea turtle remains were initially selected for ancient DNA analyses in hopes of authenticating the presence of chicken aDNA sequences. The inability to successfully amplify any sea turtle DNA instead supported the assertions of Robins et al. (2002) that aDNA could not be preserved in Tongan ‘open air’ sites. Another means was needed to investigate the differential preservation of chicken bioolecules; not dependant on the extraction of ancient DNA. The most common method for assessing this type of preservation is the measurement and quantification of specific amino acid racemizers (Poinar and Stankiewicz 1999, Bada et al. 1999, and Poinar et al. 1996). More recently Gotherstrom et al. (2002) have suggested that the preservation of ‘collagen’, as expressed as a percentage yield from ancient bone and aDNA may be coincident. Both Gotherstrom et al. (2002) and Matheson and Brian (2003) suggest this may be due to similarities in structure between the two molecules.

Poinar and Stankiewicz (1999) used Amino Acid Racemization to examine collagen content in ancient samples and found that in many samples the prominent amino acid peaks were dominant in collagen side chains; suggesting “collagen” / proteins might make an excellent proxy for DNA survivorship but would not guarantee it. A comparison of nitrogen values (a proxy for the
quantification of collagen) and histological preservation to indicate DNA survival was undertaken by Colson et al. (1997). They found only a weak correlation between protein and DNA preservation but noted that one did exist. Gotherstrom et al. (2002) tested both archaeological and artificially degraded bones for amplification of DNA products, yields of "collagen" and X-ray diffraction analysis (XRD) to assess the crystallinity of hydroxyapatite. They found there was a correlation between all three variables and concluded that the crystallinity index and overall yield of "collagen" could be used to estimate the likelihood that ancient DNA was also preserved in ancient specimens.

Radiocarbon dating had previously been successfully undertaken on contemporaneous samples of chicken, iguana and megapode bone from the site of Tongoleleka by Steadman et al. (2002b), thus suggesting that collagen was preserved within these types of faunal remains. Beta Analytic, who undertook the testing of bone for Steadman et al. (2002b), state that typical collagen yields from ancient bone are between 2 to 3% (Darden Hood, personal communication, 2003) and this level is required for successful radiocarbon dating. In order to assess the possibility of biomolecular preservation potential of sea turtle bone, two samples were submitted to the Stable Isotope Laboratory at SFU for a quantification of 'collagen' yield. When the tests showed these samples were virtually devoid of ancient 'collagen', chicken and rat samples were submitted as well in order to assess the possibility of using collagen as a proxy for aDNA.
preservation in tropical open air sites. This chapter will present the results of those analyses and a discussion of the parallel pattern of preservation observed between them.

Gross Morphology as an Indicator of aDNA Preservation

Figure 14 shows the macroscopic differences between three types of bones examined for aDNA from Tonga. Long bones from sea turtle, Vaipuna site (V3), chicken, Tongoleleka site (Li7:17) and Polynesian rat, Mele Havea site are shown. The chicken bone is thick due to the addition of medullary bone in the laying stage (King and McLelland 1984). It is clear that the sea turtle and chicken have similar macroscopic preservation, while the rat is lacking all medullary bone (the break is fresh and was made by the author).

Gross morphology of bone is not a good indicator of DNA preservation in
these burial conditions. Several of the large turtle bones were structurally intact, yet yielded no DNA. Many of the chicken bones were quite friable and those that yielded DNA were not those that would have been necessarily predicted to do so. Similar results were obtained by Haynes et al. (2002) in their study of archaeological remains of British geese. They concluded that relationships between gross and histological preservation and the survivorship of DNA is not as highly correlated in avian remains as it is in mammalian remains.

Materials and Methods: Collagen Analyses

Two samples of sea turtle (T2 – carapace fragment and H3 – long bone fragment), three samples of chicken (HC, Li7:38 and TA; tarsometatarsus fragments), and four samples of rat (FR1, MR1, MR2 and PR1; femurs) were submitted to the Stable Isotope Laboratory at SFU for “collagen” extraction (Takahashi and Nelson 2000). A modified protocol of Brown et al. (1988) was used to extract “collagen” from the samples. Powdered extracts of samples are soaked in acid, then solulized in a weak acid and finally ultra-filtered to trap high molecular weight collagen (>30 kD). See the reports produced by Takahashi in Appendix B, Takahashi and Nelson (2000) and Brown et al. (1988).

Turtle remains were the first samples subjected to “collagen” analyses. After tests of sea turtle “collagen” yield showed a similar pattern of preservation between absence of high quality “collagen” and amplifiable DNA it was decided
that chicken and rat remains must be tested as well. One chicken bone from each site was retrieved from the Ancient DNA Laboratory and submitted to the Stable Isotope Laboratory. After obtaining provenience information for rat samples tested by Robins et al. (2001), samples were sought from these sites to submit for “collagen” testing. Unfortunately, samples of rats could not be obtained for the same units and levels tested by Robins et al. (2001) and a compromise was made. Samples were taken from adjacent units which had stratigraphic integrity with those tested by the team at Auckland, assessed using level notes and profile drawings. For this test the sample from Pukotala was taken from Unit 9 at the same level, 6, and the same stratum, Ilb, which also had excellent stratigraphic integrity, as material from unit 14 or an adjacent unit was unavailable. Results of the tests on chicken and rat samples are available in Table 12 and Appendix B.

Results

Table 12 summarizes results for “collagen” yield as reported by the Stable Isotope Laboratory at SFU. Listed is provenience information, amount of bone processed and the amount of “collagen” extracted both as a total weight in milligrams and a percentage of total bone weight represented by “collagen”. The percentage (% Yield) value is typically used to assess the quality of a collagen sample. Examination of results does show a preferential preservation of
collagen in chicken remains and parallels findings of aDNA extractions, this will
be addressed in detail in the discussion section of this chapter.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Faunal Type</th>
<th>Site</th>
<th>Unit Level</th>
<th>mg Bone</th>
<th>mg &gt; 30 kD</th>
<th>% Yield (&gt;30 kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>sea turtle</td>
<td>Ha'ateiho</td>
<td>2</td>
<td>5</td>
<td>93.93</td>
<td>0.04</td>
</tr>
<tr>
<td>T2</td>
<td>sea turtle</td>
<td>Tongoleleka</td>
<td>8</td>
<td>10</td>
<td>146.15</td>
<td>0.05</td>
</tr>
<tr>
<td>HC</td>
<td>chicken</td>
<td>Mele Hovea</td>
<td>11</td>
<td>8</td>
<td>120.22</td>
<td>1.98</td>
</tr>
<tr>
<td>Li7: 38</td>
<td>chicken</td>
<td>Tongoleleka</td>
<td>5</td>
<td>2</td>
<td>154.33</td>
<td>0.00</td>
</tr>
<tr>
<td>TA</td>
<td>chicken</td>
<td>Ha'atilo</td>
<td>5</td>
<td>2</td>
<td>148.62</td>
<td>0.85</td>
</tr>
<tr>
<td>FR1</td>
<td>rat</td>
<td>Faleloa</td>
<td>17</td>
<td>7</td>
<td>78.70</td>
<td>0.16</td>
</tr>
<tr>
<td>MR1</td>
<td>rat</td>
<td>Mele Hovea</td>
<td>5</td>
<td>3</td>
<td>34.32</td>
<td>0.23</td>
</tr>
<tr>
<td>MR2</td>
<td>rat</td>
<td>Mele Hovea</td>
<td>5</td>
<td>8</td>
<td>143.33</td>
<td>0.00</td>
</tr>
<tr>
<td>PR1</td>
<td>rat</td>
<td>Pukotala</td>
<td>9</td>
<td>6</td>
<td>199.21</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 12: Results of "collagen" yield tests for the 30kD microfilter. Brackets indicate a second extraction.

Relationship Between Collagen and DNA Degradation

Figure 15 The Structure of Collagen showing covalent bonds between amino acids of the same chain and hydrogen bonds between Glycines. Also triple helix structure.

Figure 16 The Structure of DNA. Showing hydrogen and covalent bonds as well as the double helix structure.
Collins et al. (2002: 389) declare that “a key test of any screening tool is that the character being used in screening is sensitive to the same factors as the target molecule DNA.” Collagen, in conjunction with histological index have been said to register the effects of diagenesis (Hedges 2002), making it a useful tool to assess organic and molecular preservation. Similarities in the helical structure, bonding and degradation of both collagen and DNA by hydrolysis, oxidation, extremes of pH and microbial/ enzymatic action (collagenases and DNases) (Matheson and Brian 2003) also suggest that these two biomolecules will respond to site taphonomy in a like manner. Figure 15 and 16 illustrate these similarities. Both collagen and DNA have been estimated to survive at least 10,000 years in average burial environments; (see Lindahl 1993 and Bada et al 1999 for DNA and Rowley 1986 for collagen). Both are also thought to be more prone to degradation in tropical environments due to heat and humidity (Ambrose 1990; S.S. Kumar, et al. 2000; Reed, et al. 2003; Robins, et al. 2001; van Klinken 1999).

Implications of “Collagen Yield”

Measurements of amino acids and nitrogen values quantify all available collagen regardless of the level of degradation due to the sensitivity of the methods. Procedures for determining the yield of “collagen” employ the same methods used to concentrate aDNA molecules; retention in a 30kDa ultrafilter. For the purpose of this discourse emphasis is placed on the yields from the 30
Chapter 4: Collagen Analyses
Relationship Between Collagen and DNA Degradation

kD microfilters and not 10 kD even though both results are available from the lab. This is because the filters have the same molecular cutoff as the Amicons™ and Centricons™ (Millipore, Billerica, MA, USA) used in the Ancient DNA Laboratory. These should be the most informative in the comparison of products, as the quantified fragments of “collagen” and DNA are all above the cut off.

Employing high quality extraction techniques Takahashi and Nelson (2000) believe “collagen” yields must be over 1% in quantity to be considered of high enough quality to submit for radiocarbon and stable isotope analyses. Only two of the chicken samples, reported in Table 13, tested contained a satisfactory amount of “collagen”. The first, a tarsometatarsus from Mele Havea yielded 1.65% from the first extraction through a 30kD filter. The second, also a tarsometatarsus, did not yield a significant level of “collagen”, in the first extraction. The second extraction resulted in a yield of 0.98%. None of the sea turtle nor the rat show the same degree of preserved high quality protein. There is also one chicken sample Li7:38 which has a yield of zero. It is very interesting to note that sample HC has more than twice the amount of “collagen” as TA, even though the original sample weighed 28.40 mg less. DNA analysis reveals that HC produced bands of expected length twice as opposed to once for TA. This single set of results may indicate that in some situations the amount of “collagen” may be directly related to the amount of aDNA templates of suitable size for amplification. The sample size is much too small for any conclusions to
be drawn based on this observation, however, future experiments should examine any possible link between “collagen” yield and quantity of available aDNA template for amplification.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Faunal Type</th>
<th>Proxy for Bands of expected length observed</th>
<th>% Yield (&gt;30 kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>sea turtle</td>
<td>N/A</td>
<td>0.04</td>
</tr>
<tr>
<td>T2</td>
<td>sea turtle</td>
<td>N/A</td>
<td>0.03</td>
</tr>
<tr>
<td>HC</td>
<td>chicken</td>
<td>N/A</td>
<td>1.65</td>
</tr>
<tr>
<td>Li7: 38</td>
<td>chicken</td>
<td>N/A</td>
<td>0.00</td>
</tr>
<tr>
<td>TA</td>
<td>chicken</td>
<td>N/A</td>
<td>0.57 (0.98)</td>
</tr>
<tr>
<td>FR1</td>
<td>rat</td>
<td>Faleloa</td>
<td>0.20</td>
</tr>
<tr>
<td>MR1</td>
<td>rat</td>
<td>Mele Havea</td>
<td>0.67</td>
</tr>
<tr>
<td>MR2</td>
<td>rat</td>
<td>Mele Havea</td>
<td>0.00</td>
</tr>
<tr>
<td>PR1</td>
<td>rat</td>
<td>Pukotala</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 13: Comparison of “Collagen” Yield and PCR-DNA Amplification. The second extraction yield is reported in brackets for sample TA.

“Collagen” tests employed after aDNA experiments suggested differential preservation of biomolecules among taxa. As can be seen in Table 13 “collagen” results closer to the 1% yield range can be correlated with greater frequency of positive amplification of PCR products, e.g. sample HC had a yield of 1.65% and resulted in positive indicators for aDNA amplification in two separate runs of PCR. Conversely, when collagen yields were very low this could be correlated with a very low rate or complete lack of indicators for DNA preservation, e.g. sample H3 with a yield of 0.04% never produced bands of expected length after PCR. Table 13 also clearly shows that a low yield or absence of collagen is
correlated with a lack of positive indicators for aDNA preservation. The conclusion drawn from this data is that due to similarities in structure between collagen and DNA taphonomic processes such as oxidation and hydrolysis similarly degrade the chains and create situations where similarly sized fragments persist. The unique structure of avian remains may allow for the preservation of longer aDNA fragments which can be retained in a 30Kd ultrafilter. In other remains such as sea turtle, rat and some chicken the degradation is more advanced and potentially preserved fragments are much smaller, and are therefore not retained. The number of successful positive PCR amplification indicators can be correlated with “collagen” yield in the samples examined. This, therefore, provides a better indicator for probable success than observation of gross morphological preservation. While the correlation between “collagen” yield and positive indicators for aDNA amplification is not an authentication measure per se, these results are suggestive of a parallel pattern of preservation and support the findings of Gotherstrom et al. (2002).
Chapter 5: Discussion and Conclusions

Taphonomic and Technical Challenges

The successful positive amplification and sequencing of two ancient chicken samples was a welcome and largely unexpected outcome of this project, given indications from studies of contemporaneous rat from Tonga (Robins, et al. 2001). Current results potentially imply that ancient DNA information may be available in other avian samples. Before work can proceed on other ancient avian samples from open air sites the factors which contributed to the low success rate observed in these experiments requires investigation and evaluation. The low frequency of positive results is due mainly to the effects of taphonomy and a genuine lack of available template molecules for extraction. In some cases, however, technical issues may have played a role in the inhibition of successful positive amplifications. The contributing factors of each must be understood and critically evaluated to increase the chance of future success.

Taphonomic Challenges

The variable results for amplification of specific ancient DNA sequences is an indicator of the low amount of available aDNA template material. PCR products from chicken experiments banded in some instances and not in others.
While the most successful PCR conditions involved three extra units of Taq Polymerase per sample, and annealing temperatures of 60°C (most samples) and 55°C (HB and HA were particularly strong in this condition), even samples which banded strongly and often were not present in every experiment. This is mainly due to degradation of the remains, resulting in template molecules of sufficient size being low in quantity and not available in each and every run of PCR. It has been shown by Robins et al. (2001), Kumar et al. (2000) and Reed et al. (2003) that tropical environments increase the degradation rate of DNA template molecules. Yet even in this harsh environment the effects of taphonomy were not equal between or even among taxa and some template molecules were preserved in sufficient strength for amplification and sequencing.

At this stage it is unclear exactly which suite of variables are responsible for differential preservation of biomolecules. Further investigation is required but was not within the scope of this study. Testing should be done to quantify these differences over time as has been done in other studies which deal with differential preservation of faunal remains (Nicholson 1996). Particularly important is an examination of how much longer avian bone protects biomolecules as compared to other types of faunal material. The role of adhering sand or soil in preservation will also be imperative to examine in future. It would be useful to test each and every remain for both “collagen” yield and, using mass ladders or competitive PCR, quantify the available aDNA templates.
In this way it would be possible to discern if there is a strong correlation between quantity of “collagen” and amplifiable DNA and if this is species dependant. Future work should include samples of iguana, as the skeletal microstructure should be similar to chicken, reptiles and birds being closely related have like skeletal physiology (King and McLelland 1984). Radiocarbon analyses (Steadman, et al. 2002b) indicate some level of “collagen” is preserved in chicken and iguana remains from the site of Tongoleleka. The present study did not result in the successful amplification and sequencing of any chicken remains from this site nor was “collagen” of high quality observed to exist in the tested sample (Li7: 38) from that site. Samples from the same units and levels tested by Steadman et al. (2002b) should be closely examined as the indicators for successful aDNA amplification may exist.

Not all remains subjected to aDNA experiments were tested for the presence of “collagen” and this might change the observed correlation between quantity of “collagen” and number of positive aDNA amplifications. It is certainly intriguing that collagen yields varied so widely in chicken remains. Future tests on ancient Oceanic avian remains, which include “collagen” yield information as suggested above would also demonstrate if there is differential preservation among skeletal elements. In this study all chicken remains tested for “collagen” yield were tarsometatarsus fragments perhaps indicating differential preservation is not element dependant but rather site/ context dependant.
Technical Challenges

In some cases positive indicators for the presence of aDNA in chicken samples was noted and the inability to sequence these samples is not a result of taphonomy alone but also of the technical challenges for obtaining quality results. For future experiments it is important that current technological issues be explored in an attempt to find a more efficient method to extract the maximum amount of information from highly degraded samples. The following technical issues must be taken into account including: decontamination procedures, efficiency of extraction and purification protocols, primer development and human error. They will be addressed in the following sections with suggestions for remediation.

Decontamination Procedures

It is possible that the decontamination using HCl and NaOH was too strong and further degraded or destroyed existing aDNA templates. While this can be an important decontamination step, in cases of highly degraded DNA the benefits are likely outweighed by the risks of total destruction of endogenous DNA. Future studies might benefit from the removal of this step for at least a portion of the remains to see if a greater volume of amplifiable DNA results. This step also effectively removed adhering sands from HA and TD, but not completely from HB (as can be seen in Figure 21). If adhering sand plays a part
in preservation or stabilization of DNA as I suggest later this can be seen as a detrimental effect of HCl decontamination.

**Efficiency of Extraction Protocols**

A variety of protocols were instituted in hopes of increasing efficiency. Changes included the substitutions of Amicons™ for Centricons™ (Millipore, Billerica, MA, USA) in the concentration step. Unfortunately no sample run through an Amicon™ has yet produced a band of suitable length or resulted in a sequence which was that of a chicken. It is unclear why Centricons™ and Amicons™ did not produce similar results. The laboratory was equipped with Centricon™ 30 and Amicon™ 4mL Ultra-4 (30kD) which should have the same molecular weight cutoff. Therefore, it seems unlikely that there is a difference in the retention of the columns. Amicons™ were adopted for their ability to concentrate greater quantities of incubated product in less time and therefore more DNA should have been retained by this method. Sample TD did not produce bands for Amicons™ in side by side tests of the two methods yet the smaller volume run through the Centricon™ produced a sequence. As a result the Amicon™ method can be said to be much less efficient in the retention of ancient chicken DNA fragments and should not be employed in the concentration of remains with highly degraded DNA.
Efficiency of Purification Protocols

In several cases strong bands of expected length were produced by chicken samples after electrophoresis of PCR products, however, these bands did not survive the purification process to allow for submission for sequencing. Samples HA and HC were never successfully sequenced even though they produced bands of appropriate length more often than sample TD, which only presented one positive PCR amplification result. This problem became more pronounced after the addition of the evaporation step to the purification procedure. While the addition of the evaporation procedure cannot be the primary reason for a succession of failed purification experiments, it seems to be a contributing factor. In future tests the purification step should only be employed in cases where no bands of sufficient strength are observed in the original concentration of purified products. This may prove a more efficient use of materials in future experiments.

Primer Development and Application

Failure of primer sets two and three for chicken remains may be due to unsuitable development or length of designed primers. In order to test the ability of these primers to bind to chicken DNA they should be run using modern chicken DNA samples for ability to bind to target sequences and efficiency. If the primers are well designed this may indicate that they target too long a
Chapter 5: Discussion and Conclusions
Technical Challenges

Fragment for the degree of degradation in the current set of remains; primer set two targets a fragment equal to 266bp and primer set three targets a fragment equal to 248bp. These are over 100 bp longer than the target sequence of primer set one. In either case the addition of these fragments to the sequence obtained would have been very informative and should be included in future experiments.

Human Error

Human error also played a part in the inability to produce results. Two runs of PCR were performed with tubes in the wrong slots. This resulted in the evaporation of some samples due to opening of the tubes during heating and cooling cycles. Many samples survived intact and were run as normal. Also problematic was the mislabeling of two samples during the first set of chicken extractions. This was caught at the time and rectified to the best of our ability. Since these results were from tests of Amicons™, which did not result in successful DNA retention this is not an important problem. Samples from the parallel Centricon™ tests were not confused. Finally the decontamination of the bolts used to process the first set of chicken samples caused the development of a rusty residue. There is a possibility that this contributed to PCR inhibition during the second run of samples but is unclear at this juncture. Human error can never be completely removed but in future these particular mistakes can be
more carefully considered and avoided.

Implications for Ancient Biomolecular Research

Differential Preservation of Biomolecules Among Taxa

The parallels between the presence/absence of aDNA products and “collagen” yields for sea turtle, chicken, and rat, (DNA results as reported by Robins et al. (2001)), are most likely a result of bone structure and its behaviour in this particular burial environment. It is hypothesized that this correlation of aDNA and “collagen” results is a reflection of the differential preservation potentials of taxa recovered from open air sites. Rowley et al. (1986) also reported differential preservation of “collagen” in avian remains. They found that a contemporaneous sample of kangaroo and emu from the same Australian dune context resulted in greater reactivity of the emu to antibodies used to target collagen.

Davis (1997) studied the effects of organic activity or bioerosion on bird bones. He found that organismic activity resulted in “tunnels [that] do not appear to penetrate further than approximately 0.2 mm into the outer lamellar cortical layers” (Davis 1997: 397). Haynes et al. (2002) believe that avian bone is different from mammalian bone in that it is degraded from the inside out. Nicholson (1996) posits that the vascular surfaces of mammalian bone makes it more susceptible to microbial attack than that of birds. She observed that
experimentally degraded rat and bird bones had a similar appearance to the naked eye (Nicholson 1998). Yet, upon microscopic examination the rat was found to have pitting and channelling similar to that of larger mammal bones while the bird had none. She did report the observation of areas of focal destruction in avian bone but reported that they were random across larger skeletal elements such as the femur.

Mammals, sea turtles (Reptilia), and birds differ in the structure of their cancellous bone. Mammals have Haversian systems, turtles have non-Haversian vascular systems and birds have no vascular canals in their cancellous bone except in the epiphyseal disk. In the medullary bone of birds neither the hydroxyapatite minerals nor collagen fibres are oriented as they are in mammals and reptiles (King and McLelland 1984). The medullary bone of avian species also has less collagen than is found in cortical bone (King and McLelland 1984). Balzer et al. (1997) specifically refer to the role of Haversian or Volkmann canals in the ability of micro-organisms to enter and subsequently degrade bone. The lack of canals likely acts to inhibit microbial attack of avian collagens and may convey better protection to protein and DNA from damage by all factors.

In all bones the structure and orientation of collagen determines pore size. Pore size is important as it is these which allow the micro-organisms responsible for a great deal of decomposition of both bone protein and minerals to enter.
“Hole zones” (gaps) in the collagen fibril are located between the ends of molecules, while ‘pores’ are located between the sides of parallel molecules” (Sherry 1997). Pore size must be >8nm for the collagenases to enter (Nielsen-Marsh, et al. 2000). Since pores are defined as the spaces between the parallel collagen fibrils, chickens have no or at least very few pores in their bones. In this respect avian bones are at an immediate taphonomic advantage for the protection of collagen and perhaps also DNA as micro-organisms are inhibited from entering the bone matrix. This also inhibits the breakdown of the mineral matrix as the acids secreted by the micro-organisms are virtually absent. The mutual protection of collagen and hydroxyapatite also serves to protect the mitochondria and their DNA which are part of this package (Glimcher 1984). This is clearly not a perfect system, as extensive DNA damage has most certainly occurred in the samples tested from Tongan archaeological contexts. Because not all chicken remains resulted in fragments of DNA which produced sequences, positive amplifications, or good quantity “collagen” yield the structure of bird bone alone is not sufficient to ensure DNA preservation but may slow down degradation in particular burial environments or contexts.

**Binding of DNA to Mineral Surfaces**

Matheson and Brain (2003) have suggested that minerals of silica and calcium carbonate can bind to and protect collagen. Poinar and Stankiewicz
(1999: 8430) found that "molecules may be altered once they are cross-linked, adsorbed to minerals, and/or trapped within glycosylation end products resulting in preferential preservation." Lagaly (1981) reported the binding of nucleotides to mica-type layer silicates dependant on the presence of salts. DNA has also been shown to bind to clays in the presence of salts (Goring and Bartholomew 1952; Greaves and Wilson 1969, 1970). With and without salt it has been shown the DNA which is bound to a soil mineral is better protected from the effects of degradation by DNases and nucleases (Lopez and Lloyd 1997; Lorenz et al. 1981).

Romanowski et al. (1991: 1057) found that "adsorption to mineral components of sediments and soil renders DNA resistant to nucleolytic inactivation." They demonstrated this by running known amounts of DNA through columns of pure sea sand, buffered in a salt solution, and then added DNase I. Adherence to sand decreased degradation of DNA. The addition of low levels of MgCl assisted in the binding and they noted that "the low bivalent cation concentrations required for adsorption were in the range present in natural environments such as groundwater" (Romanowski, et al. 1991: 1060). Aardema et al. (1983) also found that low concentrations of NaCl in a slightly basic solution helped DNA to bind to sea sand, and this binding conferred resistance to degradation by enzymatic attack.

The conditions used in laboratories to bind DNA to silica (see Höss and
Pääbo 1993; Yang, et al. 1998) is mimicked in beach settings world wide, and thus it is notable that the Lapita peoples had a preference for back beach settlements. These environments provide sea sand in a salt rich environment and within a shell midden context which has a more basic pH overall. As Romanowski (1991) demonstrated most groundwater has sufficient MgCl\textsubscript{2} to further assist the process of mineral-DNA binding in sand.

During our experiments it was noted that three specimens of chicken, HA, HB and TD, had sand/ sediment adhering to the interior of the bone surfaces.

![Figure 17](image.png)

**Figure 17** Sample HB after decontamination procedures, sandy soil still adheres to the inside surface of the bone.

Figure 21 shows chicken sample HB after surface abrasion, liquid decontamination and exposure to UV; soil still adheres to the inside. Observations of the gross morphology of this bone would not suggest it was more likely to yield amplifiable DNA, as it was quite thin and was not a complete
or intact bone having either epiphyses to decrease exposure to agents of degradation. It seems more than coincidental that of the three chicken samples observed to have adhering sands, two were successfully sequenced and one produced very strong bands with some consistency. No turtle samples were noted as having adhering sands and none of the rat samples submitted for "collagen" yield analysis were observed to have adhering sediments. It is unknown if rat samples tested by Robins et al. (2001) had sand filled cavities.

Preservation of biomolecules is not due to the presence of sand alone, but when considered as part of an already preferential environment for preservation it may have facilitated the extraction and successful positive amplification of aDNA from chicken samples.

Implications for Archaeology

Open air sites in Oceania are characteristic of human settlement in the area and are crucial to our understanding of migration and in the region. The availability of ancient chicken DNA from these sites may provide an analogous line of evidence to other work with commensal and domesticated animals (J.S. Allen, et al. 1996; M.S. Allen, et al. 2001; Matisoo-Smith 1994; Matisoo-Smith and Allen 2001; Matisoo-Smith, et al. 1997; Matisoo-Smith, et al. 1998a; Matisoo-Smith, et al. 1998b).

It is possible that the unique set of variables found in open air, shell
midden sites may help to preserve collagen and DNA in ancient avian samples. Therefore, until this can be more fully investigated sand filled cavities should not be cleaned in hopes that the silicate minerals will help to stabilize the DNA, not only during internment but perhaps in the post excavation disturbance in equilibria. This new information may also be useful in the investigation of other sites in similar environments such as India (S.S. Kumar, et al. 2000), Sri Lanka (Reed, et al. 2003) and Africa (Ambrose 1990) where hope for DNA preservation had been all but lost.

**Chicken DNA as an Analog to Human Migration and Interaction**

The initial goal of this project was to investigate the preservation potential of ancient chicken DNA in Tongan sites and assess its utility in the tracing of human migration and interaction. The dearth of ancient faunal DNA from open air sites in Oceania (Robins, et al. 2001) is a great hindrance to developing robust models based on mtDNA reconstructions, as has been pioneered by Matisoo-Smith *et al.* (1994; 1999) using data from rats. Preliminary sequence analysis of two chicken samples shows an affinity of ancient Tongan chicken to samples from Asia. With further examination of technical challenges and the ever changing technology available to the study of ancient DNA more data may be available in the near future. The addition of open air site data may enrich understanding of relationships between island groups in prehistory and about the importance of chicken to trade and exchange in Oceanic society.
Due to the abundance of chicken remains in Oceanic sites several important questions can be addressed in future. Firstly will the widespread testing and sequence analysis of chicken remains reflect the patterns seen in rat data? If there are observed differences are these due to lineage extinctions and exclusive data (Matisoo-Smith 2002) or is it more indicative of the nature of trade and exchange of these two animals? Can the cultural values ascribed to these different animals be partially examined using genetic signatures and phylogenies? Chicken from a variety of sites and environments must be sought out and tested to examine their utility in addressing archaeological questions through application of ancient DNA data.

Conclusions

This project has evolved significantly from its original inception. An idea that began with the analysis of ancient human DNA to investigate ancestry became a study of biomolecular preservation in tropical open air sites. Complications associated with the study of ancient human DNA in terms of contamination and authentication led to the exploration of the potential of domestic chicken DNA to serve as a proxy to the same end. Work by Matisoo-Smith et al. (1997) showed the utility of faunal DNA analyses as an analog to understanding human migration and interaction, yet domestic chicken remains had yet to be tested. Rat remains from Tonga had proven to be unsuccessful as
aDNA was not preserved in sufficient quantity for amplification (Robins, et al. 2001). Suggestions that avian remains degraded differently than mammalian remains by Davis (1997) and Nicholson (1996) provided hope that aDNA might be preserved in the Tongan chicken remains despite the findings of Robins et al. (2001). After tests of chicken remains resulted in the positive amplification and sequencing of two fragments of chicken DNA another line of evidence was needed to show that these sequences were not contamination. Sea turtle samples were sought from the same site contexts and tested, but failed to show that DNA was preserved in these samples. With rat and sea turtle DNA evidence that did not support the preservation of aDNA in chicken samples a different biomolecule was needed to assess the veracity of a theory for differential preservation. Steadman et al. (2002b) had used chicken remains from the Tongoleleka site for radiocarbon dating, which demonstrated that collagen was preserved in these remains. This coupled with the findings of Gotherstrom et al. (2002) which found a level of correlation between aDNA preservation and the successful extraction of collagen and discussion by Matheson and Brian (2003) that collagen and DNA are similar in structure and response to major taphonomic events, such as hydrolysis and oxidation, led to the application of "collagen" extraction tests. These tests have resulted in a suggestive parallel pattern of preservation between aDNA and "collagen" which warrants further investigation.
Several contributions to ancient DNA, biomolecular research and archaeology have been made. These include; a new understanding of the patterning in jungle fowl mtDNA, innovations in primer design, extension of the concept for differential preservation in gross morphology to the molecular level, the role of tropical environments and beach contexts in preservation and parallels between ""collagen"" yield and positive amplification of aDNA products.

The results remain preliminary and will require further work to be fully substantiated. Supported by other researchers such as Gotherstrom et al. (2002) the coincidence of available ""collagen"" and aDNA may prove an important proxy for future consideration. Analysis of the two available chicken sequences reveals a strong affinity with Chinese domestic breeds and many more samples of ancient Oceanic chicken should be sought out and tested to validate this result and more fully investigate the utility of domestic chicken for tracing human migration and interaction.

The bulk of Lapita and later sites (over 80% as reported by Kirch and Hunt (1988)) are "open" sites located on beaches and dominated by shell midden deposits. The avian remains in these sites may provide a niche for DNA preservation. Mass extinction of many types of birds during the Lapita expansion has been of great interest to many researchers and the results of this study imply that extinct ratites and other unique avian species may yield aDNA for study. It also indicates that other "open" air and tropical sites may have remains which
are differently preserved and this should be investigated with a larger suite of faunal remains. Armed with a new insight into the suite of variables which may lead to the increased *preservation potential* of aDNA in bone from "open" air contexts, these applications may be closer than ever before.
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Appendix A
Alignment of GenBank Jungle Fowl/ Chicken Samples
For Provenience and GenBank ID Refer to Table 11
Sequences Have Been Appended to 480bp.
Appendix A

Multiple Alignments of GenBank Jungle Fowl/Chicken Sequences
## Multiple Alignments of GenBank Jungle Fowl/Chicken Sequences

### Sequence Alignments

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### Alignment Results

- **RefSeq** column shows the reference sequence.
- Columns represent different GenBank sequences: Ggdom44, Ggdom41, Ggdom40, Ggdom11, Ggdom10, Ggdom12, Ggdom14, Ggdom13, Ggdom18, Ggdom16, Ggdom15, Ggdom17, Gg24, Ggdom45, Ggdom47, Ggdom39, Ggdom46, Gg28, Gg25, Ggspad1, Ggspad6, Ggspad4, Ggspad5, Ggdom5, Gg1, Ggdw2, Ggdw4, Ggdw43, Ggdw42, Ggspad2.

### Table Structure

- Columns: `70`, `80`, `90`, `100`, `110`, `120` represent positions in the alignment.
- Rows: `Gg22`, `Gg23`, `Ggson1`, `Cc`, `Gvar3`, `Gg13`, `Gg20`, `Gg27`, `Gg28`, `Gvar1`, `Gvar2` indicate GenBank entries.

### Sequence Comparison

- `CCC TTT CCC` is a common sequence pattern across the alignments.
Appendix A
Multiple Alignments of GenBank Jungle Fowl/Chicken Sequences
Appendix A

Multiple Alignments of GenBank Jungle Fowl/Chicken Sequences
### Appendix A

#### Multiple Alignments of GenBank Jungle Fowl/Chicken Sequences

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Appendix B
Laboratory Reports for “Collagen” Analyses
To: Dongya Yang and Alice Storey  
Re: Bone Samples (H-3, T-2)  
From: Cheryl Takahashi  

Oct 15, 2003

What follows is a summary of the collagen extraction procedures performed on the above samples to determine the presence or absence of high molecular weight collagen.

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<td>H-3</td>
<td>Ha'ateiho '99, Unit II, Level 5, St III; sea turtle</td>
</tr>
<tr>
<td>T-2</td>
<td>Tongoleleka '95, Unit 8, Level 10; sea turtle</td>
</tr>
</tbody>
</table>

Sample Preparation

Samples for collagen extraction were obtained using a low-speed drilling tool fitted with a carbide bur or twist drill. Sample H-3 consisted of small fragments of spongy bone and drilling yielded a coarse, brittle powder. T-2 consisted of a single was soft and chalky in texture and drilling yielded a fine velveteen powder. About 100–150 mg of each sample was subjected to our standard collagen extraction procedure which is designed to extract high molecular weight, insoluble collagen from bone. In short, this procedure begins with a soak in weak acid to remove autochthonous bone mineral and any post-depositional mineralization. The insoluble proteinaceous remnants are rendered soluble in very weak acid by shaking at a modest temperature for several hours. The resultant solution is then ultra-filtered and freeze-dried to isolate and concentrate the high molecular-weight protein remnants. In this case both the >30 kD fraction and the >10 kD fraction were isolated and freeze-dried. The collagen extraction results are shown in the table below.

Collagen Extraction Results

<table>
<thead>
<tr>
<th>SFU ID</th>
<th>mg Bone</th>
<th>mg &gt;30 kD Extract</th>
<th>% Yield</th>
<th>mg &gt;10 kD Extract</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-3</td>
<td>93.93</td>
<td>0.04</td>
<td>0.04</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td>T-2</td>
<td>146.15</td>
<td>0.05</td>
<td>0.03</td>
<td>0.12</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Well-preserved high-molecular weight collagen has a sponge-like appearance and is usually white to off-white in colour. The extracts obtained from H-3 and T-3 appeared as faint white smears on the bottom of the sample vials. Typical collagen yields for moderately well-preserved samples range from about 5 to 10%. Samples with high molecular weight collagen yields of less than 1% are considered to be highly degraded. The poor physical appearance and the very low yields of the submitted samples, (H-3, T-3) indicate that these samples did not contain any well-preserved collagen that would be suitable for radiocarbon dating or stable isotope analysis.
To: Alice Storey
Re: Bone Samples (FR-1, PR-1, MR-1, MR-2, CH-1, CH-2, CH-3)
From: Cheryl Takahashi

Nov. 5, 2003

What follows is a summary of the collagen extraction procedures performed on the above samples to determine the presence or absence of high molecular weight collagen.

The Samples

<table>
<thead>
<tr>
<th>SFU ID</th>
<th>Submitter Sample Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR-1</td>
<td>Faleloa, Unit 17, Level 7, Rat femur, &lt;FR1&gt;</td>
</tr>
<tr>
<td>PR-1</td>
<td>Pukotela, Unit 9, Level 6, Rat femur, &lt;PR1&gt;</td>
</tr>
<tr>
<td>MR-1</td>
<td>Mele Havea, Unit 5, Level 3, Rat femur, &lt;MR1&gt;</td>
</tr>
<tr>
<td>MR-2</td>
<td>Mele Havea, Unit 5, Level 8, Rat femur, &lt;MR2&gt;</td>
</tr>
<tr>
<td>CH-1</td>
<td>Ha'afeva-HF-1, 1997, Unit 11, Level 8, Gallus gallus, tarsometatarsus UF 56710</td>
</tr>
<tr>
<td>CH-2</td>
<td>Tongolelka-1995, Li7:38, Unit 5, Level 2, Gallus gallus, tarsometatarsus</td>
</tr>
<tr>
<td>CH-3</td>
<td>Tongatapu-To5, Unit 5, Level 2, Gallus gallus, tarsometatarsus, UF 59519</td>
</tr>
</tbody>
</table>

Sample Preparation

Samples for collagen extraction were obtained using a low-speed drilling tool fitted with a carbide bur or twist drill or using a stainless steel press. The rat femurs (FR-1, PR-1, MR-1, MR-2) were all similar in physical appearance - orange-brown in colour with a hard, lustrous surface. Adhering surface contamination was removed with a wire brush and scalpel blade. These bones were too small to drill so they were crushed to a coarse powder using a stainless steel press. The chicken bones (CH-1, CH-2, CH-3) were cream-coloured, and soft and chalky in texture. These were sampled with the drill and the drillings were a fine velveteen powder. About 35–150 mg of each sample was subjected to our standard collagen extraction procedure which is designed to extract high molecular weight, insoluble collagen from bone. In short, this procedure begins with a soak in weak acid to remove autochthonous bone mineral and any post-depositional mineralization. The insoluble proteinaceous remnants are rendered soluble in very weak acid by shaking at a modest temperature for several hours. The resultant solution is then ultra-filtered and freeze-dried to isolate and concentrate the high molecular-weight protein remnants. In this case both the >30 kD fraction and the >10 kD fraction were isolated and freeze-dried.

Samples PR-1, MR-2, CH-1, CH-2, and CH-3 had large amounts of insoluble material after the standard solubilization step. These samples were ultra-filtered in the usual manner, but the insoluble material was subjected to the solubilization procedure a second time, to see if any more protein could be extracted. After this second solubilization the samples were ultra-filtered (30 kD) and freeze-dried as before. The collagen extraction results are shown in the table below.
Collagen Extraction Results

<table>
<thead>
<tr>
<th>SFU ID</th>
<th>mg Bone Extract</th>
<th>mg &gt;30 kD (&gt;30 kD)</th>
<th>% Yield</th>
<th>mg &gt;10 kD (&gt;10 kD)</th>
<th>% Yield</th>
<th>mg &gt;30 kD (&gt;30 kD)*</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR-1</td>
<td>78.70</td>
<td>0.16</td>
<td>0.20</td>
<td>0.00</td>
<td>0.00</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PR-1</td>
<td>199.21</td>
<td>0.07</td>
<td>0.04</td>
<td>0.33</td>
<td>0.17</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>MR-1</td>
<td>34.32</td>
<td>0.23</td>
<td>0.67</td>
<td>0.10</td>
<td>0.29</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MR-2</td>
<td>143.33</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>CH-1</td>
<td>120.22</td>
<td>1.98</td>
<td>1.65</td>
<td>0.13</td>
<td>0.11</td>
<td>0.38</td>
<td>0.32</td>
</tr>
<tr>
<td>CH-2</td>
<td>154.33</td>
<td>0.00</td>
<td>0.00</td>
<td>0.19</td>
<td>0.12</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>CH-3</td>
<td>148.62</td>
<td>0.85</td>
<td>0.57</td>
<td>0.06</td>
<td>0.04</td>
<td>1.45</td>
<td>0.98</td>
</tr>
</tbody>
</table>

* Refers to the >30 kD fraction of the second solubilization.

Well-preserved high-molecular weight collagen has a sponge-like appearance and is usually white to off-white in colour. While the >30 kD extracts for FR-1, MR-1 (first solubilization), CH-1, and CH-3 (first and second solubilization) had this appearance, all remaining extracts appeared as faint white smears on the bottom of the sample vials. Typical collagen yields for moderately well-preserved samples range from about 5-10%. Samples with high molecular weight collagen yields of less than 1% are considered to be highly degraded. CH-1 is the only sample that could be considered for radiocarbon dating or stable isotope analysis based on the quality of the extract and the >1% yield. All remaining samples had either poor physical appearance or very low collagen yield, or both, indicating that none contain well-preserved collagen that would be suitable for radiocarbon dating or stable isotope analysis.
Chicken Samples
Appendix C
Photos of Samples Prior to Processing
Chicken Samples
Sea Turtle Samples

F1

F2

F3

H1

H2

H3
Appendix C
Photos of Samples Prior to Processing
Sea Turtle Samples