MORPHOLOGICAL AND MOLECULAR APPROACHES TO CHARACTERISE MODIFICATIONS RELATING TO MAMMALIAN HAIRS IN ARCHAEOLOGICAL, PALEONTOLOGICAL AND FORENSIC CONTEXTS

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THIS THESIS IS PRESENTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOLOGICAL SCIENCES

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DECLARATION

I declare that this is my own account of my research and contains, as its main content, work that has not previously been submitted for a degree at any tertiary education institution.

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ABSTRACT

Mammalian hair is readily shed and transferred to persons or objects during contact; this property renders hair as one of the most ubiquitous and prevalent evidence type encountered in forensic investigations and at ancient burial sites. The durability and stability of hair ensures their survival for millennia; their status as a privileged repository of viable genetic material consolidates their value as a biological substrate. The aims of this thesis are to showcase the wealth, and breadth, of information that may be gleaned from these unique structures and address the current problem regarding the mis-identification of animal hairs.

Despite the similar appearance of human and animal hairs, the expertise required to accurately interpret their respective structures requires significantly different skill sets. Chapter Two in this thesis discusses the consequences of mis-identification of hair structures due to lack of competency or adequate training in regards to hair examiners and discusses some of the myths and misconceptions associated with microscopy of hairs.

Hairs are resilient structures capable of surviving for millennia as exemplified by extinct megafauna hairs; however, they are not totally immune to deleterious effects of environmental insults or biodegradation. There is a paucity of morphological data available illustrating the deleterious effects of biological agents on hairs. This void is filled through the comprehensive review of biodegradation of hair in Chapter 3 which showcases, for the first time, a collective visual catalogue of the destructive effects caused by an elite group of biological agents. These effects were evident in hairs from prehistoric, ancient and modern human and animal hairs.

During the course of this study hair morphologies were observed which challenged current paradigms in relation to their genesis. The results of the present study unequivocally demonstrate that hair structures, previously characterised as genetic in nature, are due to the effects of biodegradation. Furthermore, this body of work is the first to record that morphological characteristics previously ascribed to taphonomy (post-mortem insults), also occur in hairs from the living. The implications of the interpretation of hair structures in forensic investigations are discussed in greater detail in Chapter 3.
Chapter four represents the first demonstration of the advantages of adopting a multidisciplinary approach to hair examination. This chapter presents a detailed microscopical audit of extinct megafauna hair that was the remains of a larger hair sample previously consumed in more destructive analyses (molecular analyses and radiocarbon dating). One of the most significant morphological finds of this work was the presence of unusual structural features, not previously recorded, that may have been central to the survival of extinct megafauna.

Lastly, Chapter Five introduces the concept of Next Generation Sequencing (NGS also referred to as massive parallel sequencing) to investigate the forensic potential of human hairs on the basis of transfer of their respective bacterial ‘profiles or signatures’.

Taken together, this body of work presents fresh approaches to the manner in which mammalian hairs could be processed in the future and demonstrates the benefits of multi-disciplinary approaches to their examination. Bacterial DNA profiles, derived from human hair using NGS technologies, may prove to be a valuable future addition to the forensic molecular toolkit. Furthermore, this present study challenges current paradigms regarding the interpretation of microscopic post-mortem artifacts that occur on ancient and modern mammalian hairs.
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Despite my undergraduate degree being older than most of my fellow PhD candidates, I would like to thank them for accepting me into their midst, for their friendship, assistance and patience. I thoroughly enjoyed your company guys, and I will miss you. Unfortunately, being a remote student meant that I didn’t get the opportunity to get to know some of you, as well as I would have liked. It has been a truly wonderful four years which would have not been possible without Mike, thank you for your support and encouragement and for making this remarkable journey possible. Daithi thanks for your stellar computer and bioinformatics skills, but more importantly for your friendship, and great sense of humour. Megan, I remember staying with you, Steve and Harry, on my first visit to Murdoch and it’s been lovely to see your family and our friendship grow over the years. I would also like to thank Nic (the dog whisperer) and Rob for use of the ‘annex’ on many occasions and Matt, JT, Alicia and Tina for your help and support. Last but not least, to my partner Paul for his infinite patience, support and humor when it was most needed (which was pretty much most of the time).
Chapter One: Introduction

1.1 General Introduction and Thesis Outline

The chapters of this thesis are presented as a series of published papers (Chapters Two, Three, Four and Five). They are formatted in a manuscript style and so consist of their own abstract, introduction, materials and methods, results and discussion, conclusions, and reference sections. For the purposes of thesis cohesion each chapter begins with a brief preface and concludes with a chapter summary.

Due to the collaborative nature of research, all of the chapters in this thesis have contributions from co-authors. During my candidature, I have worked with national and international collaborators within the fields of ancient and modern molecular biology, bioinformatics, forensic science and microscopy. The multidisciplinary nature of this research necessitated such collaborations. The roles of co-authors who assisted with this research are listed after the section titles within the thesis chapters and others are mentioned in the acknowledgements section within each manuscript.

This thesis focuses on the morphological and molecular characterisation of mammalian hair but will make clear distinctions between the terms used to describe the hairs studied. Although paleontology and archaeology are broadly related disciplines, each may be differentiated on the basis of epochs in which the mammals lived (Garwood 2013). In this thesis the term ‘ancient’ refers to hairs derived in archaeological or paleontological contexts whilst modern hairs refer to hairs originating from extant populations. The term ‘animal’ is generally used in the vernacular to describe non-human organisms; the term ‘animal’ as used in this thesis and publications refers solely to non-human mammalian species ‘Light microscopy’ refers to transmitted visible light microscopy (TVLM). Images that are for illustrative purposes lack a scale bar. Terminology used to describe medullae, cuticle scale patterns and cross-sectional shapes of animal hairs are in accordance with the nomenclature cited in Brunner and Coman (Brunner 1974), unless otherwise stated.
Chapters Two to Five each begin with a preface that briefly describes the reasons for undertaking the research, especially in the context of the overall aims of the thesis (Section 1.6). Each chapter contains a title page and co-authors; each chapter contains a ‘Chapter Summary’ section, the aim of which is to highlight the main findings as they relate specifically to this thesis. All references cited in the chapter preface and concluding remarks sections are listed in the final discussion and future directions (Chapter Six).

The purpose of Chapter One, the general introduction, is to outline the thesis layout and to provide a general introduction into the relevance of mammalian hair in forensic applications and studies of ancient peoples and animals. In so doing, detailed expositions on mammalian hair growth, anatomy, function, colour and durability provide contextual information (Section 1.2.). The importance of selecting appropriate microscopical techniques, and microscopes, to examine and characterise hair morphology is debated in Section 1.4. This thesis bridges the morphology of hairs with molecular analyses, accordingly, Section 1.5 introduces ‘Next generation Sequencing’ (NGS) platforms specifically in relation to metagenomic audits and its application in forensic science. Finally, Section 1.6 defines the aim and scope of this thesis and its role in advocating a multi-disciplinary approach for the examination of mammalian hairs. The manuscript-based style of this thesis has the potential to become repetitive, thus, the use of cross-referencing between chapters aims to minimise repetition where possible and assist in maintaining the overall ‘flow’ between consecutive chapters.

1.2 Mammalian hair

1.2.1 Prelude

Hair is a unique and defining feature that distinguishes mammals from all other vertebrates. The evolution of these filamentous structures is reputed to have arisen from reptilian and amphibian ancestors as hair-like sensory appendages, or prototrichs (primitive hairs) (Ryder 1973). The emergence of hair, some 310 to 330 million years ago, was a significant step in the evolution of mammals (Ryder 1973). Today, due to the unique qualities exhibited by these resilient biological
structures, they are central to many forensic investigations and in studies of ancient civilisations and extinct animals.

The value of hairs as forensic evidence gained prominence in the nineteenth century with the first publication of ‘Hairs of Mammalia from the Medico-Legal Aspect’ (Glaister 1931). Hairs are excellent forensic substrates; they are readily shed, resilient and stable, consisting of microstructures that form the basis for discriminating between putative donors. Furthermore, hairs are privileged sites for genetic material that have the potential to identify the donor of the hair (using short tandem repeat assays), or provide insights into past lives and ancient migrations (using mitochondrial DNA sequencing techniques). Much of the scientific knowledge about the microscopical characteristics and structural features of human hairs today is based on extensive research conducted on sheep wool in the 1950s and 1960s (Wildman 1961) due to their importance in the textile industry. This extrapolation of knowledge is possible because all mammalian hair fibres are essentially of similar structure, chemical composition and physical behavior. In order to recognise and accurately interpret microscopical morphological features of mammalian hair, a basic understanding of the biology of hair is required.

1.2.2 Mammalian hair: growth

The genesis of hair follicles occurs during foetal skin development, following birth, when tightly regulated ectodermal/mesodermal interactions culminate in deeply invaginated skin structures or follicles (Schneider, Schmidt-Ullrich et al. 2009). Hair follicles are complex structures essential for the production of mammalian hair in which cells proliferate at the base of each dermal follicle and migrate upward. Following complex processes including protein synthesis, structural alignments and keratinisation, the fully formed hair emerges from the skin. Although all hair follicles show common morphology, they give rise to shafts with striking variability in size and shape particularly in animal hairs (explored further in Chapter Two). Both follicle and hair shaft reveal a complex architecture consisting of several compartments (Figures 1.2.1, 1.2.2).
All mammals, throughout their lives, require a constant supply of new hairs in order for continued protection against the environment, and possibly for survival. In order to achieve this constant regeneration, existing follicles undergo genetically
programmed cycles of growth (anagen), regression (catagen) and rest (telogen) (Robbins 2012).

The anagen growth phase is characterised by intense metabolic activity in the hair bulb as the growth of new hair is initiated. Hairs in this active growth phase are deeply embedded in the follicle such that removal of the structure can only be effected through the application of force. This forceful removal results in a hair containing a soft, flattened or ribbon-like root, often accompanied by the presence of follicular sheath material (Figure 1.2.3). Approximately 85% of scalp hair follicles in healthy individuals are in this active growth phase, which typically lasts 2-6 years.

![Figure 1.2.3. Examples of anagen roots.](image)

Catagen growth phase is ephemeral, lasting only for a few weeks; approximately 3% of hairs are in the catagen phase on a healthy scalp. This stage marks the beginning of programmed cell death or apoptosis of the hair follicle. Metabolic activity slows down and the base of the bulb migrates upward toward the surface of the epidermis or skin. During this upward migration the outer root sheath of the hair shrinks and attaches to the root of the catagen hair, this culminates in the formation of a club root (Alonso 2006).
Catagen hairs require progressively less force to remove, as the hair regresses the follicular sheath material, which once ‘anchored’ the hair in the follicle, gradually disappears (Figure 1.2.4).

![Figure 1.2.4. Examples of human hair catagen roots. Scalp hair (A) and pubic hair (B) catagen roots. The presence of adhering root sheath material and pigmentation indicates that these hairs are in the early transition period between anagen and catagen growth phases. Scale bar: (A) 100 microns. (Images: S.R. Tridico).](image)

The telogen phase represents the final stage of a hair’s life cycle in which all growth has ceased; the base of the hair bulb atrophies and becomes bulbous. Telogen hairs also lack sheath material (Figure 1.2.5). The telogen growth phase lasts approximately 2-3 months, which on a healthy scalp represents approximately 15% of hairs (Robbins 2012). The cessation of hair growth stimulates the follicle stem cells to ‘reset’ so that they herald the next anagen growth phase, and the genesis a new hair shaft. A new hair begins to grow beneath the atrophied telogen follicle, eventually forcing the old hair out if one is still in place. Hairs that are in this final stage are readily and easily shed and constitute the majority of hairs found in our environment.
Figure 1.2.5. Examples of telogen hair roots in human scalp hair. Telogen roots bear characteristic bulbous roots as exemplified by images A and B. Image B depicts a hair root that is in the final stages of late catagen to early telogen transition growth phase, evident by the darkened root tip and bulbous-like appearance to root. (Images: S.R. Tridico)

1.2.3 Mammalian hair: structure and function

Structurally, human and animal hair consists of the three principal components: the cuticle, cortex and medulla (Figure 1.2.6A). The outermost cuticle wraps around the entire length of the hair shaft and consists of imbricate or overlapping scales whose arrangement is akin to tiles on a roof (Figure 1.2.6A). When present, the medulla is centrally placed (Figure 1.2.6B). The medullary cells collapse in such a manner that results in cellular structures with air spaces and gaps (vacuolated), which may be prominent in some animal species (Figure 1.2.6C). The main body of the hair shaft, the cortex, is where the bulk of the pigment granules reside which are responsible for the natural colouration of the hair (Figure 1.2.6D).
Figure 1.2.6. Major anatomical structures comprising mammalian hair shafts. The outer surface of all hair shafts consists of overlapping scales (A) that protect the inner cortex. The cortex may contain an air filled central medulla (B) or one that is infiltrated with mounting medium that results in a translucent (or cleared) medulla (C), the cortex also contains pigment granules (D). (Images: A, B, D: S.R. Tridico. Image C: Courtesy of Renn Tumlison).

Mammalian hair is composed of the protein keratin, therefore human and animal hairs are similar in structure, exhibit similar chemical and physical properties and differ only in the fine detail between the species. Keratins contain a variety of amino acids of which the sulphur-rich, Cysteine (Cys) amino acid is present in far greater abundance than the other amino acids that are present. Cysteine is critical to the keratinisation (or hardening) of the hair shaft prior to its emergence through the skin. The keratinisation process is thought to occur within the hair root through the oxidation of the thiol (SH) groups in pairs of Cys molecules residing in adjacent protein chains to form a cross-linking di-sulphide bond (Figure 1.2.7). These di-sulphide bonds confer great strength and rigidity to the hair structure (Ryder 1973).
Upon extrusion of the fully keratinised hair structure through the skin, the shaft emerges as a highly stable structure, one that is highly resistant to environmental and chemical deleterious actions due to its high di-sulfide cross-linked structure (Hill, Brantley et al. 2010). These di-sulphide bonds confers such immense structural stability and resilience to the keratin proteins that mammalian hair can be retrieved from the ground, in relatively good morphological/molecular condition, thousands of years after being deposited. However, despite hairs being resilient they are not completely immune to deleterious environmental or biological insults (explored further in Chapter Three).

**Figure 1.2.7.** Diagram showing the oxidation of the thiol (-SH) moieties of Cysteine that results in the highly stable and durable, di-sulphide bonds of Cystine (circled). Valine (blue circle) is included in the diagram to represent other amino acid residues that are present in hair protein chains. (Image courtesy of K.P. Kirkbride).

Probably the most significant function of mammalian hair is the maintenance of thermal balance within the environment in which the organism lives. The evolution of hairs undoubtedly contributed to the success of mammals, enabling them to inhabit a wide range of ecological niches. Unlike humans, animals developed dense under-hair, an important evolutionary characteristic that provided them with additional insulation they required in order to survive (Ryder 1973). Irrespective of species, under-hairs invariably exhibit the same morphology: fine diameter, wavy/curly and colourless with needle-like or prominent cuticle scales, (Figure 1.2.8).
Figure 1.2.8. Scale patterns from under-hairs a brush tail possum (*Trichosaurus vulpecula*) (A) and from a European hare (*Lepus europaeus*) (B) exemplify the narrow diamond petal arrangement of cuticle scales often seen on under-hairs. (Images: S.R. Tridico)

In addition to under-hairs, animals have guard hairs that not only protect the animal from environmental conditions, but also protect the under-hairs thereby minimising heat loss and maximising retention of warmth. In addition, oily secretions (from the sebaceous gland) some of which are covalently bonded to the cuticle, are thought to enhance the natural water repelling nature of keratin, thereby further protecting the body from the cold. Taken together, these properties are critical for the survival of species living in harsh environmental conditions and most likely ensured the survival of cold-adapted prehistoric megafauna such as the woolly rhino and mammoth (the focus of Chapter Four). Conversely, hair may also protect against excessive heat, as experienced by desert dwelling diurnal animals such as camels. In hot climates, the pelage (or coat) prevents the animal from overheating by the uppermost parts of the hairs absorbing the heat, while the lower part of the coat, closest to the skin, remains close to body temperature (Ryder 1973).
1.2.4 Mammalian hair: colour

The colour of an animal’s pelage serves several functions, it may assist in sexual selection; furthermore, cryptic hair colour that matches or blends into the environment is likely to assist in evading predators or facilitate stalking prey (Figure 1.2.9).

![Cryptic colouring of predator and prey pelages.](image_url)

Colour may also be used to warn others of a perceived threat as exemplified by white-tailed deer (Odocoileus virginianus) that flash the underside of their tail to warn the herd of impending danger (Figure 1.2.10). Animals’ pelage, like human scalp hair, also serves to protect the skin from abrasion and excessive ultra-violet (UV) radiation (Kaxiras, Tsolakidis et al. 2006).
The multitude of natural shades of colour exhibited in human and animal hairs is attributable to the combination of eumelanin and phaeomelanin pigment granules. Although predominantly distributed within the cortex, these chromophores can also occur in the medulla and occasionally the outer cuticle. The size, distribution and arrangements of the pigment granules are readily visible with a light microscope. Eumelanin pigment granules are responsible for producing individuals that exhibit brown or black coloured hairs, whilst phaeomelanin granules are responsible for the production of hairs exhibiting yellow (blond) and red colouration.

However, not all red hairs result from Mendelian inheritance. Excluding the application of artificial dyes, red hair may be due to biodegradation or environmental effects (which is explored further in Sections 1.3 and 1.4). Dark, heavily pigmented mammalian hairs may exhibit orange/ red colouration as a result of the susceptibility of eumelanin pigment granules to photo-degradation, resulting from exposure to ultraviolet radiation. (Lee 2010). This process is most evident in scalp hairs in which prolonged and frequent exposure to sunlight results in lightening of the tip (or distal) portion of the hair shaft. This area of the
shaft is the oldest part of the shaft and therefore most susceptible to photo-degradation, as it has been exposed to the environment for longer periods, than the younger shaft closest to the skin. This effect is exemplified by a 100-year-old scalp hair sample that originated from a male Australian Aboriginal (Figure 1.2.11) (Rasmussen, Lohmueller et al. 2011) (co-author, Appendix B.2), and from beard hair taken from an extant, modern individual. The respective hair samples from each individual were dark brown, almost black, in colour at the root (proximal) end of the shaft. However, the colour progressively lightened along the hair shaft toward the tip end of the hair that ultimately turned red/orange in colour.

![Figure 1.2.11. Examples of hair lightening due to photo-degradation.](image)

However, photo-degradation is not the only causative agent to turn scalp hairs orange. Marko and Rowe (Marko 2001) conducted studies in which they demonstrated that exposure to humidity also causes hair shafts to become red/orange in colour, due to the proliferation of fungal hyphae. Krefft (Krefft 1969) on the other hand, observed that the red/orange colouration of hairs resulted from the breakdown of the amino acid tyrosine (Tyr) contained within
the keratin structure. Each of these observations may provide additional
information in relation to the environment in which the mammal was exposed to
or inhabited.

1.2.5 Morphological variations between human and animal hairs

Unlike animals, all human beings belong to the same species—*Homo sapiens*;
therefore all human hairs exhibit similar morphological features and
characteristics. In contrast, some 5000 animal species that inhabit the Earth today
each exhibit a variety of taxon-specific medullae configurations, scale patterns
and cross-sectional shapes (Brunner 1974). To the trained eye, human and animal
hairs may be distinguished on the basis of intrinsic macro- and microscopic
differences (Table 1.2.1 and illustrated in Figures 1.2.12 and 1.2.16).

![Figure 1.2.12 Examples of features that differentiate human hairs from animal hairs. Examples of hair structures exhibited by human hairs: homogenous colouration (A); continuous (i), absent (ii) and fragmented (iii) medullae (B). Bulbous (club) telogen root (C) and elongated flattened anagen root (D). Examples of comparable structures that may be found in animal hairs: ‘banding’ which is evident as abrupt colour changes along the hair shaft (E). Examples of globular (F, upper image) and, ‘honeycomb’ medullae (F, lower image). Examples of hair roots: elongated (G) and ‘wineglass’ roots (H) (Images A.C.H.S.R.Tridico, B FBI).](image-url)
Table 1.2.1 Morphological features that distinguish human hairs from animal hairs

<table>
<thead>
<tr>
<th>Feature</th>
<th>Human Hair</th>
<th>Animal hair</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Colour</strong></td>
<td>Relatively consistent along hair</td>
<td>Often show naturally occurring abrupt colour changes (‘banding’) along hair shaft (in addition to consistent colour). (Figure 1.2.12)</td>
</tr>
<tr>
<td><strong>Root</strong></td>
<td>Flattened or bulbous appearance dependent on growth stage of the hair</td>
<td>Variety of shapes and forms, usually distinct. (Figure 1.2.12)</td>
</tr>
<tr>
<td><strong>Medulla</strong></td>
<td>Occupies less than one third of the hair shaft. Amorphous, may be continuous or discontinuous.</td>
<td>Often present, usually occupies greater than one third of the hair shaft. Continuous, often varying in appearance along the shaft, varied morphologies (Figures 1.2.12, 1.2.16).</td>
</tr>
<tr>
<td><strong>Cuticle scale pattern</strong></td>
<td>Irregular wave, similar along the length of the shaft.</td>
<td>Variety of configurations (Figure 1.2.16)</td>
</tr>
<tr>
<td><strong>Transverse cross-sectional morphology</strong></td>
<td>Dependent on racial group of donor. May be oval, circular or flattened oval (scalp hair)</td>
<td>Variety of configurations (Figure 1.2.16)</td>
</tr>
</tbody>
</table>
Unlike the human “pelage”, close inspection of many animal pelages reveals a ‘tiered’ appearance to the types of hairs populating the coat (Brunner 1974) (Figure 1.2.13). The first tier consists of sparsely distributed hairs that are distinctly longer and thicker than the remainder of the hairs (overhairs). The second tier consists of numerous and the most prominent hairs of the pelage—the primary guard hairs. Guard hairs usually exhibit thickening of the shaft (known as the shield) that usually occurs in the distal shaft (closest to the tip); however, some animals exhibit a ‘deep shield’ in which the thickening of the shaft starts at the proximal end and extends to the distal shaft (Figure 1.2.14).

Figure 1.2.13. Major hair types present on animal hair pelages. (Image sourced from Mr. H. Brunner)

Figure 1.2.14. Diagrammatic representations of guard hairs that exhibit a ‘normal’ shield (upper image) or a ‘deep shield.’ (Image: S.R. Tridico)
The final layer contains the majority of hairs—the "underhairs", which are usually shorter and significantly finer in diameter than overhairs and guard hairs (Figure 1.2.15). To the naked eye, underhairs usually exhibit wavy, curly or kinked shaft profiles. The curliness, cuticle scales and fine diameters promote intertwining and interlocking of these hairs and the radiating body heat warms the numerous air pockets formed between the under-hairs thereby assisting in maintaining body temperature.

In addition to primary guard hairs, other shorter and finer guard hairs may also be found on the pelage. These are regarded as hybrid or transitional hairs (also referred to as secondary or tertiary guard hairs) because of their chimeric morphology, bearing shields that are typical of guard hairs but with shafts resembling under-hairs in appearance and structure (Figure 1.2.15). Like primary guard hairs, the shield region of transitional guard hairs exhibits the same taxon-specific features that are present in primary guard hairs.

Figure 1.2.15. Hair types on most animal pelages. The overhairs and primary guard hairs hair types are the most prominent hairs that cover the secondary guard hairs and under-hairs that make up the remainder of the pelage. (Imaged sourced from Ms. M. Smith).
In effecting identification or attribution of an animal hair of unknown origin to a particular taxon, primary guard hairs provide the most informative morphological characteristics (Brunner 1974). These characteristics are numerous and include a variety of morphological structures (Figure 1.2.16).

**Figure 1.2.16** Examples of the variety of scale patterns (A), medullae configurations (B) and transverse cross-sectional shapes (C) that may be exhibited by animal hair (Image courtesy of Mr. H. Brunner)

An important aspect in attributing an unknown animal hair as originating from a particular taxon is achieved through comparison with vouchered hairs (discussed in greater detail in Section 1.3.4). Vouchered hairs originate from animal pelts or whole specimens whose morphological features and traits are deemed representative of that particular taxon. Vouchered hairs play an essential role in substantiating identifications derived from biological specimens (Wheeler 2003), including hairs.
1.2.6 Relevance and significance of hair in forensic science and studies of ancient human and animal hairs

“I have seen a thousand graves opened, and always perceived that whatever was gone, the teeth and the hair remained of those who had died with them. Is that not odd? They go the very first things in youth and yet the longest to survive in the dust’ (Byron 1820).

This observation exemplifies the most significant property hairs possess that renders them as relevant biological structures encountered in forensic science and ancient sites. These complex mammalian appendages, unlike ephemeral biological structures such as tissues or body fluids, are remarkably resistant to degradative effects. This resilience is illustrated by their ability to survive, intact, for millennia. The ease in which hair is readily shed and transferred onto objects, or persons, during physical contact renders hair as one of the most prevalent types of forensic evidence encountered. The relevance and significance of hairs in forensic and archaeological/palaeontological studies is attributable to hair being a privileged repository of genetic material that has become the cornerstone of forensic DNA profiling and in the study of ancient civilisations and animal species.

1.2.7 Perspectives of hair examination in modern and ancient contexts

The microscopical examination of hairs is a venerable one dating back centuries; it is an integral part of the initial investigation of hairs retrieved from crime scenes or archaeological and paleontological sites. Transmitted visible light microscopy (TVLM) of hairs exploits the variety of microscopic, morphological features and traits exhibited by all human hairs and is frequently used to conduct a systematic audit of genetically determined characteristics such as colour and pigmentation and those that are ‘acquired,’ such as pathologies, damage and cosmetic treatments. This morphological profile may assist law enforcement
officers in providing initial avenues to explore, in the early stages of the investigation, or to provide a basis upon which ancient and modern hairs may be selected for further more detailed analyses, such as DNA profiling or sequencing. However, unlike the early pioneers of microscopical examination of hairs, today’s hair examiners have a selection of microscopes to choose from in order to examine specimens.

1.3 Microscopical examination of mammalian hairs

The expertise to accurately and correctly effect identification of animal hairs to a particular taxon is dependent on the ability of the examiner and achieved through systematic and focused microscopical examination of features (Brunner 1974, Teerink 1991); a topic that is the focus of Chapter Two. The features of interest such as scale pattern, medulla configuration, transverse cross-sectional shape and root (if present) should all be taken into account to effect identification. This tenet should be adhered to even if the taxon of origin has distinctive features such as members of Leporidae family in which rabbits and hares exhibit unique ‘multi-serial ladder’ medullae morphology that resembles ‘Sweetcorn’ as illustrated in Figure 1.3.1. Microscopical audits of all available key morphological features, in the questioned hair (animal taxon unknown), in conjunction with comparison to hairs taken from verified (voucher) specimens promote robust and credible identifications capable of withstanding scrutiny.

![Image: S.R. Tridico]

**Figure 1.3.1.** Multi-serial, or ‘sweetcorn’ medulla, that is unique to hairs from members of the Leporidae family (rabbits and hares) and spans the majority of the cortex. (Image: S.R. Tridico)
Success or failure of the morphological identification and examination of hairs, aside the competency of the examiner, is highly dependent on the selection of the instrument used to conduct the examinations. Microscopes are the mainstay for the morphological examination of mammalian hair; the choice of microscope is largely dependent upon the question that needs to be answered or the purpose of the examination. The following sections showcase the variety of microscopes available today and discuss their applicability, advantages and disadvantages.

1.3.1 Stereomicroscopes: getting the general picture

Stereomicroscopes were invented to accommodate the niche medical market, enabling pathologists to dissect bulky tissues or body parts (Nothnagle 2013). Samples examined on dissecting microscopes are usually illuminated from above (reflected light) and at modest magnification (commonly up to 40x, but some instruments may offer up to 125x). Stereomicroscopes are invaluable instruments that provide a general overview of hair samples to assess general condition, identify if roots are present and also allow samples to be triaged, in order to select hairs that warrant further microscopical examination at higher magnifications.

1.3.2 Transmitted visible light microscopes: getting the whole picture

Today, transmitted visible light microscopy (TVLM) of mammalian hairs is widely practiced and is a technique that is firmly based on principles developed in medicine, histology, physical anthropology and zoology (Houck 2005). TVLM of hairs enables external and internal morphological characteristics to be viewed along the entire length of the shaft, including the root. Microscopic examination of hairs using TVLM provides a robust basis to assess the relevance of further analytical studies (Schweitzer MH 2008). Although most light microscopes are capable of magnifications up to a 1000x, the most effective magnification in which to study hair structures lies between 100x and 200x. The use of objectives that allow magnification up to 400x and 1000x are of limited value; these magnifications are at the limit of the resolving power of the microscope. This is because TVLM cannot resolve objects that are smaller than half the wavelength of light (0.275 µm) (Cowhig 1974). In order to
microscopically view ultra-structure of hairs that are smaller than 0.275µm, another form of ‘illumination’ is required. The invention of the electron microscope in 1932 (Zewail 2010) provided this alternative ‘illumination’ required to resolve objects that are beyond the capabilities of transmitted visible light microscopy.

1.3.3 Electron Microscopes: getting a partial view of the picture

Electron microscopes (EM) depend on electrons as their ‘light source’ to view detail down to the level of nanometers. There are two main types of electron microscopes:

- Scanning electron microscope (SEM)
- Transmission electron microscope (TEM) and allied EM e.g. High resolution TEM (HRTEM).

In relation to visible light microscopy and electron microscopy, both microscopes are technical instruments that allow visualizing structures that are too small to be resolved by the naked eye and have relevance in areas of applications in biology and the materials science. However, in relation to hair examination the use of TVLM and EM is usually determined by the purpose of the examination. For routine and general examination of hairs TVLM has significant advantages over the higher magnification electron microscopes, not only in relation to the morphological examination of hair but also in relation to the technical attributes as demonstrated in Tables 1.3.1 and 1.3.2 that are present inside the hair, a region that EM cannot penetrate unless the specimen has been cross-sectioned.

However, in instances where surface details or artifacts that are too small to be resolved by TVLM, SEM is an invaluable instrument to conduct this examination and is frequently found in many forensic and research laboratories. One limitation is that SEM requires destructive sample preparation and therefore should be used judiciously. The use of TEM for hair examination outside of research is unlikely to be routinely employed in many laboratories due to the issues outlined in Tables 1.3.1 and 1.3.2.
Table 1.3.1. Comparisons of technical capabilities between transmitted visible light microscopy (TVLM) and electron microscopes (EM) regarding the examination of mammalian hair structure.

<table>
<thead>
<tr>
<th>TVLM</th>
<th>EM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Advantages</strong></td>
<td><strong>Disadvantages</strong></td>
</tr>
<tr>
<td>Small and portable can be used almost anywhere, including in the field</td>
<td>Not portable and must be operated in a laboratory</td>
</tr>
<tr>
<td>Cheap to purchase and operate</td>
<td>Expensive to purchase and operate</td>
</tr>
<tr>
<td>Preparation of hairs is quick and simple, requiring minimal training</td>
<td>Sample preparation can be elaborate, lengthy and requiring considerable expertise and complex equipment in the case of TEM</td>
</tr>
<tr>
<td>Hairs not distorted by preparation</td>
<td>Hair samples may be distorted during preparative processes.</td>
</tr>
<tr>
<td>Maintenance costs relatively low</td>
<td>Maintenance costs are high</td>
</tr>
<tr>
<td>High sampling capability: Examination of entire hair shafts possible</td>
<td>Very low sampling capability: restricted to examination of a fraction of the shaft (TEM especially)</td>
</tr>
<tr>
<td>Natural colour of hairs can be observed</td>
<td>All images are in greyscale</td>
</tr>
<tr>
<td>Non-destructive; further testing possible post microscopy</td>
<td>Destructive; further testing not possible post examination</td>
</tr>
<tr>
<td>100% sampling capabilities of internal and external features along the entire hair shaft</td>
<td>Significantly lower sampling capabilities</td>
</tr>
</tbody>
</table>

**Disadvantages**  
- Magnification capable to approx. 1000x  
- Depth of field restricted

**Advantages**  
- Magnification capabilities in excess of 500 000 x  
- Significantly greater depth of field possible.
Table 1.3.2. Comparing efficacy of transmitted visible light microscopy and electron microscopies to conduct morphological audits of mammalian hair structures. (SEM: Scanning Electron Microscope, TEM: Transmission Electron Microscope, TVLM: Transmitted Visible Light Microscope).

* Only possible if hair is longitudinally sectioned

<table>
<thead>
<tr>
<th>Examinations</th>
<th>TVLM</th>
<th>SEM</th>
<th>TEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ability to examine internal features along the entire length of the shaft</td>
<td>Y</td>
<td>Y*</td>
<td>N</td>
</tr>
<tr>
<td>Ability to examine external features along the entire length of the shaft</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ability to examine the root to determine growth phase at the time of removal</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ability to determine if the hair human or animal in origin</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ability to assess racial origin of donor</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ability to determine somatic origin of the hair</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Ability to assess and characterise pre- and post-</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Ability to screen hairs</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Ability to use hairs examined by microscopy for subsequent molecular analyses</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Ability to perform comparative examinations hairs on a side by side basis</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
1.3.4 Comparison Microscope: double vision

Developed in the 1920s (Inman 2000) the comparison microscope has become the mainstay for conducting microscopical comparisons of forensic material such as textile fibres, projectiles, questioned documents, cartridge cases and mammalian hairs. This instrument is essentially two visible light microscopes joined together by an optical bridge that enables two samples to be viewed simultaneously as illustrated in Figure 1.3.2.

![Comparison Microscope](https://proscitech.com)

**Figure 1.3.2.** A comparison microscope (A) allows simultaneous, side-by-side examination of hairs as illustrated in image B. (Image A sourced from: http://https://proscitech.com. Image B sourced from Ms B. Yates)

For decades, forensic hair examiners compared morphological features present in questioned (crime scene) hairs with those from known/reference hairs taken from a person of interest and/or victims; the aim of the examination was to exclude/not exclude individuals as the source of the hair in question. However, the advent of DNA profiling revealed shortcomings of a technique that is heavily reliant on the expertise and competence of the examiner.

In 1996, post conviction DNA testing exonerated many defendants who were incarcerated on the results of microscopical human hair comparisons. These exonerations were largely due to testimony in which the evidentiary significance of the results were based on ‘un-validated science,’ such as probabilistic data and over stating the significance of a ‘match’ between features observed in the victim’s hair with that of the suspect’s hair (Massie, 2013). In contrast, animal
hair identifications are less about ‘matching’ the unknown to an individual but more about species-level identifications. Ultimately, the ‘weight of evidence’ that can be placed on data derived from microscopy of hair relies upon a variety of factors such as the specific question being addressed and the experience/competency of the examiner.

Taken together, the comparison microscopy of hairs remains an essential addition to the forensic microscopy toolkit, with human hair ‘matches’ now being considered as an adjunct to other more empirical evidence or data such as DNA profiling. In relation to animal hair identification, comparison microscopes are an essential tool in the ascribing an unknown, or questioned animal hair, to a particular taxon. Side by side examination of the questioned animal hair with an appropriate vouchered reference hair assists in identifying the taxon of origin of the questioned animal.

An example of this comparative process is illustrated in Figure 1.3.2, in which the image on the right shows two hairs being compared side by side: one hair is from a known (vouchered) animal source, the other represents a hair from an unknown animal. Furthermore, microscopy also provides a morphological audit of microscopic structures and characteristics that may provide additional information regarding the natural history of the animal of origin e.g. hair nits, or post-mortem artifacts.

1.3.5 Scanning Laser Confocal Microscope (SLCM) of Hair-virtual reality

Physical, transverse cross-sections of hairs are of value in the morphological identification of animal hairs due to characteristic morphologies exhibited by many taxa (Brunner 1974, Teerink 1991). Furthermore, transverse cross-sections of human hair may assist with the determination of racial origin of the donor (Seta 1988). Compared with physical cross-sections, SLCM has the following advantages:

- Non-destructive, which is a major consideration in relation to forensic hair samples, ancient hair samples or museum hair specimens or apparel containing mammalian hair.
• Special mounting techniques not required; hairs previously mounted for conventional microscopy require no further treatment.
• Enables the examiner to image the cross-section at any point along the shaft.
• Successfully produces virtual cross-sections of hairs too short to be processed using physical cross-sectioning techniques such as claw hairs or hair fragments that measure less than one centimeter in length.
• Enables transverse cross-sectional profiles to be obtained from hairs that are too fragile to be physically cross-sectioned, such as those recovered from ancient sites.
• Can be reliably applied to a variety of hairs, except those that are heavily pigmented.

Scanning laser confocal microscopy allows the production of transverse, and longitudinal, cross-sectional images of a wide range of hairs and fibres (Kirkbride 2010). An example of a virtual hair cross-section and demonstration of the fidelity of transverse cross-sections obtained using SLCM is illustrated in Figure 1.3.3.

Figure 1.3.3 An example of the fidelity of transverse cross-sections of dorsal guard hairs from the brush tail possum (*Trichosaurus vulpecula.*) using confocal microscopy. The virtual cross-section, which is taken where the cross hairs meet, (left panel), shows an eye-shaped transvers cross-section that is compatible with that obtained from a physical transverse cross-section as indicted by the arrow (right panel) (Image: S.R. Tridico).
1.4 Generations of DNA Sequencing

Genomics has been a field in constant evolution and revolution since Watson and Crick unraveled the structure DNA in the 1950s (Watson 1953). In the 1970s, Sanger and Coulson (Sanger, Nicklen et al. 1977) continued to further elucidate the structure of DNA through the development of the di-deoxy chain termination method for deciphering the sequence of DNA. In the mid-1980s, the ability to replicate DNA in vitro resulted in the genesis of the polymerase chain reaction, PCR) which irrevocably and dramatically changed the field of molecular biology (Inman 2000). Each of these molecular milestones provided the foundation upon which automated sequencing instruments of today are built.

The most recent and significant molecular innovation was introduced in 2000s which enabled sequencing DNA at unprecedented levels and speed (Shendure and Ji 2008). This new technology, known as Next Generation Sequencing (NGS), was harnessed and embraced in order to explore a much wider scope of applications than previously possible with Sanger sequencing. NGS processes, enables thousands of genomic sequences to be determined in parallel, which gave rise to new, sequencing terminology-massively parallel sequencing. These new capabilities revolutionised many fields of biological studies - none more so than microbiology. For the first time, 99% of planet’s bacteria, which could not be cultured or identified, using conventional techniques, could now be characterized through their DNA. NGS, almost instantaneously, obviates the requirement to culture bacteria in order to study them. This unrivalled access to environmental bacteria gave rise to a new tool in the microbial toolkit- ‘metagenomics’. This term was first coined by Handelsman et al (Handelsman 2004) as the genomic analysis of microorganisms by direct extraction from their environment; since being first coined, the term has become synonymous with culture-independent molecular analyses.

Over the last decade, the inception of NGS platforms and improvements in bioinformatic capabilities, two sequencing strategies have gained prominence in deciphering microbial genomes utilising amplicon-based sequencing or whole
genome shotgun sequencing (WGS). The choice of sequencing strategies, used to audit mammalian genomes, is dependent on the sample material and the objective of the study.

1.4.1 Amplicon sequencing using NGS methods

Amplicon sequencing focuses on targeting specific regions of DNA; following DNA extraction the samples are subsequently pooled together and massive parallel sequencing of the samples is conducted on the chosen NGS platform (a more detailed exposition of this process is presented in Chapter Five). Amplicon sequencing is a cost-effective approach to process samples on NGS platforms because it is conducive to multiplexing. In order to enable disparate samples to be pooled together, a method to separate and identify post-sequenced data into their original samples must be employed. This ‘deconvolution’ is made possible through the use of unique multiplex identifier (MID) tags that are attached to the gene specific primer (Binladen, Gilbert et al. 2007). In addition, a MID-tagged PCR product also requires the attachment of a specific sequencing adapter (unique to each NGS platforms). This attachment of which occurs following PCR amplification of the target fragments, or is attached to the gene-specific primer together with the MID tag (to form a ‘fusion primer’) (Roche 2009). A summary workflow diagram for amplicon NGS is represented in Figure 1.4.1.
Figure 1.4.1 Example of a next generation sequencing (NGS) amplicon-based workflow (Diagram: S.R. Tridico).

Since its inception, next generation amplicon sequencing has been applied to many, and disparate environments (Venter JC 2004, Cox-Foster, Conlan et al. 2007, Dewhirst, Chen et al. 2010). Subsequent to these studies, a number of tentative steps have been taken to explore the forensic potential of metagenomic analyses of a variety of environment such as soils (Sensabaugh 2009), identification of biological warfare agents (Budowle and Williamson 2009) or to differentiate between individuals on the basis of skin bacteria (Fierer, Lauber et al. 2010). However, the forensic potential of this technique has yet to be fully explored, or exploited.

1.4.2 Whole genome shotgun (WGS) sequencing

Whole genome shotgun sequencing represents the most straightforward approach using NGS capabilities. WGS offers a global view of the bacterial communities
in the environment of interest, for example it enables the phylogenetic diversity
of microbial denizens to be assessed in the environment of interest, and has the
potential to discover novel bacterial genes (Chen and Pachter 2005).

In relation to hairs, WGS has been used to successfully decipher whole genomes
of permafrost preserved extinct Mammoth hairs using the genome of the modern
elephant as a scaffold (Miller, Drautz et al. 2008). In relation to deciphering
ancient human genomes, WGS has also been used to extract molecular data from
permafrost preserved scalp hairs of an individual from an extinct Saqqaq culture
(Rasmussen, Bertalan et al. 2010) (Co-author, see Appendix B.1) and from a
lock of hair sampled from an Australian aboriginal male, that was ‘archived’ in a
desk drawer for a century (Rasmussen, Lohmueller et al. 2011) (Co-author,
Appendix B.2).

Whilst WGS is capable of supporting the broadest coverage of the genome, it
also requires the largest sequencing and interpretation of data efforts Forensic
investigations however, may be benefit from targeting more specific regions of
the DNA to best answer forensic questions.

1.4.3 Applications of Next generation Sequencing

The development of NGS has impacted on most fields of molecular analyses, but
arguably few have benefited as much as the field of ancient DNA (aDNA)
studies Early molecular forays into aDNA studies concentrated on extraction of
DNA on bones, however, Gilbert et al. (Gilbert MTP, Shapiro et al. 2004,
Gilbert MTP, Campos et al. 2007) discovered that, in contrast to bone, hair shafts
contained privileged sites of relatively intact ancient DNA. In forensic contexts,
NGS is tentatively being explored as a potential means to improve traditional
STR profiling systems (Van Neste, Van Nieuwerburgh et al. 2012, Fordyce,
Ávila-Arcos et al. 2011) and as an addition to the forensic wildlife toolkit
(Coghlan, Haile et al. 2012). Currently, human DNA profiling is the main
molecular technique to provide associative evidence between victim and offender.
However, the future of forensic science may involve microbial DNA evidence to
augment more traditional DNA profiling endeavors.
The development of metagenomics (culture independent microbiology) was born as a result that the vast majority of bacteria could not be cultured (Handelsman 2004). The advent of NGS provided the means to study bacterial DNA taken directly from the environment at greater sequence depths and more cost effective levels, than was previously possible. Since the term was first coined, the scope of metagenomic analyses has expanded to include descriptions of microbial denizens extracted from diverse and disparate environments such as the human gut, (Sweeney and Morton 2013) or honeybee colonies (Engel, Martinson et al. 2012).

1.5 Morphological and molecular approaches to characterise hairs in archaeological, paleontological and forensic contexts: Aims and scope of thesis.

The ultimate aim of this thesis research is to promote novel approaches to the future examination of mammalian hairs in order to:

1) Demonstrate the value of a multi-disciplinary approach to the examination of hairs,
2) Highlight the value of formal training in the interpretation of microscopical structures of hairs, and demonstrates the consequences of misinterpretation of microscopic morphological structures by the unwary or ill trained,
3) Present a comprehensive overview of the biodegradation of hairs.
4) Introduce the concept of bacterial metagenomic analyses for forensic analyses of human hairs using deep sequencing technologies.

Chapter Two focuses on establishing a set of guidelines for the morphological identification of animal hairs. As part of this thesis research, this paper set out to assemble a set of world experts in animal-hair identification with the aim of reversing some of the misconceptions and misrepresentations that have recently appeared in the literature (Aim 2).
Chapter Three provides, for the first time, a comprehensive, in-depth microscopical catalogue of morphological ‘signatures’ caused by the actions of biodegradation of mammalian hairs (Aims 2 and 3).

Chapter Four investigates the hair of extinct megafauna, the woolly rhino (*Coelodonta antiquitatis*) and woolly mammoth (*Mammuthus primigenius*). Using a variety of microscopical approaches this manuscript explores what these rare hairs reveal and how the structures and pelage is conducive to life in a cold environment (Aim 1).

Chapter Five introduces the concept of metagenomic analyses of bacteria colonizing human hairs. Addressing Aim 4, this manuscript demonstrates the potential of NGS-based metagenomic analysis as a future molecular addition to the forensic molecular toolkit.

Collectively the chapters presented in this thesis are designed to span a spectrum of approaches seeking to re-define best-practice investigation of mammalian hairs, across applications in archaeological, paleontological and forensic contexts.

**References**


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Chapter Two

Splitting Hairs: A critical analysis of microscopical approaches applied to the examination of human and animal hair

‘Not only his fingerprints, or footprints, but his hair... bear mute witness against him. Physical evidence cannot be wrong, it cannot perjure itself; it cannot be wholly absent. Only its interpretation can err. Only human failure to find it, study and understand it, can diminish its value’

Paul Leland Kirk ‘Crime Investigation’ 1953

2.1 Preface

Chapter Two. The impetus to write this review article was brought about by an increase in the frequency of publications that appeared to misinterpret microscopic structures when examining animal hair. The work resulted in a published manuscript titled ‘Morphological Identification of Animal Hairs: Myths and Misconceptions, Possibilities and Pitfalls’ (Forensic Science International, 2014). The content of this chapter is the same as in the published article found in Appendix A; apart from minor changes made to incorporate in-thesis referencing.

Human and animal hairs are frequently encountered in forensic investigations and at archaeological/paleontological sites. It is typical for these hairs to be microscopically examined (TVLM) in order to determine if hairs are human in origin, or to attribute unknown animal hairs as originating from a particular taxon. In forensic investigations, human hairs (recovered from the crime scene) are microscopically compared with reference (known) hairs to assess common origin of the hairs being compared. In contrast, the skills and knowledge to identify an animal hair as originating from a particular taxon require the recognition and interpretation of key signature microscopical hair characteristics.

At the onset of this research I, together with a number of other forensic hair practitioners in the field (see co-authors below), was becoming increasingly
concerned by the quality of interpretations of microscopic features exhibited by animal hairs. Collectively we recognised that this may, in part, be because there is no critical analysis of the field – one that highlights some of the pitfalls and misconceptions. The specific aim of this chapter is to produce a ‘position-paper’ for researchers and forensic practitioners that discusses the potential of animal hair identification but raises some of the common problems that were becoming increasingly common in the literature. Specifically this paper set out to stress the importance of competency and proficiency learned from theory and practice required by practitioners engaged in the identification of animal hairs, based on their microscopical characteristics; principles that are also espoused by the Scientific Working Group for Wildlife Forensics (SWGWild).

2.1.1 Statement of Contribution

The concept for this review paper was borne out of my experience as a practitioner and reviewer in the area of animal hair identification. The motivation to write the first ever published ‘position paper,’ that addressed shortcomings in current practices in relation to the microscopical identification of animal hairs. I recruited appropriately qualified co-authors and wrote the majority of the content. I wrote the first and last versions of the manuscript and received editorial input from the listed co-authors.

Silvana R. TRIDICO
2.2 Morphological Identification of Animal Hairs: Myths and Misconceptions, Possibilities and Pitfalls

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2.2.1 Summary

The examination of hair collected from crime scenes is an important and highly informative discipline relevant to many forensic investigations. However, the forensic identification of animal (non-human) hairs requires different skill sets and competencies to those required for human hair comparisons. The aim of this paper is not only to highlight the intrinsic differences between forensic human hair comparison and forensic animal hair identification, but also discuss the utility and reliability of the two in the context of possibilities and pitfalls. It also addresses and dispels some of the more popular myths and misconceptions surrounding the microscopical examination of animal hairs. Furthermore, future directions of this discipline are explored through the proposal of recommendations for minimum standards for the morphological identification of animal hairs and the significance of the newly developed guidelines by SWGWild is discussed.
2.2.2. Introduction

The morphological identification of animal (non-human) hairs (MIAH) is based on fundamental aspects of microscopy, biology, and zoology. The purpose of MIAH is to categorise the animal source of an unknown hair sample to a particular taxon based on well-defined, genetically based features that are characteristic to that group. The breadth of knowledge required to identify mammalian hairs from all potential taxa is extensive but may be relatively simple in certain contexts, for example identification of mammal hairs as encountered in biological fieldwork, in museum curation, or in the textile industry. In contrast, the forensic examination of hair involves knowing not only the range of expression of mammalian hairs within taxa, but also being aware of other structures that may resemble hairs, such as man-made wig fibres and faux fur fibres, insect seta, and plant tendrils. The forensic context is thus wider and more complicated than a controlled mammalian orientation.

This complexity is compounded because forensic hair examiners typically are examiners of human hair. Unlike MIAH, the human hair practitioner is dealing with hairs from a single species, Homo sapiens, and answering a quite different series of questions which may include (but not limited to):

1. Is it a human hair?
2. From what area of the body did it originate?
3. Is there damage, disease or treatment evident in the hair?
4. Are the hairs suitable for forensic nuclear DNA profiling?
5. Does the hair contain sufficient information for comparison to a putative human source or sources?
6. Could the hair have originated from one of those sources?
7. What is the broad ethnic origin of the donor of the hairs? (i.e., Caucasian, Mongoloid or African)

Although questions 1-3 may also be relevant to anthropology, questions 4–7 are purely ‘forensic’ in nature and address a concept specific to forensic methods, i.e. source attribution. In fact, categorisation and source attribution represent the core and enduring questions asked of a forensic investigation: “What is this material?” “Where did it come from?” and “Does it confirm or reject associations between people, places, and things involved in criminal activities”.

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The first part, categorisation or identification, is common enough among sciences; what sets forensic science apart is its core intention of sourcing where the identified item came from (the victim, suspect, their environments or the scene).

The composition and origins of materials lend themselves to a greater or lesser specificity of sourcing. Hairs, because of their complex matrix and variable expressivity, are limited by their “intra sample variations (which) can be nearly as large as variations between certain samples from different sources...the results of a hair comparison (are) far less than certain” (Deforest 1987). The process of human hair comparison is widely considered as fundamentally ‘subjective’ in the context that results and conclusions are not quantifiable but based on opinion. This practice is not unique to forensic analyses; it is also relevant in areas of the medical profession such as histology (e.g. identifying cancer cells) and anthropology/palaeontology (e.g. identification of human/animal remains on the basis of bone or teeth morphology).

Typically, three conclusions can be drawn from a human hair comparison, given suitable samples:

1. The questioned hair exhibits the same microscopical characteristics as the known sample and therefore could have come from the person from which the known was taken,
2. The questioned hair exhibits different microscopical characteristics as the known sample and therefore could not have come from the person from which the known was taken,
3. The questioned hair exhibits both similarities with, and differences to, the known sample and therefore no conclusion can be drawn as to the source of the questioned sample.

In some instances positive associations deduced from this comparative process have been afforded more probative value than is scientifically warranted resulting in individuals being wrongfully incarcerated (Hsu 2013). As a consequence, criticism has been leveled at forensic human hair comparison, which may tarnish related or similar disciplines, especially MIAH. However, there is a fundamental difference between comparative examinations between
human hairs to infer an association to a particular individual (sourcing) and MIAH, which is an exercise in taxonomy to identify an animal hair to a particular taxon and not to a particular animal. Therefore, criticisms leveled at the former are not relevant to the latter.

This paper is primarily aimed at raising awareness levels of what can go wrong for inexperienced, unwaried or inadequately trained practitioners attempting to microscopically identify animal hair. The paper also discusses the future of MIAH in the context of accreditation of the discipline and its practitioners.

2.2.3 Morphological Identification of Animal Hairs

All mammalian hair is composed of the protein keratin. Mammalian hairs are all similar in their chemical composition and major structural features but they do differ to a greater or lesser extent in morphology at varying taxonomic levels. Mammalian hair consists of three layers: an outermost cuticle, an inner cortex, and a central core or medulla as illustrated in Figure 2.2.1. Mammalian hairs bear morphological features characteristic for a particular taxon that may be phylogenetic in origin or functionally derived, these are:

1. the configuration of cells in the medullae of guard hairs,
2. cuticle scale patterns,
3. transverse cross-sectional shapes.

Additionally, mammals exhibit somatic variation in hair morphology that must be taken into consideration for taxonomic identification. Whilst the examination of animal hairs takes into consideration gross morphological features such as colour (banded or uniform), length and general profile, these are not, in general, taxon specific. However, these features may assist in excluding animals from a particular taxon as sources of the hair in question if a number of taxa share similar microscopical morphological characteristics.
2.2.4 Myths and Misconceptions

Several popular myths and misconceptions exist regarding MIAH that demonstrate ‘a little knowledge is a dangerous thing’ when exercised without any competence in MIAH.

2.2.4.1 Myth: Cat (Felis catus) and Dog (Canis familiaris) hairs can be reliably identified solely on root shapes

Hairs from cats and dogs are undoubtedly the most commonly encountered animal hairs in forensic (crimes against the person) examinations. There are a number of forensic publications that state that the identification of these two species may be effected solely on the basis of their root shapes (Hicks 1977, Deedrick 2004). It is generally accepted in the scientific community that hairs from these two species can be distinguished, and identified, on the basis of the shape of their hair roots, i.e., dog hairs exhibit spade-shaped roots, and cat roots are fibrillated (Figure 2.2.2). However, both of these root shapes can occur in both species (Tridico 2005) and other species. In order to effect an accurate identification, and one that withstands scientific scrutiny, the examiner must
consider details of the medulla and scale pattern throughout the length of guard hairs in order to distinguish between each of these species - not solely the root shapes. Furthermore, the examiner must query the aggregate morphological characteristics in order to consider what other animals might exhibit similar features in all aspects, i.e. medulla pattern, cuticle pattern, and in some instances, cross-sectional shapes.

![Figure 2.2.2](image)

**Figure 2.2.2** Images of root morphologies that may occur on cats (*Felis catus*) and dog (*Canis familiaris*) hairs. **Top panel:** Cat guard hair with fibrillar root (bar 50 mm) (A) and an overhair with spade shape root (B) (bar 200 mm). **Lower panel:** Dog guard hair with fibrillar root (C) (bar 100 µm) and a coarse guard hair with spade shape root (D) (bar 200 µm).

Some early work by Peabody *et al.* (Peabody 1983) indicated that medullary index (i.e. the ratio of the medulla diameter to the hair diameter) could be used as a basis for discriminating domestic cat (*Felis catus*) hairs from dog (*Canis familiaris*) hairs. Although this work was original and important, we believe that it is of limited forensic value. Identifications were effected by comparing data derived from reference hairs of unknown body origin with questioned hairs of unknown body origin. We believe a more scientifically valid approach would have been to produce different data sets derived from hairs from known body
areas, for comparison with data derived from the questioned hairs from unknown body areas. This is because morphological characteristics of animal hair varies in relation to somatic origin i.e. body area (Brunner 1974). In addition, Peabody et al. (Peabody 1983) attempted to corroborate their quantitative findings with scale pattern analysis. Unfortunately these authors compared scale patterns from what they believed to be domestic cat hairs (Felis catus) (based on their medullary index) with the images of cat hairs produced by Appleyard (Appleyard 1978). However, the cat hairs cited in Appleyard (Appleyard 1978) came from ‘Felis ocreata catus’ (African wild cat), not a domestic cat (Felis catus). Each of these felid species exhibit different scale patterns as illustrated in Appleyard (Appleyard 1978) and Brunner and Coman (Brunner 1974).

2.2.4.2 Misconception: Pig (Sus scrofa) hairs may be mistaken for human hairs

Not infrequently forensic scientists need to identify hairs recovered from environments such as forests, beaches, or caves to determine whether the hairs are human or animal in origin. If human, authorities may be looking for an injured or deceased person and law enforcement personnel need a timely, accurate identification of these hairs in order to determine an appropriate course of action.

Whilst it is accepted that pigskin is commonly used as a surrogate for human skin, and pig corpses are used in taphonomic studies in lieu of human cadavers, the hairs of these two species are absolutely distinguishable as demonstrated in Figure 2.2.3, which depicts features exhibited in dorsal hairs of adult pigs. An additional feature characteristic of adult porcine hairs is that the tips of pig guard hairs are split (commonly referred to as ‘flagged’) in most instances.
Figure 2.2.3. Images demonstrating Pig (*Sus scrofa*) hairs may be readily distinguished from human scalp hairs, based on at least two morphological characteristics. Top Panel shows a close ripple scale pattern, with close margins (A), which contrasts with the irregular wave, normal separation of smooth scale pattern of human scalp hair (B). The medullae configurations exhibited by pig body guard hairs (C) compared with human scalp hair (D) allows differentiation between these species (Scale bars: (C) 100 µm; (D) 50 µm).

This myth highlights the necessity of forensic animal hair examiners to be competent and capable of correctly identifying animal hairs from wild and ‘domesticated’ fauna in their particular geographic location.

2.2.4.3 Misconception: Scanning Electron Microscopy (SEM) is more effective than Transmitted Light Microscopy (TLM) in animal hair identification

It is widely espoused (e.g. Sato, Miyasaka *et al.* 1982, Chernova 2001, Chernova 2002, Furskin Company 2011, Kumar 2013) that by using high magnification and sophisticated digital microscopy more details will be revealed that will provide more power of observation and therefore more exactitude in MIAH - this is unfounded. Although SEM can certainly deliver high magnification and depth of field (much higher than transmitted light microscopy), it is a monochromatic,
surface-imaging technique that cannot provide details of colour or internal structure. As noted by Rowe (Rowe 1997) ‘...SEM for hair examinations is limited because most the morphological features used to identify species of animal from which the hair originated and used to compare evidentiary and exemplar hairs are within the hair, not on its surface’.

Transmitted light microscopy is the recommended and most widely used method for examining internal features and cuticle scale pattern along the entire length of the hair, as well as hair cross-sectional morphology. This provides the examiner with a comprehensive view of the specimen and allows study of all available taxonomic features that may be critical to effect an accurate identification.

2.2.4.4 Myth: Polar bear (Ursus maritimus) hairs are hollow

The most prevalent and widely cited myth, which appears to be universally accepted on the Internet and in peer-reviewed literature (Lewin 1979, Armstrup 2003), is that polar bear hairs are hollow. Polar bear hairs have been described by Morioka (Morioka 2005) as having a shaft that resembles an ‘end-capped straw,’ implying that the shaft is like a hollow tube. Furthermore, Morioka (Morioka 2005) also states that polar bear hairs lack medullae.

Each of these assertions is demonstrably incorrect as shown in (Figure 2.2.4). The medulla or core of the hair shaft is composed of air filled cells and vacuoles, which, under transmitted light appears dark; however, if the hair shaft integrity is compromised, mounting medium may seep into the hair and fill the medulla cells and vacuoles. The result is that the entire hair becomes translucent and apparently devoid of a medulla using transmitted light microscopy.

It is possible that inexperienced researchers, concluding that polar bears as hollow, may have based this observation on hairs with a cleared medullae (Figure 2.2.4). However, as Morioka (Morioka 2005) did not provide the images from which he derived his conclusion it is impossible to ascertain what is was that led him to his “hollow hair” conclusion.

1 Using Google, a search of the Internet using the string ‘polar bear hair hollow’ returned in excess of 450,000 ‘hits’ that supported this premise.
Figure 2.2.4 Transverse cross-sections of polar bear (*Ursus maritimus*) dorsal overhairs (A) and guard hairs (distal shafts) and human beard hair, (B) each showing a dark, central air filled medulla in unpigmented cortex. Polar bear guard hairs showing air filled medulla (C) and) translucent or ‘cleared’ medulla filled with mounting medium (D). (Scale bars (C) and (D) = 100 µm.

2.2.5. Possibilities in MIAH

Assuming a competent practitioner conducts the identification process, the taxon level to which the animal hair in question can be attributed is dependent on the following criteria

1. The hair type
2. Condition of the hairs
3. Availability of reference hairs from known, vouchered specimens for comparison with the morphological characteristics from the questioned hair

As discussed in Section 1, guard hairs are recognized as the hair types that contain the most diagnostic features upon which a microscopical identification may be made. If the condition of the hair in question is such that insufficient morphological characteristics are present (e.g. short, broken hair fragments or hairs that have been degraded by environmental processes) identification may only be possible to a higher taxonomic level such as Order, rather than at a lower
level such as Family or Genus. Confirmation of the identification necessitates the comparison of the characteristics exhibited by the questioned hair with relevant hair(s) from a vouchered animal reference specimen. MIAH cannot attribute the source of a questioned hair to an individual animal; however, some studies suggest limited associations may be possible (Suzanski 1988, Suzanski 1989).

2.2.6. Pitfalls

This section discusses common pitfalls witnessed by the authors, either through reviewing literature or reviewing work conducted by inexperienced or inadequately trained animal hair examiners.

2.2.6.1 Training

MIAH, like any other scientific discipline, is only as good as its practitioners, the equipment, and the reference materials they use. Pertaining to practitioners, Bisbing and Houck state: “Training and qualification of forensic hair examiners is crucial to the quality and reliability of forensic hair examinations. Many of the weaknesses in forensic hair examinations…are a result of inadequate training of forensic hair examiners and a lack of understanding about the fundamental nature of the examination of hairs” (Houck 2005). Although this was written in relation to human hair examinations, the tenet is equally applicable to MIAH. A practitioner seeking to identify an animal hair needs to have knowledge of key morphological features from many different species as opposed to knowledge of only one species, as is the case of human hair examination, or a target species. For MIAH, there needs to be awareness of somatic, inter- and intra-species morphological variations, as stated by Lobert et al. (Lobert, Lumsden et al. 2001); ‘We emphasise the need for practitioners to gain considerable personal experience of the technique, the diagnostic characteristics used to identify hair of different species and intra-specific, in order to maximize the reliability of identification results’.
2.2.6.2 Forensic Human and Animal Hair Competencies

A significant pitfall in relation to morphological animal hair identification is the assumption that a practitioner competent in morphological human hair comparison is equally, and automatically, competent in MIAH. However, both examinations have different goals and as such necessitate different competencies in order to accurately conduct each type of analysis. Ogden (Ogden 2010) expresses these sentiments: “... it is generally easier to teach a wildlife geneticist to do forensic (human based DNA) casework than it is to convert a human forensic DNA specialist into a wildlife DNA forensic scientist. A human (sic) forensic scientist attempting to learn the range of scientific techniques and underlying biological assumptions involved in different wildlife identification enquiries is faced with a very large, diverse body of knowledge to attain”.

Morphological identification of animal hairs is an exercise in classification that relies on the recognition and interpretation of defined, genetically determined features present in all hairs from animals belonging to a particular taxon. In contrast, human hair examinations rely on the comparison of subjective, albeit genetic, characteristics (e.g. colour, pigment type, and distribution) and acquired characteristics (e.g. damage, artefacts, chemical treatments) in order to exclude, or not exclude, an individual(s) as the possible source of the questioned hair. Therefore, forensic practitioners solely trained and experienced in human hair comparisons do not automatically achieve competency in morphological identification of animal hairs; the same logic applies to those solely trained in MIAH, who would not be competent in human hair comparison.

2.2.6.3 Atlases and Literature

Whilst standard reference works (Wildman 1961, Brunner 1974, Appleyard 1978, Teerink 1991) serve as excellent examples to illustrate morphological features useful for MIAH, it is crucial that the practitioner, experienced or otherwise, is aware that these are not definitive or exhaustive works, either in regards to the range of animals covered or in regards to all of the morphological features present in each hair type. As Brunner and Coman (Brunner 1974) state in the preface to their animal hair atlas, “It is important to realize that the photographs...represent only some of the multitude of structures observed in...
Atlases are of considerable use in training hair examiners as they illustrate the diversity of morphological characteristics present in animal hairs. However, as a sole basis for identification, atlases are of limited utility as they offer ‘snapshot’ images of only one part of the hair; furthermore, it is not uncommon to find that morphological features of hairs, from the same species, differ in different atlases. Therefore, the use of these pictorial references should not be used as substitutes for knowledge and information derived from the examination of vouchered hairs, from a well-stocked reference collection. As Wildman (Wildman 1961) observed ‘. . . although books and photographs are useful as guides, there is not reliable short-cut method for identifying animal hair fibres by simply ‘matching up’ the microscopical appearance of an unknown fibre with a photomicrograph’.

In relation to keys or other classification schemes that attempt to assist in the identification process, Kirk noted: “Such schemes have a certain value when used with the reservations imposed by experience and study, but their value even in this sense is limited. Experience in examining hair and study of its characteristics will supply far more information than can be obtained by study of any stereotyped classification scheme”(Kirk 1953). Although this was in relation to classification of human hair types, this tenet is equally, if not more, applicable to MIAH for reasons outlined above.

2.2.6.4 Taxonomy and Binomial Nomenclature

Binomial nomenclature is universally understood. It not only crosses linguistic and cultural boundaries, but it also ensures that there is no doubt as to the identity of the animal in question. In a wildlife forensic context, an indictment is predicated on determination of the taxon represented by the evidence and its legal listing as endangered or threatened. The pitfall of referring to the animal in question solely by its common, or vernacular, name is likely to result in misunderstandings or confusion in relation to the real identity of the animal being discussed. Reference hair collections, or questioned hairs, identified with vernacular names are likely to result in mis-identifications. For example, a sample labelled as dog may be hairs from domestic dog (Canis familiaris) or raccoon dog (Nyctereutes procyanoides), which is a wild species used in the
industry. Fur apparel labelled as ‘dog’ may be mistaken as originating from a domestic dog instead of a farmed raccoon dog, which may lead to accusations that furriers are using domestic dogs in fur coats. In presenting testimony, we recommend the use of the common name and binomial scientific name when the animal in question is first mentioned and thereafter refer to the animal or taxon in question by its common name (a good example of the confusion that can arise is exemplified by the work of Peabody et al. (Peabody 1983) where *Felis catus* (domestic cat) was confused with *Felis catus ocreata* (African wild cat). Unfamiliarity with taxonomy and/or binomial nomenclature of animals cannot justify the sole use of common or vernacular names; in a forensic context the onus of unambiguously identifying the animal of origin of the questioned hair(s) solely relies on the scientist presenting the evidence, not the jury, legal counsel or the judiciary. In the provision of investigative leads, we would advocate the use of common names as law enforcement personnel are likely to be non-specialists in relation to animal taxonomy, except if there is a risk of misleading the investigators.

### 2.2.7 Future Directions in MIAH

#### 2.2.7.1 Promoting Best Practice

A significant recent direction in MIAH, and other forensic wildlife disciplines, is in the formation of the Scientific Working Group for Wildlife Forensics (SWGWILD). Founded in 2011 with affiliation to the Society for Wildlife Forensic Science (SWFS), SWGWILD brings together world wildlife forensic experts to promulgate best practice across diverse species and evidentiary material unique to this field through the provision of standards, education, and certification starting with the disciplines of DNA and Morphology. The production of these guidelines and recommendations for wildlife forensic practices is the first of its kind. As such, it is a significant milestone in formalizing practices and standards for this discipline to ensure practitioners and laboratories are appropriately qualified, accredited and competent to be regarded as experts in the MIAH.

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2http://www.wildlifeforensicscience.org/swgwild/
2.2.7.2 DNA analyses and Microscopy

Over the years the molecular analysis of animal DNA has steadily increased in the investigation of poaching and trafficking in CITES listed mammals (CITES), animal cruelty cases and crimes against the person in which animal hairs are submitted for examination (Tridico 2005). However, whilst these analyses are routine in specialized forensic wildlife laboratories, this is not the case for many forensic laboratories, which usually deal with crimes against the person. In cases in which animal hairs are critical to investigations, species identification is commonly outsourced to specialist laboratories. However, the costs are prohibitive for regular or routine use of these services. Therefore, it makes good economic and efficiency sense to subject unknown animal hair specimens to morphological analysis first in order to establish whether molecular techniques are even required. Based on a global benchmarking process for forensic laboratories, FORESIGHT (Houck, Riley et al. 2009) the average cost per case for a human DNA analysis is $2,255 in 2012; if one or more items can be excluded from analysis by a simple microscopical examination, the cost savings to the laboratory can be significant. For example, it can be quickly decided whether the hair in question is human, animal, plant, or textile fibre in origin. If it is assumed that DNA analysis of animal hairs involves a similar cost then MIAH as the first step also makes economic sense for the same reasons. This is likely to remain the case until such time as NGS is routine, simple, validated for forensic purposes and more cost effective.

Research has shown the value of combining DNA analyses with the morphological examination of human hairs (Rasmussen, Bertalan et al. 2010, Rasmussen, Lohmueller et al. 2011) and there is no reason to doubt that the two techniques will be also be complementary in regards to animal hair identification as illustrated in the work conducted by Shajpal et al. (Sahajpal, Goyal et al. 2009) in relation to wildlife forensic cases. Whilst DNA sequencing can identify the origin of an unknown animal hair and in time might even allow individualization within a species, MIAH in addition to providing a highly reliable screen can provide additional value in relation to mode of removal, effects of taphonomy, and identification of artefacts and treatments. For example, in a hypothetical case, a large clump of ‘big cat’ hairs is found in the back of a
suspected poacher’s vehicle but further microscopical examination shows the presence of post-mortem banding. This means that the hairs could only have originated from a decomposing body, which opens up the possibility that the suspect may have merely picked up a dead body rather than poached it.

2.2.8 Conclusions

Morphological identification of animal hairs is a robust and valid forensic technique; however, the integrity of the results is wholly dependent on the availability of type/or vouchered reference specimens and on the proven ability of the practitioner to accurately identify the animal of origin of unknown animal hair based on morphological characteristics and to present appropriate testimony. Budowle et al. (Budowle 2005) in their recommendations for animal DNA forensic and identity testing state “It is important to operate under a set of minimum guidelines that assures that all service providers have a template to follow for quality practices that can withstand legal scrutiny”. In this vein, from our experience in the field of MIAH (which amounts to over 60 years total just for two authors), we propose the following recommendations for legal practitioners, investigators, journal editors, and forensic scientists to consider when producing or reviewing MIAH statements, publications or reports.

- Microscopy. Scale patterns, medullae configurations, and root shapes (if present) must be recorded and appraised using representative samples of each hair type present in the sample. Scale patterns and medullae configurations should be determined along the length of the hair shafts.
- Images. Images used to record MIAH must contain scale bars that are clearly visible, the exception being scale cast patterns where it is inappropriate to include scale (since the entire diameter of the shaft may not be in contact with the medium). Image legends must include information on hair type, where on the hair the image was taken, and the somatic origin of the hair (if known). All images should clearly and unambiguously demonstrate the feature of interest.
- Descriptors. Nomenclature describing medullae and scale pattern configurations should include the reference from which the descriptors are taken.
• Comparative analyses. Confirmation of identification must result from a comparative analysis between the characteristics shown by the questioned hair and relevant hairs taken from a vouchered specimen and the points of comparison recorded.

• Taxonomic identification. Common names must be accompanied by binomial nomenclature i.e. scientific (Latin) names (at least at first mention)

2.2.9 References


2.2.10 Chapter Summary

Chapter Two represents a significant advancement for the field of microscopical examinations of hairs. For the first time the chapter dispels a number of widely held myths and misconceptions, in relation to the microscopical examination of hairs, promulgated in the literature and on websites. It also raises the awareness of the pitfalls untrained or ill trained practitioners may encounter in attempting to identify (and interpret) diagnostic features on hairs.

Identification of animal hairs via microscopical characteristics is not an insignificant undertaking and one that requires years, rather than months, to achieve competency and proficiency. However, due to ever decreasing budgets, employers are reluctant to divert funds to long-term training, particularly in the era of hair analyses by molecular analyses and DNA sequencing. Structured formal training of potential hair examiners appears to have been replaced by comparison of microscopical features present in the hair of unknown origin with snapshots present in hair atlases or, through the use of keys. Each of these methods is a poor substitute for formal training with a qualified mentor. Hair atlases are appropriate as aide memoirs, training aids, or to showcase the variety, and variation, of microscopical features that are exhibited in animal hairs. However, these pictorial atlases should not used as substitutes for knowledge and information gained from the systematic audits of hair features from vouchered hair specimens from a well stocked collection. The technological advancement of the ‘Internet’ has resulted in ‘online’ pictorial atlases of animal hair morphology that more often than not, lack information that ‘validates’ their atlases, such as where on the body (somatic origin) the hair originated from, the hair type e.g. primary or secondary guard or where on the shaft the image was taken from; to use these images as substitutes for mentored training, to attribute an unknown animal hair, to a particular taxon is unlikely to yield to result in a reliable identification. In relation to the use of keys Packer (Packer, Gibbs et al. 2009) sums up the efficacy of this approach as ‘Keys are written by those who don’t need them for those who can’t use them.’
Mis-identification of features exhibited by human and animal hairs may have serious consequences in forensic investigations or result in misguided distribution of funds in mammalian conservation projects. Prior to the publication of this thesis research (published in Forensic Science International, Appendix A.1), the distinct and separate skill sets required for conducting microscopical human hair examinations and animal hair identifications was not acknowledged or addressed in the literature. It is hoped that this publication may raise awareness of the minimum standards hair examiners should adhere to, in order to promote best practice in this field of study. It is also the first publication to discuss the future of the microscopical identification of animal hair in the context of accreditation of not only the discipline, but also its practitioners. The issues of practitioner competency and expertise in the microscopical identification of animal hairs broadly is one that is driven internationally by the Society Working Group for Wildlife Forensic Science (SWGWILD) (Forensics 2012). The aim of this group is to ‘...standardise and promulgate best practice in the diverse fields and evidence types of that are intrinsic in the unique (forensic wildlife) field’

In forensic, archaeological and paleontological contexts hairs can be in ‘pristine’ condition when examined, but this is not universally the case. Hairs of humans and animals are modified by various environmental ‘insults’ from specialised biological organisms capable of dismantling hair. In the following chapter (Chapter Three) the importance and significance of identifying these features is explored more fully.
Chapter Three

Pre and post-mortem changes to mammalian Hair: Life after death

‘From each sad remnant of decay, some forms of life arise’
Charles Mackay (1814-1889)

3.1 Preface

Chapter Three. This chapter builds upon the concepts introduced in Chapter Two regarding microscopical skills required to accurately interpret morphological characteristics of hair. This current chapter showcases a variety of morphological signatures produced by environmental factors and/or specialised biological agents capable of dismantling and digesting hairs. This work resulted in a published manuscript titled ‘Interpreting biological degradative processes acting on mammalian hair in the living and the dead – which ones are taphonomic?’ (‘Royal Society Proceedings B 2014, 281:1796’.)
The content of this chapter is the same as in the published article found in Appendix A.2 apart from minor changes made to incorporate in-thesis referencing.

Mammalian hairs are subject to a variety of influences that affect their structure and function. One of these influences is exerted by the actions of a group of biological organisms; although disparate in nature, each of these organisms shares the unique capability to digest keratin and systematically dismantle hair. The identification of pre- and post- mortem modifications to hairs caused by these biological agents may assist forensic investigators in recreating the scene of a crime or enable archaeologists to gain glimpses into ancient lives.

Although biodegradation of mammalian hairs has been reported previously, work published in this area is ad hoc, incomplete, and at times misleading. This present study addresses these shortcomings; it is the first holistic, in depth microscopical study of the effects of biodegradation of hairs caused by environmental factors and/or disparate biological agents. The aim is to showcase a suite of microscopic morphological signatures attributable to a variety of environmental insults and biological agents capable of digesting keratin. This present study also aims to address misconceptions in the current literature in
relation to the effects of biodegradation of hairs. Furthermore it echoes the sentiments expressed in Chapter Two regarding the importance of accurate interpretation of microscopic features and the consequences of misinterpreting biodegradation as genetically acquired structures.

3.1.1 Statement of Contribution
As the lead author of the following manuscript, I conducted all microscopical examination of hairs and interpretation of microscopic morphological characteristics. I also acquired all images presented in the manuscript. I wrote the first and last versions of the manuscript and received editorial input from the listed co-authors.

Silvana R. TRIDICO
3.2 Interpreting biological degradative processes acting on mammalian hair in the living and the dead – which ones are taphonomic?

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3.2.1 Summary

Although the taphonomic (\textit{post-mortem}) degradation processes relevant to teeth and bones have been well described, those taking place with regards to mammalian hairs have not been characterized to the same extent. This present article describes, in detail, microscopic changes resulting from the actions of biological agents that digest and degrade hairs. The most noteworthy and prevalent agents responsible for the destruction of hair structure are fungi, which use a range of strategies to invade and digest hairs. One of the most important finds to emerge from this study is that taphonomic structures and processes can easily be interpreted by the unwary as ‘real,’ or as class characteristics for a particular animal taxon. Moreover, under certain conditions ‘taphonomic’ processes normally associated with the dead are also present on the hairs of the living. This work will improve the reliability of hair examinations in forensic, archaeological and paleontological applications. In addition, the finding has relevance in the protection of mammalian collections susceptible to infestation. This article also addresses the popular myth that ancient peoples were often red-haired and discusses phenomena responsible for this observation. Insights gained from detailed characterisation of taphonomic processes in 95 hairs from a variety
of species demonstrate the range and breadth of degradative effects on hair structure and colour. Lastly, the study demonstrates that hairs often tell a story and that there is value of extracting as much morphological data as possible from hairs, prior to destructive sampling for biomolecules.

3.2.2 Introduction

Mammalian hair is one of the most frequently encountered biological materials in forensic investigations and may be associated with human remains at ancient and modern burial sites. In addition to bodily remains, mammalian hairs may also be present in artifacts such as textiles and cordage (Appleyard 1978, Rowe 2010). Hair is essentially composed of three layers, namely the outermost cuticle, an inner cortex and a central core or medulla (Figure 3.2.1). The medulla consists of vacuolated cells filled with air and although they have been reported as only occurring in ‘larger’ hairs (Bengtsson, Olsen et al. 2012), medullae also occur in hairs of medium and fine diameters (Teerink 1991). The cuticular scales are arranged along the hair shaft (akin to slates on a roof), with the free edges always protruding towards the tip end of the hair. During the extrusion of the hair shaft from the follicle the hair becomes keratinised (hardens) and the cortical cells become tightly fused to the scales or cuticle. The cortex makes up the bulk of the hair shaft in most mammalian hairs and is mechanically the most significant component of the hair. It is made up of tightly packed, elongated spindle shaped cells which are oriented parallel to the axis of the shaft. Dispersed within this densely packed structure are the melanin pigment granules, the number of which and their distribution pattern determine the colour of the hair. Hairs have their medulla centrally placed in the shaft.
Figure 3.2.1 Mammalian hair structure and agents of hair degradation. Pigment granules (A), medulla (B) and cuticle scales (C).

Hair can withstand the test of time, often surviving for millennia due to the resilience of the keratin biopolymer. Central to this stability and resilience of keratin is the presence of numerous disulphide bonds that form between the thiol moieties of cysteine (amino acid) residues in adjacent protein strands. These stable bonds endow hairs with the ability to withstand exposure to diverse and extreme conditions such as freezing, burial and mummification. However, despite this resilience, hair is not immune to the effects of pre-mortem or post-mortem (taphonomic) degradative processes.

Traditionally associated with palaeontology (Lyman 2010) the term ‘taphonomy’ is today used in a broader sense and is synonymous with post-mortem degradation processes on organic remains- whether prehistoric, ancient, or contemporary in origin.
Post-mortem degradation of hairs is evident as microscopic morphological changes caused by biological agents that share the unique capability to degrade and digest keratin (Figure 3.2.1). The ubiquity and unique abilities to exploit a variety of niches make microorganisms the most significant agents of hair degradation. The most prevalent of these are filamentous fungi that have the natural capacity to break down and digest keratin (Kunert 1989).

Fungi that can attack hair are described as ‘keratinophilic’ and ‘keratinolytic’ and these descriptors have been used interchangeably despite clear distinctions between their meanings. Keratinolytic describes a group of microorganisms capable of decomposing and digesting keratin through the production of boring hyphae (fungal structures with appendages capable of penetrating hairs) and subsequent enzymatic digestion of keratin (Marchisio 2000, Blyskal 2009), whilst ‘keratinophilic’ describes fungal species capable of only utilising easily degraded substances such as partially decomposed keratin or materials related to keratin (Sharma 2003, Blyskal 2009). Species from each of these fungal types mainly reside primarily in soils (geophilic) with variable distribution patterns that are dependent on a variety of factors, one of which includes a preference for co-location with mammals (Deshmukh 1985, Garg 1985). Keratinolytic fungi represent an ecologically significant group of fungi that recycle one of the most abundant and highly stable mammalian proteins on Earth-keratin.

The aim of this present study was to microscopically examine ancient and modern mammalian hairs in order to document the effects of biological degradation commonly regarded as taphonomic (occurring after death). An additional aim was to demonstrate that accurate interpretation of mammalian hair morphology is technically demanding; as such it is important that forensic, archaeological and other practitioners are aware of potential artifacts in structure, colour and composition that can occur in hairs both pre- and post-mortem. An appreciation for what information can be gained from studying the effects of biological degradation of hair will ensure they are recorded and imaged prior to destructive analyses.
3.2.3. Materials and Methods

3.2.3.1 Materials
Approximately 450 hairs, from a variety of species, were examined and 95 hairs were chosen as exemplars of the spectrum of taphonomic characteristics; these were examined in detail. These exemplar hairs included extinct megafaunal hairs and ancient human hairs that were the subject of previous studies (Rasmussen, Bertalan et al. 2010, Rasmussen, Lohmueller et al. 2011); others were modern human and animal hairs. Table 3.2.1 provides further details in relation to each of the samples examined. The ages cited in relation to hairs relating to the “Somerton Man”, unidentified female, Norse Kal male, Borum Eshøj male, polar bear and Asian red-cheeked squirrel were provided on documentation accompanying the samples. Hairs were selected on the basis of microscopic morphological features that best articulated the effects of biodegradation or environmental alterations.
Table 3.2.1. Sources, age and depositional environments of hairs examined in the present study

<table>
<thead>
<tr>
<th>Hair Sources</th>
<th>Hair type</th>
<th>Age of specimens</th>
<th>Depositional environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woolly Mammoth (Jarkov)</td>
<td>Coarse</td>
<td>20380 +/- 140BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Woolly Mammoth (Yukagir)</td>
<td>Coarse</td>
<td>18560 +/- 50BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Woolly Mammoth (M25)</td>
<td>Coarse</td>
<td>59300 +/- 2700BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Woolly Rhino (Churapcha)</td>
<td>Coarse</td>
<td>19500 +/- 120BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Woolly Mammoth (Yuka)</td>
<td>Coarse</td>
<td>39440-38850 BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Human (Saqqaq Male)</td>
<td>Scalp</td>
<td>4044 +/- 31BP</td>
<td>Permafrost</td>
</tr>
<tr>
<td>Human (Norse Kal Male)</td>
<td>Scalp</td>
<td>Circa 14th Century</td>
<td>Wrapped in cloth and buried in soil</td>
</tr>
<tr>
<td>Borum Eshøj ‘young man’</td>
<td>Scalp</td>
<td>Circa 14th Century</td>
<td>Oak coffin buried in barrow</td>
</tr>
<tr>
<td>Aboriginal (Male)</td>
<td>Scalp</td>
<td>100 years</td>
<td>Stored in office drawer</td>
</tr>
<tr>
<td>‘Somerton Man’ unsolved homicide</td>
<td>Chest</td>
<td>60 years</td>
<td>Removed from plaster cast of body</td>
</tr>
<tr>
<td>Murder victim</td>
<td>Scalp</td>
<td>30 years</td>
<td>Tropical environment</td>
</tr>
<tr>
<td>Asian Red-Cheeked Squirrel (Dremomys rufigenis)</td>
<td>Base of tail</td>
<td>9 years</td>
<td>Curated Mammalian Collection (M32566)</td>
</tr>
<tr>
<td>Polar bear (Ursus maritimus)</td>
<td>Flanks, belly</td>
<td>7 years</td>
<td>Zoo specimen (M10983)</td>
</tr>
</tbody>
</table>
3.2.3.2. Methods

To ensure that each hair sample was comprehensively examined and to maximise the amount of morphological data obtainable, the following methodologies were adhered to:

- Initial examinations with low magnification (6-40x) stereo/dissecting microscopy. These enabled gross morphological features to be recorded e.g. colour, length and profile and enabled selection of hairs for further, more detailed examinations.
- Detailed examinations conducted at higher magnifications using transmitted visible light microscopy (TVLM) at 100x-400x. This enabled internal and external morphological traits to be examined along the entire length of the shaft.
- Scanning electron microscopy (SEM) was used to examine surface artifacts or details in much greater detail than possible with TVLM.

3.2.3.2.1 Stereomicroscopy

Samples were examined with a Wild M3Z Type-S stereomicroscope (Heerbrugg, Switzerland).

3.2.3.2.2 Transmitted Visible Light Microscopy (TVLM)

Hairs were mounted using Safe-T-Mounting permanent mounting medium (FRIONINE Pty Ltd, refractive index ~1.52). Hairs were mounted between conventional glass microscope slides and cover slips (0.17mm thick). Microscopy was performed on Leica DME compound transmitted visible light microscope equipped with UPLFL20x Semi apochromatic, UPLANO40x Apochromatic objectives. Images were acquired with Q imaging camera and associated software.

3.2.3.3 Scanning Electron Microscopy (SEM)

Each hair was affixed to double sided adhesive tape attached to a 12.6 mm diameter aluminium stub then coated with a 90nm layer of gold in a Balzers Union Ltd. Sputter coater (Liechtenstein) before being examined and
photographed in a Philips XL20 Scanning Electron Microscope (the software for image capture is part of the microscope operating software).

3.2.3.4 Scale cast pattern

Scale cast patterns were produced in accordance with the methodology of Brunner and Coman (Brunner 1974). Briefly, a cover slip was coated with clear nail polish and the hair was placed on the wet polish; once hardened the hair was removed leaving an impression of the cuticle in the nail polish.

3.2.4. Results and Discussion

3.2.4.1 Fungal invasions and digestion of hairs

We observed that the prequel to fungal infiltration of the hair is the envelopment of the structure by mycelia (a mat of filamentous fungal threads or hyphae) (Figure 3.2.2A); this is a process that takes place irrespective of whether the attacking fungi are keratinophilic or keratinolytic (English 1963). Hyphae on the woolly mammoth hair (Figure 3.2.2A), unlike the hair itself, may not be thousands of years old but a reflection of modern fungal invasion; either post-excavation or post-thaw. Keratinolytic fungi produce eroding fronds and penetrating/perforating organs that attack the cuticle by lifting or eroding the protective cuticle or scales (Figure 3.2.2 B,C) Perforating or penetrating organs (Figure 3.2.2.D) may be connected to filamentous hyphae (Kanbe and Tanaka 1982) (Figure 3.2.2 E) or produced from a mycelial mass (Figure 3.2.2 F) (English 1963, English 1968)
Figure 3.2.2. Fungal invasion of hairs. Fungal invasion of hairs. Woolly Mammoth (Jarkov) hair engulfed by hyphae (A), partial removal of cuticle (arrow) and dissolution of cuticle (bracketed) on woolly mammoth (Jarkov) hair, (B, C). SEM image of a penetrating organ (arrow), embedded in a woolly rhino hair (D). SEM image of a lateral fungal hypha with an eroding frond (arrow) (E). Mycelial mass (arrow) on shaft of woolly mammoth (M10) hair (F).

Once inside the hair shaft, keratinolytic digestion of hairs resulted in the following distinctive and varied morphological features:

- Thin thread-like hyphae (Figure 3.2.3 A,B), some of which spread laterally (Figure 3.2.3 B-D)
- Deeply penetrating or perforating organs (“borers”), which bore into the cortex (Figure 3.2.3E), Electronic Supplementary Material (ESM) Figure 3.2.4.1 S1a-d and Figure 3.2.4.1 S2 A-C)
- Fungal ‘blooms’, which appear to feed at a superficial level with negligible penetration into cortex (Figure 3.2. 3f and Figure 3.2.4.1 S2D-F).
Figure 3.2.3. **Fungal penetration of hair shafts.** Fungal penetration of hair shafts. Thin hyphae invading ancient human scalp hair (Saqqaq) and woolly mammoth (Yukagir) hair (lateral fronds are circled) (A, B). Lateral hyphae in woolly rhino hair (C). Extensive mass of lateral hyphae in polar bear flank hair. (D). ‘Needle’ (i) and ‘fissure’ (ii) type damage, to the shaft of woolly mammoth (Yukagir) hair (E). Surface fungal ‘bloom’ on M26 woolly mammoth hair (F).

In some instances, the high number and concentration of fungal hyphae present in the cortex may be so severe as to cause the shaft to bulge and distort (Figure S3.2.3A,B).

We also observed apparent fungal preferences in relation to their *modus operandi* in their destruction of hairs, which are discussed in detail in the ensuing sections.

3.2.4.2 **Fungal infiltration - from the outside in**

Fungal hyphae engulf the hair shaft (Figure 3.2.2A) and eventually compromise the cortical structure such that it disintegrates, as illustrated in Figure S3.2.4A,B. The efficacy with which the keratinolytic fungi invade and ultimately destroy hairs is mostly due to the production of keratinase – an enzyme that specifically dismantles keratin (Oyeka 2000, Blyskal 2009). Human scalp hairs from a 4000-year-old Palaeo-eskimo (Rasmussen, Bertalan *et al.* 2010) and hairs from woolly rhino each showed superficial fungal invasion
by thin hyphae. These hyphae grew transversely along the hair axis and some produced hyphae that travelled laterally (Figure 3.2.3a,b). DeGaetano et al. (1992) also reported similar thread-like fungal structures in human scalp hairs from buried remain relating to a forensic investigation. The thin hyphae observed here and by DeGaetano et al. (1992) closely resemble those produced by *Curvularia ramosa* (English 1963) and *Alternaria* spp (English 1965); these mildly keratinophilic fungi are environmental in origin and are found in soil and decaying plant matter (Thomma 2003). However, Shaw et al. (1967) identified the presence of *Alternaria* spp on the scalp hair of healthy subjects. The finding that these fungal species colonize the hair of the living and not just the dead has implications for forensic science. For example, a person is missing and their hairs are found in the premises of a suspect; the hairs exhibit fungal structures. Hitherto, it would be concluded that the missing person is dead and that the suspect has been involved with moving the body after it had been buried in soil or leaf litter; a search for a clandestine grave would begin and police would question the suspect as to their involvement in moving the body. In reality, the missing person might still be alive, albeit with an infection of fungus.

3.2.4.3 *Fungal infiltration - from the inside out*

Penetrating hyphae, once inside the cortex, in some instances appear to preferentially invade the medullary canal rather than digesting the cortex (Figure S3.2.5A), an observation previously noted by Kanbe (1982). It appears that fungal hyphae might find it easier to digest vacuolated medullary canals in preference to the much denser cortex. In essence the fungus digests the hairs from the inside out. We observed this type of destruction in hairs from the woolly mammoth ‘Yuka.’ This medulla to cuticle invasion is clearly visible as finger-like or stellate projections emanating from the medulla and penetrating the cortex toward the cuticle (Figure S3.2.5B).
3.2.4.4 Keratinolytic fungal invasion of hairs on the living

Keratinolytic fungal pathogens (dermatophytes) are capable of invading hairs of living humans and animals (non-humans) (English 1978, Papini 2008) as well as those associated with the dead. Dermatophytes may be divided into 3 broad epidemiological groups (Ellis 2014):

i) Geophilic: these fungi live in the soil; some species may invade skin, hair or nails of animals and humans following contact with the soil.

ii) Zoophilic: these fungi are primarily parasitic on animals.

iii) Anthropophilic: these fungi are primarily parasitic on humans.

Ecological sites inhabited by keratinolytic (and keratinophilic) fungi are diverse, and favor areas that are frequented by humans and/or animals e.g. playgrounds, stables, zoological gardens (Garg 1985, Deshmukh 2006). Lewin et al. (1981) investigated a reasonably common phenomenon that involved healthy polar bears (Ursus maritimus) kept in zoological gardens undergoing a ‘greening’ of their hair. In addition to algae (the source of the green colouration), images also show ‘stellate’ medullae and what appear to be fungal bores on the surface of the shaft. We also noted stellate medullae in belly hairs of polar bears kept in a zoo (Figure S3.2.5C). An additional phenomenon was noted in several of the polar bear hairs we examined, which was manifested as the complete hollowing out of the medullary canal (Figure S3.2.5D). However, based on the data here, it is possible Lewin et al. (1981) may have misinterpreted the stellate medullae as normal characteristics of hairs, rather than features arising from fungal attack. Whether this fungal invasion occurs in polar bears in the wild, as well as in captivity, has yet to be determined.

Brunner and Coman (1974) and Brunner and Triggs (2002) depict stellate medullae in hairs from an Australian marsupial - the common wombat (Vombatus ursinus). These authors reported this medulla type as a genetically determined, class characteristic for this species and therefore a suitable taxon-specific marker. However, based on the findings of the present study we believe that this type of medulla is likely due to the actions of keratinolytic fungi. The invasion most likely results from repeated and continuous exposure to geophilic keratinolytic fungi; wombats lead a largely subterranean existence. This
observation demonstrates that mis-interpretation of fungal artifacts can result in erroneous identification of hairs in faunal research applications and forensic investigations.

The presence of keratinolytic fungi and keratinophilic fungi on hairs that have been buried may reveal information in relation to the season or time of year interment occurred. Rowe (1997) states ‘the presence of the (fungal) tunnels is consistent with the burial taking place at a time of the year when it was warm enough and there was sufficient moisture available for the growth of the fungi’. The presence of these fungi in mammalian hairs most likely reflects burial in warm conditions. This is important in an archaeological context as an indication of the season of death may be inferred. In addition to indicating the season of death, which has obvious importance to an investigation, the observation of fungal tunneling can have other relevance. Firstly, fungal tunneling may preclude the positive identification of a hair as being human in origin and prevents its comparison against hair known to be from a missing person. Secondly, if hairs exhibiting fungal tunneling are found associated with a suspect, the implication is that the suspect has been in contact with hairs (and possibly therefore a body) that have been buried.

3.2.4.5 Keratinolytic bacteria

Compared to research into the biodegradation of keratin through fungal invasions, there appears to be a relative paucity of work conducted on the effects of keratinolytic bacteria. This may reflect the general lack of recording of or recognition of the effects bacteria may exert on hairs, or difficulty in culturing bacterial colonies for identification purposes. Instances in which bacteria have compromised the structure of hairs are presented and discussed below.

3.2.4.5.1 Keratinolytic bacterial invasion of hair shafts

Appleyard (1972) found that bacterial activity on wool fibres created numerous small pits on the surface of hairs, which were similar to pits found in extinct megafauna hairs in the present study (Figure S3.2.6A). Rod-like bacilli were found in tail hairs taken from an Asian red-cheeked squirrel (*Dremomys rufigenis*) from a curated natural history collection (Figure S3.2.6B), in which
the devastation of internal medulla structures is obvious in comparison to a healthy, unaffected hair (Figure S3.2.6C). The medulla of the infected hair has been digested resulting in a ‘hollowed out’ cortex (Figure S3.2.6B). The hollowed out medulla compromises the structural integrity of the shaft, to the extent that the affected hairs collapse under the pressure of obtaining the cross sections (Figure S3.2.6D). In comparison to fungal invasions, bacterial invasion of hairs may appear to be significantly less pronounced and superficial (McBride, Freeman et al. 1970). However, bacterial invasions of mammalian hairs have the potential to devastate curated skins and specimens. Appleyard (1972) also observed that bacterial invasion of wool fibres not only occurred in detached fleece, but also occurred on the fleece of living animals. In relation to humans hairs Shelley (1984) identified and recorded the destructive nature of pathogenic corynebacteria colonies on axillary (armpit) hairs of otherwise healthy humans.

3.2.4.5.2 Post mortem banding

Post-mortem banding (PMB) is a post-mortem degradation process that has received attention over the years due to its potential relevance in forensic investigations. The most recent studies conducted by Koch et al. (2013) suggest that post-mortem banding formation is accelerated in warm and humid conditions but retarded in colder ones. Biodegradation appears to occur solely in roots that are actively growing (anagen and catagen roots) at the time of death, rather than in roots in which growth has ceased (telogen). The preferential invasion of anagen and catagen roots is attributed to these roots being softer and more easily degraded, unlike telogen roots that are bulbous and hardened through the keratinisation process (Koch, Michaud et al. 2013). Although the precise mechanism responsible for PMB of hairs remains unknown, Linch et al. (2001) suggest that microbial and autolytic activities may be of importance.

PMB is observable by TVLM as a darkened band at the proximal (root) end of the hair (Figure 3.2.4A,B). This phenomenon has been observed not only in ancient hairs that are hundreds, or even thousands of years old, but is also observed in contemporary hairs (Koch, Michaud et al. 2013) as illustrated by the examples in Figure 4. Post-mortem banding occurs irrespective of the environment in which the hairs or remains were deposited. This is evident from
the examples shown in Figure 4, in which the depositional environments ranged from warm and humid (tropical) to frozen soil (permafrost). As the decomposition advances, the root will eventually break off at the darkened area and leave the proximal end with a pointed or a brush-like appearance (Figure 3.2.4 C,D) This loss of the band and root may clarify the erroneous assertion of Wilson (2008) who states ‘... postmortem root banding. . . is not noted in older hairs from archaeological remains’.

Koch et al. (2013) concluded that due to extensive environmental variables, PMB cannot be used to determine post-mortem interval (PMI), but it can determine whether an individual was dead when the hair was pulled or detached from the scalp. This determination can be instrumental in forensic investigations. For example Tafaro (2000) was able to unequivocally associate a suspect with a body that was undergoing decomposition; a finding which was instrumental in eliciting a confession to murder from the suspect.

![Figure 3.2.4. Post-mortem banding. ‘Somerton man’ chest hair (A). Pointed root of scalp hair from a murder victim. (B). Pointed root of Norse Kal male scalp hair (C). Brush root of woolly mammoth (Jarkov) hair (D). Scale bar: a = 50 µm, b=200 µm, c = 100 µm, d = 500 µm.]

3.2.5 Natural or acquired hair colour?

Archaeological hair samples, human and animal, frequently exhibit red colouration (Wilson 2001, Tridico S.R 2014). Post-mortem or post depositional colour change to hairs may be attributed to a number of factors such as photo-
degradation of pigment granules in sunlight (Lee 2010) or the oxidation of melanin pigment granules over millennia (Lister 2007). Microscopical examination of a 100-year-old tress of scalp hair from an Aboriginal youth (Rasmussen, Lohmueller et al. 2011) revealed hairs that most likely showed the effects of photo-degradation of pigment granules. Pigment granules in the oldest part of the hair (tip end or distal shaft) were significantly lighter in colour in comparison to the younger part of the hair (Figure S3.2.7A). In an ante-mortem context other factors can impact on bleaching of pigment granules (e.g. chemicals), but in the case of the Aboriginal youth (Rasmussen, Lohmueller et al. 2011) it is reasonable to assume that the older parts of the hair have had more exposure to sunlight.

Wolfram et al. (2006) noted that whilst all pigmented hair lightens when exposed to sunlight, the affect was most noticeable at low latitudes and in high humidity environments. Rowe (1997), in his study of bio deterioration of hair colour, observed that exposure of white, un-pigmented dog hair to relatively high humidity resulted in the development of a distinct yellow-orange colouration. Further investigation of these hairs revealed the presence of brown fungal hyphae and spores on the surface of the hair; a phenomenon also seen in extinct megafauna hairs that caused the shaft of the hairs to become orange/red in colour (Tridico S.R 2014). Although the chemical reactions and their affects on hair pigments undoubtedly account for some of the red colouration of hairs, they cannot be the sole cause. Krefft (1969) found that hairs devoid of pigmentation also exhibited this red colouration, which he attributed to the alteration of the chemical structure of keratin, as demonstrated in animal ancient and modern animal hairs (Figure S3.2.7B,C). Irrespective of the cause, red hair on or near remains should be thoroughly investigated to determine if the colour is natural or acquired. Naturally red (ginger) scalp hair, unlike brown or black hair, usually shows medial (central) distribution of pigment granules within the shaft (Figure S3.2.7D).

3.2.6 Insect damage

Curated mammalian hair collections represent important and valuable scientific resources. Mammalian furs from these collections are often used for species
identification of animal hairs of unknown origin. Structural integrity of these collections may be compromised not only by keratin-digesting fungi and bacteria but also by the action of insects. The implications of this destruction are that these exemplar hairs are no longer reliable as comparative resources from which unknown animal hairs may be identified.

The most commonly encountered insects able to utilize keratin are larvae of clothes moths (*Tineidae*) and carpet beetles (*Dermentidae*) (Appleyard 1972). Although these larvae are usually associated with the invasion and destruction of museum and other curated mammalian specimens, they may also affect forensic and archeological hair samples as illustrated in Figure 3.2.5a-d.

**Figure 3.2.5.** Insect larvae damage. Woolly rhino coarse hair (A). Borum Eshøj ‘young man’ scalp hair (B). Red deer (*Cervus elaphus*) dorsal hair. (C) Chest hair from deceased ‘Somerton Man’ (D). Images a, b and d show cuspate bite marks caused by larval mandibles. Scale bars: a, b, d = 100 µm, c = 200 µm

3.2.7 Taphonomy of hair and survival of DNA

Hair has, in recent years, gained more prominence as a substrate for ancient genomic analyses. Extraction of DNA from hair is often preferable to drilling bone as it has been reported to contain a higher proportion of endogenous DNA relative to contaminating microorganisms (Gilbert MTP, Campos et al. 2007). The presence of nDNA in the roots of ‘modern’ hairs is beyond the scope of this study. DNA preservation in hair shafts is less well established and more relevant to hair discovered in archeological/paleontological and in some forensic contexts
as a result of taphonomic or physical severance of the root. Whilst mtDNA is present in abundance in the hair shafts, nDNA appears to be present at significantly lower, and often variable, levels (Butler 2010). Some researchers propose nDNA is present within the outer layer (cuticle) of the hair shaft (McNevin 2005) whilst others challenge this hypothesis by proposing that nDNA is present within the cortex and in the cuticle (Amory, Keyser et al. 2007).

Irrespective of the location of DNA within hair shafts, assessment of hairs for their suitability for molecular analyses should take into account the depositional environment from which the hairs were removed, rather than the elapsed time since they were deposited. Burger et al. (Burger, Hummel et al. 1999) also agree with this proposition stating, ‘Recent publications indicate that environmental conditions have more influence on DNA preservation than does time’ and they conclude that there is no general correlation between the age of the sample and the preservation of DNA. Bengtsson et al. (Bengtsson, Olsen et al. 2012) illustrate this premise by demonstrating successful extraction of DNA in ancient hairs (permafrost), some of which exceed the limit of radiocarbon dating; however, attempts to detect DNA in hairs from considerably younger ‘bog bodies’ failed. Perhaps the moist, bog environments accelerated DNA degradation to a much higher rate than that exerted by storage in permafrost. Alternatively, bog environments may contaminate hairs with powerful PCR inhibitors e.g. humic acids, phenolics or tannins. It is unclear without further research, if morphological features (e.g. microbial attack) on hair shafts are a predictor of the abundance of preserved DNA.

3.3. Conclusion

This study is, to our knowledge, the first to describe holistically the factors responsible for the biodegradation of mammalian hairs. Of significance is the finding that degradation processes previously reported as wholly post-mortem also occur in hairs from living mammals. Our data also reveal that taphonomic processes are not well understood and we found internal structures resulting from fungal infiltrations that have previously been interpreted as class characteristics upon which taxonomic identifications have been made. Collectively this study provides key information for fields where mammalian
hairs play a central role including, but not limited to, paleontology, archaeology, ancient DNA studies, forensic investigations and conservation biology. Unlike other degradative effects on hairs, we propose that post-mortem banding is the only hair modification process that unequivocally takes place after death and then only in catagen and anagen hairs. This finding can be of use in cases involving missing persons, mass disasters or even when analyzing hair in archaeological/paleontological contexts. Although post-mortem banding cannot determine post-mortem interval (PMI), it may, however, help investigators to reconstruct events and corroborate (or refute) statements and theories. In archaeological specimens the presence of PMB indicates that the environment in which the remains decomposed were sufficiently conducive for this phenomenon to occur.

The present study demonstrates the importance of familiarity and expertise in the recognition of normal and abnormal microscopical characteristics of mammalian hairs; an ideal that is discussed in greater depth by elsewhere (Tridico S.R 2014). Microscopical examination of hairs for post-mortem degradation may reveal the types of causative agents and conditions at the time of burial or death. There is much to be gained from examining hairs with transmitted light microscopy prior to more destructive techniques, such as molecular analyses or electron microscopy. The consequences of not performing detailed transmitted light microscopical examinations on hairs prior to more destructive analyses are irremediable damage to the hair and the likely loss of critical or key pieces of information or evidence.

3.4 Acknowledgements

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OR, USA for the provision of hairs from the Asian red-cheeked squirrel and polar bear (respectively).

3.5 Ethical statement

All hairs collected and analysed in this study were ethically obtained and permissions sought, and granted. For the most part samples were from museum (Tridico S.R 2014) and/or reference collections established over many years by SRT. In relation to the ancient human Palaeo-Eskimo and aboriginal hairs permissions are documented in previous publications (Rasmussen, Bertalan et al. 2010, Rasmussen, Lohmueller et al. 2011).

3.6 References


3.7 Supplementary Images

**Figure S3.2.1 Fungal perforating organs (borers).** Perforating organ on woolly mammoth (Jarkov) hair (circled) (A). Increased magnification of borer reveals and auger-like terminus (B). Borer, which has penetrated hair shaft (square). (d) Longitudinal view of an an in situ borer (arrow) (C). Scale bar: a,c =20µm
Figure S3.2.2 Fungal penetration of hair shafts. SEM images of woolly mammoth (Jarkov) hair, showing deep penetration of borers resulting in pits (A, B). Scale cast pattern of hair invaded with borers (C); SEM images of woolly mammoth (M10) showing superficial damage to shaft due to non-boring hyphae (D, E). Scale cast pattern of hair invaded by surface fungal blooms (F). Scale bar: a=100 µm, b= 5 µm, d= 50 µm, e= 20 µm.

Figure S3.2.3. Internal disruption of hair shaft caused by fungal mycelia on polar bear (belly) hair. Transmitted light image (A) and SEM image (B). Scale bar: a= 200 µm, b= 50 µm
**Figure S3.2.4. Fungal hyphae enveloping**: woolly mammoth hair shaft in which the separation of the cortex is evident (circled) (A) and human hair shaft in which the destruction is more advanced (B). Scale bar: a= 100 µm, b=50 µm.

**Figure S3.2.5. Fungal digestion of hair from the inside out.** Fungal hyphae invading medulla of woolly mammoth (Yukagir) hair (A). Cone shaped borers emanating from the medulla into the cortex of woolly mammoth (Yuka) hair (B) and polar bear belly hair (C). Arrows in Yuka hair indicates lateral hyphae extending from the main fungal structures. Polar bear forehead hair showing hollowed out medulla (D). Scale bar: a=20µm, b= 100 µm, c, d= 50 µm.
Figure S3.2.6. Keratinolytic bacteria invasion of hairs. SEM image of pitted surface of a woolly mammoth (Jarkov) hair (A). 'Hollowed' out Asian red-cheeked squirrel (ARCS) (Dremomys rufigenis) base of tail hair due to bacterial invasion (B). (Non-infected ARCS tail hair showing a healthy, air-filled medulla (C). Collapsed transverse cross-sections of hollowed out ARCS tail hairs (arrow) (D). Scale bar: a = 10 µm, b = 20 µm, c, d = 50 µm.

Figure S3.2.7. Effects of genetic and environmental influences on hair colour. Comparison of photo oxidised pigments in the tip area of an aboriginal scalp hair resulting in an orange colouration, with the un-affected markedly darker lower shaft (A). 'Acquired' environmental discolouration of hairs lacking pigment granules in (B). Woolly mammoth (Yuka) hair and (C) polar bear hair shafts (dark entities in the centre of the shaft are air filled vesicle not pigment granules). Human scalp hair with medial pigmentation that is commonly exhibited by individuals with genetically determined red hair (D). Scale bar: a, d = 50 µm, b = 200 µm, c = 100 µm.
3.8 Chapter Summary

Chapter Three collectively described unique morphological signatures caused by the actions of a variety of biological agents on hairs. The goals of this chapter were not only to showcase these signature morphological effects, but also to demonstrate that a variety of niche-specific, biological agents capable of degrading hairs, do so irrespective of specimen age, mammalian origin or the environment from which the hairs were recovered. The hairs studied and imaged in this study were those that best exemplified the morphological signature of the biological agent responsible for their destruction.

The present study is the first to identify that only one effect arising as a result of biodegradation of hair may be wholly considered as occurring post-mortem. ‘Post mortem banding’ (PMB), or ‘putrid root’ was solely found in hairs that had been present in decomposing corpses or remains, both human and animal. This phenomenon was found to occur in prehistoric, ancient and modern human and animal hairs suggest that the evolution of microorganisms capable of degrading hairs spans millennia. The significance of post-mortem banding in hairs is one that will have ramifications in scenarios involving missing persons. For example, if police find hairs suspected to originate from (or known to have originated from, as a result of DNA typing) a missing person, the presence of PMB on those hairs would indicate that further efforts would be towards seeking a corpse rather than mounting a rescue of a living person.

Chapter Three also demonstrates, for the first time, that fungal degradation of hairs can also occur on hairs from the living, as well as the dead. This finding is of particular significance in forensic investigations, as the presence fungal tunneling does not necessarily reflect that the hairs have originated from a deceased individual. Fungi that digest hairs have close association with soil, therefore hairs originating from animals that forage or live close to the ground such as voles and wombats (Vombatidae) are found to exhibit fungal tunneling. In a forensic context the interpretation of fungal tunneling in loose human hairs requires careful consideration of all circumstances surrounding the discovery. This chapter also demonstrably dispels some popular myths that are promulgated about polar bear (Ursus maritimus) hairs, namely that the shafts are hollow.
Furthermore, stellate medullae may be erroneously reported as genetic features rather than the result of fungal degradation (exemplified in Figure 1.2.17C penultimate image, bottom row). The potential negative consequences of mis-identifications of mammals not only promulgate incorrect interpretations, but also may have negative faunal and fiscal impacts in relation to conservation efforts or ecological studies.

The effects of biodegradation on hairs has broad applicability, it has relevance in forensic, investigations, archaeological and palaeontological applications curated mammalian collections and ecological surveys. Chapter three also presents, for the first time, the evolutionary longevity of biological agents capable of demolishing prehistoric, ancient and modern hairs, irrespective of animal origin or environment from which mammalian remains were ultimately retrieved. This chapter also reiterates aspects first raised in chapter 2 regarding core competencies required to accurately interpret microscopic features of hair.

The following chapter, not only builds further on the concept of biodegradation on extinct megafauna hairs first illustrated in this chapter, but also includes the morphological consequences of environmental insults on these permafrost preserved hairs. Chapter four also introduces the many benefits afforded by a multidisciplinary approach to the examination of hairs.
Chapter Four

Ancient History: microscopical examination of permafrost preserved extinct megafauna hairs

At midnight in the museum hall,
The fossils gathered for a ball.
There were no drums or saxophones,
But just the clatter of their bones,
A rolling, rattling carefree circus,
Of mammoth polkas and mazurkas.
Pterodactyls and brontosauruses
Sang ghostly prehistoric choruses.
Amid the mastodonic wassail
I caught the eye of one small fossil,
"Cheer up sad world," he said and winked,
"It's kind of fun to be extinct."

Ogden Nash, 1949

4.1 Preface

Chapter Four builds upon the study of biodegradation of hairs first introduced in Chapter Three, and introduces the concept of a multi-disciplinary approach to the examination of extinct megafauna hairs. This work resulted in the published manuscript titled ‘Megafaunal split ends: microscopical characterisation of hair structure and function in extinct woolly mammoth and woolly rhino’ (Quaternary Science Reviews, 2014,83:68-75). The content of this chapter is the same as in the published article found in Appendix A.3 apart from minor changes made to incorporate in-thesis referencing.

The morphology of woolly mammoth and woolly rhinoceros bones, teeth and carcasses have been extensively studied and documented; however the coats for which they are renowned have received significantly less attention and scrutiny. This present chapter attempts to redress this imbalance by presenting the first in-depth and comprehensive microscopical audit of microscopic features exhibited by permafrost-preserved woolly mammoth (Mammuthus primigenius) and woolly rhino (Coelodonta antiquitatis) hairs. It is also the first study to include
extinct megafauna hair samples that were originally part of a larger sample consumed for molecular analyses; the results of which are the subject of separate publications (Gilbert MTP, Campos et al. 2007, Gilbert, Drautz et al. 2008).

The aims of this present chapter are to:

(i) Present results from an in depth and comprehensive microscopical audit of morphological features and characteristics present in permafrost preserved extinct megafauna hairs,

(ii) Investigate possible relationships between the structures observed on extinct megafauna and the environment in which they lived,

(iii) Explore the benefits of a multi-disciplinary approach to the examination of hairs especially before they are destructively sampled for molecular analyses.

4.1.1 Statement of Contribution

As lead author of the following manuscript I prepared all hairs for transmitted visible light microscopy (TVLM) and confocal microscopy (CM). I had assistance from Mr. Gordon Thomsen regarding preparatory work for samples examined using scanning electron microscopy (SEM). I interpreted all of the morphological characteristics present within each of the hairs examined. I also conducted all photomicrography, with the exception of the CM image, which was taken with the assistance of Mr. Paul Rigby. I had comments, edits and input from the listed co-authors.

Silvana R. TRIDICO
4.2 Megafaunal Split Ends: Microscopical characterisation of Hair Structure and Function in Extinct Woolly Mammoth and Woolly Rhino

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4.2.1 Summary
The large extinct megafaunal species of the Late Pleistocene, *Mammutthus primigenius* (woolly mammoth) and *Coelodonta antiquitatis* (woolly rhino) are renowned for their pelage. Despite this, very little research has been conducted on the form and function of hair from these iconic species. Using permafrost preserved hair samples from seven extinct megafaunal remains, this study presents an in-depth microscopical characterisation of preservation, taphonomy, microbial damage, pigmentation and morphological features of more than 420 hairs. The presence of unique structural features in hairs, from two extinct megafauna species, such as multiple medullae and unparalleled stiffness suggests evolution of traits that may have been critical for their survival in the harsh arctic environment. Lastly, despite popular depictions of red-haired and/or uniformly coloured mammoths, a closer examination of pigmentation reveals that mammoth coats may have exhibited a mottled/variegated appearance and that their ‘true’ colours were not the vivid red/orange colour often depicted in reconstructions. Insights gained from microscopical examination of hundreds of extinct megafauna hairs demonstrate the value of extracting as much morphological data as possible from ancient hairs prior to destructive sampling for molecular analyses.
4.2.2 Introduction

Mammalian hair predominantly consists of the protein keratin, which due to its chemical structure is highly durable. This resilience is responsible for the survival and preservation of hair for millennia in remains that have been exposed to diverse and extreme conditions such as freezing, burial and mummification. Hair preserved in archaeological and palaeontological contexts is now sought after as a source of “pure” preserved ancient DNA (Gilbert, Drautz et al. 2008, Rasmussen, Lohmueller et al. 2011); however there is much to be gained from the morphological analysis of hair before it is destructively sampled.

Mammalian hair is essentially composed of three layers consisting of the outermost cuticle, an inner cortex and a central core or medulla (Figure.4.2.1). Close inspection of animal pelts reveals the presence of three distinct types of hair: overhairs, guard hairs and underhairs. Overhairs are the most prominent and coarsest of hairs on the pelage (coat) and are commonly circular in cross-sectional shape. Guard hairs are coarser and larger than underhairs; guard hairs exhibit an array of medullae morphologies, scale patterns and cross-sectional shapes that may be diagnostic for a particular taxon (Teerink 1991). The underhairs are shorter and much finer; they range from being wavy, lightly curled to tightly curled and commonly show circular cross-sections. In most mammalian hairs there is a gradation from one hair ‘type’ to another. This gradation is not abrupt as shown by the presence of ‘transitional’ hair types, which bear ‘hybrid’ features.

All mammalian hair shares similar chemical and physical composition and structure. Cross-sectional shapes, medullae morphologies and scale pattern not only differentiate human hair from animal but may also assist in differentiating animal hairs that originate from different taxa. Furthermore, mammalian hairs exhibit intra- and interspecies variance in profile and morphological characteristics depending on the somatic origin (body area that hair originates from) (Brunner 1974, Teerink 1991). While many extant taxa have been studied with regards to hair form and function, for obvious reasons, extinct species have received much less attention.
Figure 4.2.1. Schematic diagram of generic mammalian hair (centre) that consists of three major components. (A) The outermost cuticle (A), the central core or medulla (B) which may be continuous (left) or interrupted (right) and the cortex that contains pigment granules (melanins) which may be uniformly distributed across the hair shaft (left) or medially distributed (right) (C). (Images: S.R. Tridico)

The woolly mammoth (*Mammuthus primigenius*) is probably the most iconic and charismatic of all the extinct northern megafauna and is renowned for its size and hairy coat. Vast numbers of these animals roamed Eurasia and North America in the Pleistocene becoming extinct on the mainland some 10,000 years ago. The species clung to existence until the last known individuals, comprising a dwarf island population on Wrangel Island, vanished some 4,000 years ago (Vartanyan, Garutt *et al*. 1993). The causes underlying the extinction of woolly mammoth still remain elusive – a complex interplay of climate and anthropogenic influences is currently proposed (Lorenzen, Nogues-Bravo *et al*. 2011). Despite becoming extinct a few thousand years ago a great deal is known about the woolly mammoth, and it is arguably one of the best-understood representatives of the extinct megafauna. Their relative abundance and wide geographic range increased the probability of discovering their remains; their demise and subsequent entombment in a natural freezer ensured exceptional preservation.
In contrast, the woolly rhinoceros (*Coelodonta antiquitatis*) is less well understood. This is probably due to the paucity of mummified remains (compared to woolly mammoth) that have been discovered, which may reflect the more restricted geographic distribution of this species (it was absent from large areas of the high Arctic, for example) and possibly lower population density, relative to that of the woolly mammoth.

The morphology of woolly mammoth and woolly rhinoceros bones, teeth and carcasses have been extensively studied and documented contributing a wealth of knowledge with regards to their natural history and adaptations to surviving cold temperatures (Boeskorov 2004). Woolly mammoth were also among the first species to be investigated using PCR of ancient mitochondrial (Paabo, Higuchi *et al.* 1989) and nuclear DNA (Greenwood, Capelli *et al.* 1999). The advent of next-generation sequencing enabled researchers to sequence short, fragmented strands of mammoth DNA using the elephant genome as a scaffold (Miller, Drautz *et al.* 2008). Significantly, the substrate used for this genome was mammoth hair due to the high levels (relative to contaminating environmental sequences) of endogenous mammoth DNA compared to bone (Gilbert MTP, Campos *et al.* 2007, Gilbert, Drautz *et al.* 2008) The survival of woolly mammoth hair entombed in permafrost for millennia is testament to the resilience of the biopolymer keratin to withstand harsh environmental conditions and insults. In contrast to the woolly mammoth’s genome and skeletal morphology, hairs comprising the thick woolly coat, for which this species and (woolly rhino) are famously known, have received little detailed morphological examinations. The objective of the current study is to conduct detailed and comprehensive microscopical examination of hairs from these extinct megafauna in order to investigate possible relationships between hair structure and the environment these animals inhabited and study the effects of taphonomy.

4.2.3. Materials and Methods

4.2.3.1 Materials

A total of six woolly mammoth (Jarkov, Yukagir, Dima, Fishhook, M25 and M26) and one woolly rhinoceros (Churapcha) hair samples were examined. The
original geographic locations in which the remains of these megafauna were found and specimen details are presented in Figure 4.2.2, and in more detail in other publications (Gilbert MTP, Campos et al. 2007, Gilbert, Drautz et al. 2008).

**Figure 4.2.2.** Sites of recovery of woolly mammoth hair and woolly rhino hair which were used in this present study, detailing identification details, radio carbon dated ages, sex and age for each hair sample used in the present study. (Image: J. Haile)

Adult African elephant (*Loxodonta africana*) hairs were obtained from the United States Fisheries and Wildlife Forensic Laboratory and Aalborg Zoo, Denmark. Adult Asian elephant (*Elephas maximus*) hairs were obtained from Copenhagen Zoo, Denmark. Somatic origins of *Loxodonta* hairs were flank and lower leg/top of foot area, and head, flank, dorsum and lower leg/foot area of the *Elephas* individual. All extant animal hair samples were obtained in accordance with the relevant legislation for the importation of samples from animal species listed in Appendix I of CITES. Megafauna samples used in this current study may not have contained representatives of all hairs types present on the living animal.
4.2.3.2 Methods

Preliminary examinations of each hair sample were conducted macroscopically (naked eye) and at low magnification (6-40x) using a stereomicroscope. Hair types were assigned in accordance with Brunner and Coman classification (Brunner 1974). Representative hair types from each sample were subsequently selected for detailed examinations and microscopic analyses at higher magnifications using transmitted light microscopy (100-400x magnification), scanning electron microscopy and confocal microscopy. A total of approximately 420-450 hairs were examined in both macro- and microscopic detail.

4.2.3.3 Scale cast pattern and cross-sections

Scale cast patterns and cross-sections were produced in accordance with the methodology of Brunner and Coman (Brunner 1974). Briefly, a cover slip was coated with clear nail polish and the hair was placed on the wet polish; once hardened the hair was removed leaving a scale impression. Cross-sections were obtained by placing hairs in acetate fibres vertically in holes drilled into a stainless steel plate. A razor blade was used to cut the protruding hair and acetate bundle. Accurate shaft diameters were obtained from whole mounts and cross-sections. Scale bars are not included for scale cast images as the entire hair shaft may not be in contact with the medium.

4.2.3.4 Transmitted Light Microscopy (TLM)

Hairs were permanently mounted using Safe-T-Mounting permanent mounting medium (FRIONINE Pty Ltd, refractive index ~1.52); all were mounted between conventional glass microscope slides and cover slips (0.17mm thick). Microscopy was performed on an Olympus compound transmitted light microscope equipped with UPLFL20x Semi apochromatic, UPLANO40x Apochromatic objectives. Images were acquired with an Olympus DP 70 camera and associated software.


4.2.3.5 Confocal Microscopy

Confocal microscope images were collected using a modification of published methodology (Kirkbride 2010). A Nikon A1RMP equipped with a Nikon PlanApo VC 60x oil immersion NA 1.40 objective was used for all imaging. Multiphoton imaging was used employing 800nm laser excitation and detection through 450/50nm, 525/50nm, 595/50nm and 704/32nm bandpass filters. Z stacks were collected through the entire hair thickness typically using z steps of 1µm. Image data sets were processed using Nikon NIS Elements and Nikon NIS Viewer.

Each hair sample was affixed to double sided adhesive tape attached to a 12.6 mm diameter aluminium stub then coated with a 90nm layer of gold in a Balzers Union Ltd. Sputter coater (Liechtenstein) before being examined and photographed in a Philips XL20 Scanning Electron Microscope (the software for image capture is part of the microscope operating software).

4.2.4 Results and Discussion

4.2.4.1 Morphological features of permafrost preserved hair

Like most mammals, woolly mammoth and woolly rhino coats comprised multiple hair types each of which were different in regards to structure, colour and microscopic characteristics. Hairs from each megafauna species were categorised on the basis of their macroscopic appearance into overhairs, guard hairs and underhairs in accordance with Brunner and Coman (Brunner 1974). Macroscopically, overhairs and guard hairs exhibited a variety of colours, ranging from colourless, to dingy yellow, bright red/orange and brown. In contrast, underhairs were either colourless or dingy yellow. Microscopic examination of each hair type revealed unique structures and a variety of post-mortem/taphonomic artifacts.

4.2.4.2 Preservation and Damage

Although hair is remarkably resilient it is not immune to post-mortem degradation processes – the hairs reported upon here were no exception despite
being predominantly frozen since death. Notably, Jarkov, Dima and M26 woolly mammoth hairs exhibited a phenomenon known as *post-mortem* banding (or putrid root) (Figure S4.2.3). *Post-mortem* banding has been studied extensively in human hairs and it solely occurs at the proximal (root) end of hairs that are attached to decomposing bodies; this process is thought to occur from bacterial action and appears to be accelerated in warm and humid conditions and retarded in colder ones (Koch, Michaud *et al.* 2013).

Figure 4.2.3. Examples of ante-mortem and taphonomic (*post-mortem*) artifacts present on extinct megafauna hair shafts. Jarkov (woolly mammoth) underhair bearing normal root (A). Jarkov underhair with the centrally placed, dark *post-mortem* banding in the shaft at the proximal (root) end (B). Perpendicular needle-like fissures caused by keratinophilic fungal invasion of Jarkov (woolly mammoth) overhair (C). Conical fissures caused by keratinophilic fungi invasion of M26 (woolly mammoth) guard hair (D). SEM image showing circular surface degradation and/or points of entry, by keratinophilic fungi, in Jarkov (woolly mammoth) overhair (E). Woolly rhino underhairs with the *ante-mortem* deposition of a hair louse egg case (F). Cuspate, insect bite-marks on woolly rhino guard hair shaft (G). (Images: S.R. Tridico)

The presence of *post-mortem* banding reveals that the bodies of Dima, Jarkov and M26 mammoths underwent some degree of putrefaction before being frozen. To the best of the authors’ knowledge the presence of this *post-mortem* artifact in animal hairs and ancient animal hairs has not been previously published and as such represents a novel and significant finding.
Evidence of insect activity was found on woolly rhino hairs in the form of cuspalate markings (or “bite marks”) (Figure 4.2.3) but whether this artifact occurred as a result of ‘ancient’ taphonomy or ‘modern’ taphonomy (e.g. during storage) is unknown. Evidence of ante-mortem insect activity is also apparent as nit (hair lice) sacs were observed on woolly rhino hair (Figure 4.2.3); lice lay eggs on hair shafts close to the skin, as body heat is required in order for the eggs to hatch.

Hairs buried in soil are susceptible to degradation by keratinophilic fungi that live in soil. They obtain nutrients from digesting keratin containing biological matter such as hooves, horns and hair. Fungal digestion of hairs is well studied and reported in the literature (Blyskal 2009). Evidence of fungal damage was variable in the permafrost preserved hair with widespread fungal growth in some hairs (e.g. M25) and negligible growth in others (e.g. Dima); this may reflect the environment in which the animal carcass was interred i.e. keratinophilic fungi are strictly aerobic and would not survive in an anaerobic environments. Examples of fungal invasion of hairs are illustrated in Figure 4.2.4 and Figure S4.2.1 and Figure S4.2.2.

In woolly mammoth and woolly rhino hairs that did not show evidence of keratinophilic fungal activity the multiple medullae-like structures retained their fine, narrow parallel ‘track-like’ appearance. This contrasted with the situation in hairs that were infected by fungi, where the medullae-like structures were enlarged and dark Figure S4.2.3. It would appear that fungal hyphae find it easier to digest areas such as medullary canals once they have entered the shaft, as illustrated in Figure S4.2.1B; in essence these keratinophilic fungi digest the hair from the inside out, starting with the medullae. An observation also noted by Mary P. English (English 1963) ‘As soon as the fungus reaches the medulla hyphae begin to grow along it. Growth is much more rapid than through the cortex’
The degree of bacterial, fungal and insect activity on a hair sample may be a valuable indication of its ‘purity’ for future genetic and isotopic studies that are complicated by post-mortem contamination by microorganisms.

4.2.3.3 Roots

Although most of the hairs studied were fragments (i.e. root absent), a significant number of hairs bore intact roots. The majority of hairs with roots were underhairs with the remaining roots being present on coarser guard hairs (additional information and images provided in Figure S4.2.4). The large number of hairs indicated that these hairs most likely became detached from the body as a result ‘skin slippage,’ a phenomenon that commonly occurs in the early stages of decomposition, rather than becoming detached from mummified or frozen remains. The number of hairs bearing roots confirms that the detachment of these hairs was the result of skin slippage rather than from mummified skin. Mummified skin is leathery and the removal of intact hairs (i.e. bearing roots) would be almost impossible to achieve without breaking the shaft. The premise that some of the bodies were decomposing is further supported by the presence of post-mortem banding in some of the hairs as illustrated in Figure 4.2.3.B.

4.2.3.4 Surface features and scale patterns

Woolly mammoth and woolly rhino guard hairs exhibited comparable surface scale patterns (Figure S4.2.5 which alternated from irregular wave/mosaic pattern and broad petal (nomenclature according to Brunner and Coman (Brunner 1974)). The overall appearance of the cuticles, which were not prominent, was that the cuticle edges were broadly curved or straight. By analogy with extant mammals that have similar scale patterns, this indicates that individual hairs would not easily interlock, but may freely ‘slide’ over each other, ensuring these hairs remained separate. This may represent an adaptation to discourage matting or tangling of these hairs (see further discussion below).

The scale arrangements in the finer underhairs were broad petal, with rounded,
non-prominent edges. This arrangement, like the overhairs and guard hairs, would have discouraged the hairs from becoming matted, but would have encouraged the hairs to become loosely intertwined, thereby facilitating the formation of insulating thermal air-pockets.

4.2.3.5. Internal structures-Medullae

The medulla, when present in modern mammalian hairs is, almost exclusively single and placed centrally in the hair shaft. Notable exceptions occur in human coarse and stiff beard-, sideburn- and moustache hairs, which may exhibit a double medulla. Our present study revealed two additional mammalian species that exhibit multiple medullae in some of their hairs; *Loxodonta africana* (lower leg/foot hairs) and *Elephas maxima* (dorsal and head hairs) as illustrated in Figure. S4.2.6.

The most significant characteristic of all woolly mammoth and woolly rhino overhairs was the presence of multiple medullae-like structures, which were often present in greater numbers than that seen in samples from extant mammals previously discussed. These structures were manifested as numerous parallel lines that occurred at many radial positions throughout the axis of the shaft (Figure. 4.2.4).
Figure 4.2.4. Examples of multiple medullae-like structures prevalent in extinct megafauna hairs. Transverse cross-section of Jarkov (woolly mammoth) overhair showing dark multiple medullae-like structures throughout the shaft (A). Longitudinal TLM image of cross-sectioned hair (A) showing multiple medullae-like structures (B). Confocal virtual cross-section of woolly rhino overhair (approximately 210μm diameter) showing multiple medullae-like structures throughout the shaft (C). These structures are parallel in the longitudinal view (left image) and as small spots in the virtual transverse cross-section (arrow). Scale bars (A) 100μm, (B) 200μm (Images: S.R. Tridico & P. Rigby)

The greatest number of these structures occurred, without exception, in the coarsest overhairs. In regards to the guard hairs however, an apparent correlation exists between shaft diameter and number of ‘medullae’ present. Only single medullae were found in the finer guard hairs. Multiple medulla-like structures were not seen in the fine underhairs (Figure. S4.2.7). In comparison to woolly mammoth and woolly rhino hairs, and Loxodonta hairs, the majority of Elephas hairs microscopically were opaque due to the heavy concentration of pigment granules within the cortex (Figure. S4.2.6). Therefore, it is possible that dense pigment granules may mask multiple medullae-like structures, if present. In
addition, compared to their hirsute elephantid progenitors, extant elephants possess a very sparse pelage and their hairs are mostly coarse and bristle-like.

Gilbert et al (Gilbert MTP, Campos et al. 2007) and Lister and Bahn (Lister 2007) depict transverse cross-sections of woolly mammoth hair with multiple dark structures in the cortex. Although these structures are reported as nuclear remnants (Gilbert MTP, Campos et al. 2007) or pigmentation (Lister 2007) they are so similar to the structures we observed in the current study (Figure. 4.2.A) that we suspect they are neither pigment, nor nuclear remnants. Our findings demonstrate that longitudinal views of these features show them to be elongated parallel lines running along the length of the shaft (Figure 4.2 B, C) this observation does not support premises of these structures being nuclear remnants or pigmentation. Nuclear remnants are significantly smaller than the structures depicted and pigmentation is granular and scattered throughout the shaft. We hypothesize that these medullae-like structures are a cold adaptation that assists their survival in Arctic conditions. Support for this hypothesis is explored in the following section.

4.2.3.6 Form and function

Through the course of the Pleistocene, megafauna had to adapt and change in order to survive harsh environmental conditions; Campbell et al. (Campbell, Roberts et al. 2010) describe an adaptive physiochemical adaptation of woolly mammoth haemoglobin that aided in its survival in cold conditions. We suggest that multiple medullae-like structures in hairs from two extinct megafauna species may result from convergent evolution of structures that, in combination with the density of their coats, may have been critical for their survival. Like ‘rods’ of reinforcing metal in concrete, multiple medullae may have strengthened the hairs in order to maintain shape and orientation and resist distortion. It was noted that woolly mammoth and woolly rhino overhairs were very strongly resistant to being bent and manipulated, and were noticeably ‘springy’ and very smooth, almost slippery, to the touch. These attributes probably prevented the long overhairs and coarsest guard hairs becoming intertwined and/or matted. Matted hair is likely to be less efficient at channeling moisture/water and snow
away from the body, which would have proved fatal in the depths of an arctic winter. The ‘springiness’ of overhairs, might also be attributed to a different type of keratin in these hairs, which is currently being investigated.

The discovery of sebaceous glands in mummified woolly mammoth remains by Repin et al. was significant as ‘…sebaceous glands are a sign of cold adaptation’ (Repin, Taranov et al. 2004). These glands secrete an oily/waxy substance (sebum), which lubricates the skin and hair surface and acts as natural water repellant. Given the similarity in morphology and texture of woolly rhino and woolly mammoth hairs it is not unreasonable to assume woolly rhino skin also contained sebaceous glands which served the same purpose as those found in the woolly mammoths. The waxy/slippery feel to the overhairs may have arisen by the presence of sebum. This too is currently under investigation.

Mammalian underhair (or underfur) acts as an insulating layer that assists thermoregulation by forming insulating air pockets between the intertwined hairs. Woolly mammoth and woolly rhino underhairs were comparable to modern, extant mammal underhairs.

Woolly mammoth underhairs exhibited uniform shaft diameters (which measured approximately 20-100µm); all were wavy but in addition the numerous hairs were tightly coiled and difficult to separate. Woolly rhino underhairs whilst exhibiting wavy and lightly curled hairs similar to those found on the woolly mammoth, did not exhibit the tightly coiled underhairs and as consequence were easier to separate. Woolly rhino underhairs measured approximately 20-100µm in diameter. The profiles of the thickest underhairs differed to those from woolly mammoth in that they were ‘buckled’ along the length of the shaft (Figure S4.2.8). It is reasonable to assume that like coarse human beard hairs, or pubic hairs, these ‘buckled’ shafts would not have lain flat but may have afforded the animal a ‘puffier’ or bulkier appearance than the woolly mammoth whose hairs were not buckled.

Each of the above proposed structural adaptations to woolly mammoth and woolly rhino pelage may have increased the effectiveness of their woolly coats,
‘Effective pelage can extend a little further the meager calories in winter food…. Woolliness can mean the difference between life and death.’ (Guthrie 1990).

4.2.3.7. Colour and Pigmentation

Mammalian hair colouration is one of the most conspicuous phenotypes; in some animals it plays diverse and significant roles such as sexual attraction, sexual dimorphism and camouflage. However, on the basis of the results of this study, there is no indication that any of these functions applied to woolly mammoth and woolly rhino. Hair colour, length and type appeared to be equally represented in each of the samples, irrespective of the age and sex of the specimen they were taken. Macroscopically and microscopically, woolly mammoth and woolly rhino overhairs, guard hairs and underhairs varied in colour from colourless, to dingy yellow, red/orange and brown (which ranged from pale brown to dark brown, almost black). The majority of overhairs and thicker guard hairs from the woolly mammoths and woolly rhino were vivid red/orange colour or ‘fox red’ as described by Krefft (Krefft 1969). Close examination of woolly mammoth and woolly rhino hairs revealed that their colours could be attributed to either natural pigmentation, or ‘acquired’ colouration (discussed below).

4.2.3.8. Natural Pigmentation

The diversity of mammalian hair colour is attributed to the quality, quantity and ratio of two melanins (pigment types), eumelanin (predominant in dark brown/black hairs) and phaeomelanin (predominant in red and blonde hairs) (Ito and Wakamatsu 2003, Lister 2007). Pigmentation in hairs is usually found as granules in the cortex of the hair shaft; its distribution may be uniformly or medially distributed (around the central axis of the shaft). In hairs from some animals (but not humans) a unique feature is one in which the hair shaft shows natural, abrupt colour changes (commonly known as banding). These hairs may be bi-or tri-coloured along the length of the shaft. If present in sufficient quantities these hairs may give the pelage a mottled or speckled appearance. Microscopic examination of woolly mammoth and woolly rhino hairs revealed visible pigment in many guard hairs and underhairs, but absent in overhairs (Figure. 4.2.5A-C). Where present, pigment distribution was either uniformly
distributed or medially distributed as illustrated in Figure. 4.2.5D-F; however, medial pigmentation was the most prevalent distribution in hairs from both extinct megafauna species, as is also the case in extant elephantids. Like extant elephantids, Yukagir, Jarkov, M25 and M26 woolly mammoths also exhibited bi-coloured hairs (Figure. S4.2.9); bi-coloured mammoth hairs are also noted by Lister and Bahn (Lister 2007). These hairs were coarse and bristle-like, similar to both species of extant elephantids. No bi-coloured hairs were evident in the woolly rhino sample.

Underhairs from woolly mammoth and woolly rhino were comparable exhibiting colourless, pale yellow or pale brown hairs. Pigment granules in coarser underhairs were sparse and uniformly distributed within the shaft. Guard hairs from Yukagir woolly mammoth were notably darker and more heavily pigmented compared with the samples from other woolly mammoths and woolly rhino. This may be due to the pelage of this animal being significantly darker than the hairs of other megafauna studied or the hairs originated from a different somatic origin (body area).
Figure 4.2.5. Examples of natural and ‘acquired’ colouration in overhairs and guard hairs from two extinct megafauna species. Images A-C represent natural colouration of overhairs, which are devoid of pigmentation. Images D-F show the distribution of pigment in guard hairs, which were either uniformly pigmented (D, E) or medial (F). Scale bars: A, C, E, G, H, J, L: 200 µm; B, I: 50 µm; E: 200 µm and K: 100 µm.

The image of the woolly mammoth (to the right in Figure 4.2.5) shows a ‘deconstructed’ view of the distribution of the hair types (shown in the left panel) comprising woolly mammoth (and woolly rhino) pelages. Images G-I show ‘acquired’ colouration present on the inside of hair shafts devoid of pigmentation. The homogeneous red-orange colouration throughout the hair is evident in transverse cross-section (H). Images J-L reveal red/orange colouration due to ‘debris’ on the outer surface of hair shafts. The woolly rhino overhair (J) shows breaches in the surface debris reveal three underlying colourless areas hair shafts (thick arrows) and faint multiple medullae are apparent (fine arrows).
4.2.3.9. ‘Acquired’ Colouration

Current literature attributes red/orange colour of extinct megafauna overhairs and guard hairs to the oxidation of melanin pigment granules as a result of interment over millennia (Lister 2007). It is generally accepted that eumelanin and phaeomelanin pigment granules are susceptible to photo degradation via UV in sunlight (Krefft 1969, Lee 2010). However, although this chemical reaction undoubtedly accounts for some of the red/orange colouration seen in these megafauna hairs, it cannot be the sole cause because hair totally lacking pigment granules also showed this colour that was more vivid than seen in pigmented hairs.

Krefft concluded that multiple processes were acting upon hairs each resulting in colour changes. He acknowledged the effects of photo oxidation of pigments and found that the red/orange (‘fox-red’) colouration not only occurred in pigmented hairs, but also in hairs totally lacking pigmentation; he concluded that this could be attributed to the breakdown of tyrosine residues in keratin. This process resulted in colouration that was homogenously distributed throughout the entire hair shaft (Krefft 1969). We observed a number of homogenously coloured ‘fox red’ hairs from both extinct megafauna species, predominantly in overhairs and coarsest guard hairs as illustrated in Figure. 4.2.5 G-I. On the basis of the work conducted by Krefft it is likely that this colouration may be attributed to the chemical breakdown of keratin.

However, many overhairs bore red/orange debris or a ‘sheath’ encasing the shaft (Figure. 4.2.5 J-L). This may be due to a fungal deposit. The present study supports the premise that the natural coat colour of an individual animal was probably not uniform and certainly not red/orange in colour. Instead, the results of this current study strongly indicate that woolly mammoth and woolly rhino pelages may have exhibited a variety of colours comprising hairs of different colours from different somatic origins and/or hair type. A modern day example of just such a pelage is present on the musk oxen (*Ovibos moschatus*), whose pelage is likened to that of the woolly mammoth, which has white hair on its muzzle, top of head, forelocks and saddle. This is in stark contrast to the
remainder of the body on which the hairs are rich red/brown in colour.

Workman *et al* (Workman, Dalen *et al*. 2011) assert that ‘light coloured woolly mammoths probably were very rare, or even non-existent.’ The current study of woolly mammoth and woolly rhino hairs does not support this premise as we found an abundance of colourless hairs being in all samples and on both species. It does, however, suggest that woolly mammoths and woolly rhino pelages comprised light and dark coloured hairs with lighter hairs predominating, especially amongst underhairs. On the basis of the mixture of pigmented, non-pigmented and bi-coloured hairs found in each sample examined woolly mammoth and woolly rhino coats were likely to have exhibited heterogeneity in colour rather than homogeneity. The arrangement of hair types comprising the pelages would be colourless, long overhairs covering a mixture of pigmented and non-pigmented guard hairs all of which covered predominantly colourless underhairs, for both species of extinct megafauna (Figure 4.2.5). Furthermore, it is possible that woolly mammoths and woolly rhinos may have shown a mottled (‘salt and pepper’) appearance to their coats if bi-coloured hairs occurred en masse; perhaps further genetic studies on hairs, for which the phenotype is self-evident, may further elucidate extinct megafauna pelage colouration.

4.2.5. Conclusion

The results of the present study demonstrate new insights into woolly mammoth and woolly rhino hairs and their preservation in permafrost. In particular, regarding the structure and colour of woolly mammoth and woolly rhino pelages, detailed microscopical examinations enable development of a more accurate picture of pelage appearance, form, function and colour than currently exists. This study challenges the current view that pelages of these two species were uniform in colour; the findings indicate that they were likely to exhibit a variegated colouration with long colourless overhairs covering a mixture of bi-coloured, uniformly coloured brown or red/brown and colourless guard hairs, and innumerable colourless underhairs. The presence of multiple medullae-like features in two extinct megafauna species is suggestive of convergent evolution of traits that, together with their woolly coats, may have helped them to survive
the thermally, and in winter nutritionally, challenging environments of the Pleistocene glaciations. Future morphological examinations of woolly mammoth and woolly rhino hairs taken from known areas of the body would undoubtedly shed further light on the colouration and distribution of hair types on their pelages. The present study demonstrates the importance of familiarity and expertise in the microscopical, morphological examination of hairs to reveal aspects of megafauna hairs that might have remained hidden. We advocate that there is much to be gained from morphological and microscopic examination of hair prior to any destructive sampling for molecular analyses. A multidisciplinary approach to the examination of extinct megafauna remains can only continue to enhance our knowledge of these iconic species.

4.2.6 Acknowledgements

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4.2.7 References


4.2.8 Supplementary Information

Figure S4.2.1. Additional examples of keratinophilic activity on extinct megafauna hair. (A) Large fungal ‘blooms’ on the surface of a woolly rhino guard hair devoid of visible pigmentation but with a feint single medulla in the centre of the shaft. (B) Fishhook (woolly mammoth) overhair exhibiting fine keratinophilic fungi hyphae ‘targeting’ medullae (arrow). (C) Severe keratinophilic fungal destruction of the integrity of M25 (woolly mammoth) guard hair shaft resulting in the exposure of the underlying cortex (arrow). (D) Scale cast pattern of M25 hair, which reveals imprints of a severely damaged shaft, stripped of cuticle and two areas of exposed cortex (arrows). Scale Bars: (A) 200µm, (B) 100µm, (C) 200µm (Images: S.R.Tridico)
**Figure S4.2.2** The extent of the destructive nature of surface feeding keratinophilic fungi is evident in the TLM image of Fishhook overhair with extensive surface keratinophilic fungal damage masking internal features (left). The extent of the surface damage is evident in the irregular outline of the transverse cross-section (in which dark multiple medullae-like structures are visible) shown top right (**Top panel**). In contrast, a woolly rhino overhair (**bottom panel**), which has not suffered from keratinophilic fungal attack. The TLM image shows faint multiple medullae-like structures (blue arrows) and a cross-section that bears smooth outline and discrete black multiple medullae-like structures throughout the cortex. Hairs from both species were devoid of pigmentation. All scale bars 100µm (except lower left, bar 200µm)  (Images: S.R. Tridico)
Figure S4.2.3. The effect of keratinophilic invasion on medullae. (Top left) Woolly rhino overhair unaffected by keratinophilic fungal activity exhibits medullae-like structures as feint parallel lines in the cortex (black arrows) bar=100 µm. The corresponding transverse cross section presented at top right in which central medullae-like structures appear diffuse (bar 200 µm). (Bottom left) Jarkov woolly mammoth overhair in which medullae-like structures are black and enlarged as a result of keratinophilic fungi invasion (bar 200 µm) (Bottom right) The corresponding transverse cross-section in which the black multiple medullae-like structures are markedly darker and more obvious in comparison to the top right image (bar 100µm).(Images: S.R. Tridico)
Figure S4.2.4. Examples of roots from woolly mammoth underhairs (top panel) guard hairs. Underhairs exhibited elongated roots, whilst guard hair roots were shorter and wider. (Images: S.R. Tridico)

Figure S4.2.5. Examples of scale patterns commonly found on woolly mammoth and woolly rhino guard hairs. (Top) Irregular wave-like pattern at the proximal to mid-shaft region of the hair. (Centre) Irregular mosaic-like pattern at mid-shaft region of the hair. (Bottom) Irregular wave-like pattern at distal shaft of the hair. (Note: smooth ‘cylindrical’ features are excess casting material). (Images: S.R. Tridico).
Figure S4.2.6. Multiple medullae-like structures evident in Loxodonta foot and lower leg hairs (A) and guard hair of Fishhook woolly mammoth hair (B). Example of the opaque nature of many *Elephas* hairs due to heavy pigmentation (bar = 200µm) (C). Single central medulla visible in the lighter part of an *Elephas* bi-coloured (banded) head hair (D) (bar = 100µm). Possible multiple medullae-like structures in an *Elephas* bi-coloured (banded) dorsal hair (E) (as indicated by arrows). This image over-exposed in order to visualise these structures) (bar = 100µm). (Images: S.R. Tridico)
**Figure S4.2.7.** Fishhook woolly mammoth overhair showing numerous medullae -like structures, in cross-section in the left panel (scale bar 100µm) and longitudinal TLM image of the hair (scale bar 200µm) (A). Jarkov woolly mammoth guard hair few medullae-like structures in cross-section in the left panel (bar 100µm) and longitudinal TLM image of the hair (scale bar 200µm) (B). (Virtual cross-section of Dima finer guard hair showing a single medulla (left panel) and the longitudinal image of the hair showing a single, central medulla (arrows) (bar 100µm) C). Transverse, physical cross-sections of woolly rhino (D) and Yukagir (E) fine underhairs, which, like the majority of mammalian underhairs, are circular and devoid of medullae (D and E). (Images: S.R. Tridico)
Figure 4.2.8. Images that compare underhair profiles, in woolly mammoth (left panel) and woolly Rhino (right panel). Both megafauna species exhibited comparable underhairs in size and appearance with the exception of the majority of coarsest woolly rhino underhairs consistently showing uneven shaft diameters caused by ‘buckling’ as illustrated in the four images on the right panel. (Lowest image on RHS panel: scale bar 50µm, Top three images scale bars 100µm) (Images: S.R. Tridico).
Figure S4.2.9. Examples of bi-coloured (banded) guard hairs. TLM image showing a darker pigmented proximal half (root end) of a Yukagir woolly mammoth hair shaft. The image on the right is the lighter portion of the mid-distal hair shaft (A). M25 bi-coloured hair showing heavy pigmentation in the mid-shaft area and the right image showing medial pigmentation of the distal part of the shaft (B). (Scale bars 200µm) (Images: S.R. Tridico)

4.2.9 Chapter Summary
This current body of work may be regarded as a significant milestone in advancing our knowledge regarding the natural history of two iconic extinct megafauna. For the first time, specific and unique adaptations to their hairs were identified. These adaptations, together with the remainder of hairs comprising their voluminous coats, may have conferred sufficient thermal advantage to enable woolly mammoth and woolly rhino to survive extended periods of exposure to sub zero temperatures. Future morphological studies may benefit from hairs recovered from additional woolly rhino remains in order to shed further light on their ‘true’ coat colour. This chapter also revisits the effects of pre- and post mortem influences on hairs detailed in Chapter 3 and echoes the sentiments in Chapter 2 regarding the judicious selection of microscopes to best reveal the feature in question. One of the most surprising morphological finds was the first recorded presence (as far as we know) of discharged nit sacs evident on a few woolly rhino hair shafts.

This chapter is the first to not only introduce the concept of a multi-disciplinary approach but also demonstrate the scientific value of such an approach in relation
et al (Gilbert MTP, Campos et al. 2007) (Gilbert, Drautz et al. 2008). Taken together, the morphological data presented in Chapter Four and the molecular data presented by Gilbert et al, provide a much more comprehensive and ‘richer’ picture of extinct megafauna natural history than either study alone and, in doing so exemplifies the tenet ‘the whole is greater than the sum of its parts’ (Aristotle).

Hair has become somewhat of a preferred substrate when constructing aDNA genomes – the mammoth (Gilbert MTP, Campos et al. 2007) and two human genomes (Rasmussen, Bertalan et al. 2010) (Rasmussen, Lohmueller et al. 2011) (see also Appendices A and B) used hair as the biological substrate. The focus on hair for aDNA genomes is due primarily to the observation that hairs appear to have a lower proportion of contaminating microorganisms relative to bone (Gilbert MT, Shapiro et al. 2004, Gilbert MTP, Campos et al. 2007). Despite this higher proportion of endogenous DNA it is clear that there is still a significant number of microorganisms present on, or within, hair both modern and ancient. Moving away from extinct species and back into the realms of forensic science, chapter 5 explores the probative value of microbial DNA profiles recovered from hair.
Chapter Five

Bugs in the system: exploring the potential of bacterial metagenomic analyses of hairs in forensic applications

‘A male’s reproductive system is arid
But with microbes a female’s abounds
With lactobacilli and with other genera
Their variety astounds’

Michael Wilson, Bacteriology of Humans

5.1 Preface.

This chapter builds upon the concept introduced in chapter four in relation to microorganisms colonising ancient hair shafts, by investigating the potential of bacteria present on modern hairs for use in forensic investigations. This work resulted in a published manuscript titled ‘Metagenomic analyses of bacteria on human hairs: a qualitative assessment for applications in forensic science’ (Investigative Genetics 2014, 5, (1), 1-13). The content of this chapter is the same as in the published article found in Appendix A.4 apart from minor changes made to incorporate in-thesis referencing.

Metagenomics, or the culture-independent study of bacterial genomes, has been widely applied to identify bacteria inhabiting the human body in health and disease (Cho and Blaser 2012) or to identify bacteria from a variety of environmental niches (Carew, Pettigrove et al. 2013, Daniel 2005, Venter JC 2004). This field has benefited greatly from the advent of NGS (next-generation sequencing, also known as massive parallel sequencing or high throughput sequencing) which allows all bacterial genomes, from any given environment, to be simultaneously interrogated at unprecedented depths and levels.

However, the current contribution of metagenomics to forensic investigations is still in its infancy; some have explored the applicability of microbial evidence in a number of disparate areas such as of bioterrorism (Budowle and Williamson 2009), discriminating between individuals on the basis of their skin bacteria...
(Fierer, Lauber et al. 2010) or to provide associative evidence between illicit drug seizures or crime scene and offender based on soil microbes (Waters, Eariss et al. 2012). However, to date, there is no published data in relation to the evaluation of metagenomic analyses of one of the most frequently encountered forensic evidence types; human hairs. This ‘gap’ in knowledge was the impetus for the research presented in this chapter.

Hairs are one of the most ubiquitous traces recovered in forensic investigations, however in the absence of violent removal, these hairs commonly possess telogen roots (hairs that have ceased to grow). Hairs bearing telogen roots typically contain sub-optimal levels of nuDNA; these roots either fail to produce a DNA profile or produce an incomplete DNA profile (Szabo, Jaeger et al. 2011). This results in a concomitant reduction in the statistical value of the profile. Furthermore, these substrates also bring low-template DNA considerations, which is an area with its own pitfalls and controversies (Buckleton 2009). In these instances mitochondrial DNA (mtDNA) may be extracted and profiled; however due to its uni-parental (matrilineal) inheritance, statistical analyses are of limited probative value in comparison with nuDNA profiling (Melton T 2012). The impetus to conduct metagenomic analyses of hairs was to investigate whether populations of bacteria colonising human hairs may provide an adjunct to current molecular analyses.

The aims of this current chapter were to investigate whether:

1) microbial colonisation of human scalp and pubic hairs enables differentiation between individuals,
2) scalp and pubic hairs can be differentiated on the basis of microbial composition,
3) bacterial 16S profiles on hair shafts are stable or variable over time,
4) hair metagenomics has potential for further development as a component of the forensic toolkit.
5.1.1 Statement of Contribution

As the lead author of the following manuscript, I was responsible for the concept of this inaugural study. I extracted DNA from all hair samples and conducted all qPCR in order to ascertain the amount of total DNA extracted for each hair sample. I conducted the fusion-tagged amplicon generation and assisted with 454 sequencing, I assisted D. Murray (second author) in post-sequencing deconvolution of samples and in the bioinformatic analyses of the data. Human ethics approval was sought and granted by Murdoch University Research Ethics and Integrity Office prior to commencing this study (Project number 2011/159). I had comments, editing and input from the listed co-authors.

Silvana R. TRIDICO
5.2 Metagenomic Analyses of Bacteria on Human Hairs: A qualitative assessment for applications in forensic science.

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5.2.1 Summary

Background: Mammalian hairs are one of the most ubiquitous types of trace evidence collected in the course of forensic investigations. However, hairs that are naturally shed or that lack roots are problematic substrates for DNA profiling; these hair types often contain insufficient nuclear DNA to yield STR profiles. Whilst there have been a number of initial investigations evaluating the value of metagenomics analyses for forensic applications (e.g. examination of computer keyboards) there have been no metagenomic evaluations of human hairs – a substrate commonly encountered during forensic practice. This present study attempts to address this forensic capability gap, by conducting a qualitative assessment into the applicability of metagenomic analyses of human scalp and pubic hair.

Results: Forty-two DNA extracts obtained from human scalp and pubic hairs generated a total of 79,766 reads, yielding 39,814 reads post control and abundance filtering. The results revealed the presence of unique combinations of microbial taxa that can enable discrimination between individuals, and signature taxa indigenous to female pubic hairs. Microbial data from a single co-habiting couple added an extra dimension to the study by suggesting that metagenomic analyses might be of evidentiary value in sexual assault cases when other associative evidence is not present.
Conclusions: Of all the data generated in this study, the NGS data generated from pubic hair held the most potential for forensic applications. Metagenomic analyses of human hairs may provide independent data to augment other forensic results and possibly provide association between victims of sexual assault and offender when other associative evidence is absent. Based on results garnered in the present study, we believe that with further development bacterial profiling of hair will become a valuable addition to the forensic toolkit.

5.2.2 Background

Over the last decade the development of bacterial culture-independent approaches, (metagenomics), based on 16S rRNA genes (hereafter referred to as 16S) sequences has become the cornerstone of microbial ecology (Parkhill 2013). The advent of NGS technologies and platforms capable of generating millions of sequences per sample facilitated assessments of microbial communities between body sites and individuals (Costello, Lauber et al. 2009, Peterson, Garges et al. 2009). The increased sequencing power stimulated the development of robust computational programs capable of processing large, complex sequencing data sets (Kuczynski 2010) and enabled phylogenetic analyses of human and environmental genomes (Venter JC 2004, Peterson, Garges et al. 2009).

Studies on the human microbiome (the collective genomes present in the human body) suggest that there are significant differences in bacterial composition not only between different body sites but also between individuals (Costello, Lauber et al. 2009, Grice, Kong et al. 2009, Peterson, Garges et al. 2009). The potential that individuals may harbor unique bacterial species is of significance to forensic investigations.

For centuries, associative hair evidence relied solely on comparative microscopy based on qualitative features such as pigmentation (Deedrick 2004, Scientific Working Group for Materials Analysis 2005, Houck 2005). The advent of PCR in the mid-1980s initiated a paradigm shift in the forensic examination of hairs. For the first time, DNA profiles could complement qualitative microscopical
observations (Houck 2002). However, the success of the highly discriminatory STR profiling is dependent on hairs bearing anagen roots (actively growing hairs) that are rich in nuclear DNA, (nuDNA) and to a lesser extent, hairs that are in the quiescent (catagen) growth phase (Linch, Smith et al. 1998). However, the majority of hairs recovered in forensic investigations are shed hairs (i.e. those in their telogen phase); these hairs have ceased to grow and contain little or no nuDNA (Opel, Fleishaker et al. 2008). STR profiling of these hair roots typically yields trace amounts of, often degraded, human DNA and can require the use of low-template DNA strategies and the complications that accompany such approaches (Butler 2011). In these instances mitochondrial DNA (mtDNA) analysis is routinely conducted. However, due to its common matrilineal inheritance and haploid nature, mtDNA typing yields modest exclusionary capability, which lacks the statistical power afforded by STR profiling (Nilsson 2012). However, low yields of human nuDNA from forensic hair samples does not equate to the absence of other sources of DNA that could assist in the individualisation of hair. Indeed, metagenomic analyses of hairs unsuitable for nuDNA profiling may provide a microbial fingerprint to augment other forensic results such as mtDNA analyses. This would not involve extra or additional extraction procedures, as DNA isolation procedures for human DNA will also ‘collect’ microbial DNA.

Conventional forensic hair examination, using either morphological or molecular techniques, is contingent upon the deposition and recovery of hairs; however, despite Locard’s adage that ‘every contact leaves a trace’ (Hanson, Haas et al. 2012), this may not always be the case. Research in relation to the transfer of pubic hairs in forensic investigations involving sexual assault cases, discovered limited transfer (4%) of male pubic hair to female genital area during sexual intercourse (SI) (Mann 1990). In addition, the present study demonstrated that no female pubic hair transfer to male genital area took place.

The utility of metagenomic analyses for forensic applications has been explored since the inception of next-generation sequencing (NGS); for example Fierer et al. (Fierer, Lauber et al. 2010) conducted preliminary work to explore the potential to link individuals to computer keyboards and mice on the basis of
transferred of skin bacteria. However, one of the most ubiquitous of evidence types- human hair - has yet to be evaluated in the context of forensic metagenomics. To the best of our knowledge this present study is the first to qualitatively assess the viability of metagenomic analyses of hairs in a forensic context. The three aims of the research reported here were to assess:

1) whether human scalp and pubic hairs can be differentiated on the basis of their 16S microbial composition,
2) whether individuals can be differentiated on the basis of microbial taxa colonising scalp and pubic hairs and
3) whether bacterial 16S profiles on hair shafts are stable over time

Overall, the objective of this initial study was to establish whether further development of the technique is warranted.

5.2.3 Methods

5.2.3.1 Sample Collection

Bacterial communities, associated with human scalp and pubic hair, were surveyed using a multiplex barcoded sequencing approach from seven healthy Caucasian individuals of both sexes (two of whom were in a de facto relationship), ranging in age from 23 and 53 years old. The health status of each volunteer was self-reported with each individual stating that antibiotics were not taken at least 8 months prior to the collection of hairs used in the study. Each individual self-collected a number of hairs cut from the scalp and pubic areas, at three time points; initial collection in addition to 2 and 5 months thereafter, referred to as T0, T2 and T5 respectively. Replication is important in NGS amplicon sequencing workflows - due to the investigative nature of this study and limited availability of resources, we selected to investigate multiple time points (temporal replicates) in lieu of multiple extractions at each time point (sampling replicates).

Each volunteer was provided with a hair collection kit consisting of labeled clip-seal plastic bags, sterilized scissors, ethanol wipe, latex disposable gloves and disposable forceps. Hairs were cut close to the skin and from approximately the same area at each time point; volunteers were asked to clean the scissors with
ethanol wipes between sampling to avoid contamination. The rationale of severing the hairs close to the skin, rather than plucking the hairs was to ensure the bacterial taxa identified were more likely to originate from the hair shaft rather than the skin. Hairs taken from the head were labeled as female scalp hair (FSH) or male scalp hair (MSH), similarly, female and male pubic hairs were marked FPH or MPH. Once sampled, these hairs were placed in separate labeled clip-seal plastic bags and stored at room temperature after being catalogued. Hairs were sampled and processed within 24 hours of collection and unused hair samples were returned to their original packaging and stored at room temperature; these hairs were not further sampled or examined. The effect of storage and storage conditions on bacteria was not in the scope of this present study; however Lauber et al. (2010) investigated the effects of storage conditions on bacteria and concluded that bacterial community composition is unaffected in the short-term.

Each volunteer was made aware of the nature of the study and gave written, informed consent. Information regarding the sexual habits or orientations of the volunteers was not sought. The project was approved by, and conducted in accordance with, Murdoch University Human Research Ethics Committee Policies and Guidelines (Project Number 2011/139).

5.2.3.2 DNA Extraction and Quantification
Three hairs from each body area were cut into approximately 1cm lengths and placed into 1.5ml Eppendorf tubes. The contents of each tube were digested overnight using 1ml of hair digest buffer containing; 10mM Tris pH 8 (Sigma, MO, USA), 10mM NaCl (Sigma, MO, USA), 5mM CaCl2, (Sigma, MO, USA), 2.5mM EDTA pH 8 (Invitrogen, CA, USA), 1mg/ml ProK (Amresco, OH, USA), 40mM DTT (Thermo Fisher Scientific, MA, USA) 2% SDS (Invitrogen, CA, USA) and milliQ water (Sigma, MO, USA) to make the remaining volume. The samples, including extraction and laboratory environmental controls, were secured on rotating arms (to ensure total immersion) and digested overnight in a 55°C oven.
All samples were then centrifuged for 2 minutes at 13000rpm. To concentrate the DNA a total of 600 µl of supernatant was transferred to Vivaspin ultrafiltration spin columns with a 30,000 MW cut-off (Sartorius Stedim Biotech, Germany) and centrifuged at 30,000rpm to leave 50-100 µl of supernatant. Concentrated supernatant was subsequently combined with five volumes of PB buffer (Qiagen, CA, USA) and transferred to a Qiagen silica spin column and centrifuged at 13,000rpm for 1 minute. Two wash steps followed (Qiagen AW1 buffer and AWII buffer) prior to elution of DNA from the spin column with 60 µl of 10mM Tris-Cl pH 8 buffer. The DNA extracts were subsequently quantified via real-time quantitative polymerase chain reaction (qPCR; Applied Biosystems StepOne) using SYBR green and the bacterial 16S F515/Bact 16S (V4 loop)_R806 primers, (Table 5.2.1).

**Table 5.2.1. Details of primers used in in the amplification of 16S RNA region of bacterial mitochondrial genome**

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primer Sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward bacterial primer (Bact_16S_F515)</td>
<td>GTGCCAGCMGCCGCGGTAA</td>
<td>(Turner 1999)</td>
</tr>
<tr>
<td>Reverse bacterial primer (Bact_16S_R806)</td>
<td>GGACTACHVGGGTWTCTAAT</td>
<td>(Caporaso, Lauber et al. 2011)</td>
</tr>
</tbody>
</table>

Extracts were analysed using qPCR for neat extracts in addition to 1/10 and 1/100 dilutions, in order to determine if extractions were successful and to identify samples with low-template DNA (defined as those with CT values >32). The possible presence of PCR inhibitors was also determined by qPCR. The 16S qPCR assay was conducted in 25µl reactions using a 2X ABI Power SYBR master mix (Applied Biosystems) together with 2µl of extracted DNA with primer concentration at 0.4µM (IDT) cycled for 95 °C for 5 minutes followed by 50 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 45 seconds, with 1 °C melt step and a 10 minute final extension at 72 °C. The optimal DNA concentration free of inhibition was used for all subsequent analyses. Each hair sample bacterial extract had CT values less than 32 PCR cycles indicating the presence of sufficient 16S template copy number for robust NGS amplicon sequencing.
5.2.3.3 Fusion-tagged 16S V4 Amplicon Generation

Bacterial F515 and R806 (Table 5.2.1) 16S primers (targeting the V4 region) used in the initial qPCR extract screen, giving a size variable product minus primers of ~250 base pairs, were modified into fusion primers for the generation of amplicon products for subsequent sequencing. Each fusion primer consisted of a GS FLX Titanium (Lib-A) adapter A or B on the 5’ end followed by a unique 6 bp MID (Multiplex Identifier) tag and the template specific forward or reverse primer at the 3’ end of the primer (Roche 2009) giving a final size variable product of ~350 bp including primers and additions. A single-step, uniquely tagged fusion PCR approach was employed to minimise the contamination associated with the multiple PCR steps used in NGS workflows.

For each time point, each extract was assigned a unique 6bp MID-tagged fusion primer in preparation for amplicon sequencing. MID-tagged amplicons were generated in triplicate (i.e. PCR replicates) in 25 µL reactions containing: 1X PCR Gold Buffer (Applied Biosystems), 2.5mM MgCl₂ (Applied Biosystems (ABI), CA, USA), 0.1 mg/ml BSA (Fisher Biotech, WA, Aus), 0.25 mM of each dNTP (Astral Scientific, NSW, Aus) 0.4 µM of forward and reverse primer (IDT), 0.2 µL1 unit of Taq DNA polymerase (AmpliTaq Gold™, Applied Biosystems (ABI), CA, USA), 1:80,000 (final concentration) of SYBR Green ‘gel-stain’ (Life Technologies, S7563,) and DNA extract. The same processes were performed on PCR negative/reagent controls for each PCR plate run, pre- and post addition of DNA extracts.

All PCR amplicons were purified using the Agencourt AMPure XP™ Bead PCR Purification protocol (Beckman Coulter Genomics, MA, USA). Solely for the purpose of sequencing coverage, purified amplicons were electrophoresed on 2% agarose gel to obtain roughly equimolar ratios of each sample. Where extraction/environmental controls or PCR negative/reagent controls showed positive qPCR results these were also pooled, purified and sequenced. The final pooled library was quantified using qPCR to determine the appropriate volume of library to use for emulsion PCR (emPCR) prior to amplicon sequencing on the GS Junior™ as described in Murray et al. (Murray, Bunce et al. 2011), using reaction conditions in Murray et al. (Murray, Haile et al. 2013)). All emPCR,
bead recovery and amplicon sequencing procedures were carried out according to Roche GS Junior™ protocols for amplicon sequencing (Lib A).

5.2.3.4 Bioinformatic Analysis
Amplicon sequence reads obtained from the GS-Junior™ (hereafter referred to as sequences) were sorted into batches based on MID tags assigned to each extract allowing for no mismatch in MID tag DNA sequences. Additionally, template specific 16S bacterial primer sequences were annotated and trimmed from all sequences allowing for no mismatch in base composition or primer sequence length. Sequences that failed to meet these criteria were discarded. The aforementioned steps were conducted using Geneious™ v7.0.6 (Kearse, Moir et al. 2012).

Once batched and trimmed, sequence fasta files were imported into QIIME V1.8.0 (Caporaso, Kuczynski et al. 2010) and merged into a single fasta file. Chimeric sequences were identified and removed on a per individual sample basis using the usearch61 (Edgar 2010) de novo method passing --split_by_sampleid. Following this, OTUs (Operational Taxonomic Units) were identified using an open reference OTU picking method using usearch61 with a 97% clustering identity, using the most abundant sequence within each OTU as the representative sequence and the Greengenes 13.8 database release (McDonald, Price et al. 2012). Representative sequences for each OTU were aligned using PyNAST (Caporaso, Bittinger et al. 2010) against the Greengenes 13.8 pre-aligned database, the alignment filtered and phylogeny built using FastTree (Price, Dehal et al. 2010) in QIIME. Additionally, any OTUs found within the control samples of specific time points (i.e. T0, T2, T5) were removed from samples contained within the respective time point. Following the removal of control OTUs, each individual sample was filtered to remove low abundant OTU clusters. In each case all singleton OTUs were discarded and any OTU whose abundance was below 0.2%, an estimated error rate associated with 454 sequencing (Loman, Misra et al. 2012), of the total number of filtered sequences in that sample were removed. OTUs remaining post-filtering were taxonomically identified using the BLASTn option within QIIME’s assign taxonomy script against the Greengenes 13.8 database. Moreover, OTUs were taxonomically
assigned using RDP (Wang, Garrity et al. 2007) and UCLUST (Edgar 2010) options, again against the Greengenes 13.8 database (McDonald, Price et al. 2012). To determine at a gross level if there was clustering of samples according to sex and/or somatic origin a principal co-ordinate analysis (PCoA) plot was constructed using filtered sequences negating whether or not sequences were part of the core microbiome.

Following taxonomic identification and PCoA construction the core microbiome for each sex/somatic origin (SSO) grouping (i.e. FSH, FPH, MSH and MPH) was determined using QIIME. The ‘core’ microbiome was defined in accordance with that established by Shade et al. (Shade, Hogan et al. 2012); all OTUs that occur in two or more (i.e. the majority) of the recorded time points for each of the SSO groupings. Any OTU’s occurring in only one of three time points is classed as ‘transient’ (Tr). In addition to this, the number of OTUs that were unique to an individual was determined; these were defined as all OTUs occurring solely in that individual across at least two time points irrespective of whether it was found to be a core OTU in the above SSO groupings. Upon identification of personalised OTUs it was determined whether or not said OTUs occurred in pubic hair, scalp hair or in both. Finally, the number of OTUs shared solely by two individuals was identified to examine whether the number of OTUs between the cohabiting couple was greater in comparison to other non co-habiting participants.

5.2.4 Results and Discussion

Forty-two pools of DNA extracts obtained from human scalp and pubic hairs were used to interrogate their microbial composition by next-generation sequencing. A total of 79,766 reads were generated, yielding 39,814 reads post control and abundance filtering. On average the coverage per sample was 1899 reads pre-filtered and 948 post-filtered. While this depth of coverage is less than ideal given the advancement in NGS technology (i.e. Illumina and Ion torrent platforms) these 454 data are still sufficient to explore the potential of hair microbial forensics for future development. Like all novel forensic techniques, metagenomic analyses of hairs will ultimately require robust evaluation and
validation to ensure these analyses are fit for purpose and able to withstand scientific scrutiny. Part of this validation should take into consideration; replication (spatial, temporal and PCR replicates); persistence of hair bacteria not only once they are transferred or deposited (during contact and stability during storage), and prevention of contamination during processing hairs in the laboratory. Budowle et al. (Budowle, Connell et al. 2014) outline and discuss in detail the future validation criteria for metagenomic analyses in relation to microbial forensic applications, which they believe will require international participation. However, such an undertaking is beyond the scope of this initial evaluation into just one of many applications of forensic metagenomic investigations.

There are many ways to present metagenomic data such as generated here; the sections below explore the data using Principal Coordinates Plots (PCoA), Taxonomy and OTU’s focusing on the value of the data in forensic applications. OTUs taxonomically assigned using RDP or UCLUST options revealed little to no difference in assignment to the rank of family. For this reason, all assignments refer to BLASTn taxonomic assignments.

5.2.4.1 Principal Coordinates Plot (PCoA)

Of all the data generated in this study the NGS data generated from pubic hair held the most potential for forensic applications. A general dichotomy was observed between taxa (OTUs) harbored on male and female pubic hair shafts (Figure 5.2.1).

In general, males clustered close to the PC2 axis along the PC1 axis while females were more evenly spread along the PC1 axis and further from the PC2 axis than the males. Data relating to two individuals, who were a cohabiting couple, presented some interesting results. The red dots in the yellow ellipse at high PC1 represents the taxa present on the female partner of the couple at T0 and T2; while the two orange dots enclosed by small blue circles at low PC1 represent the taxa from the male partner at T0 and T2. The lilac circle encloses one red dot (taxa from the female at T5) and one orange dot (taxa from the male at T5). Microbial taxa extracted from the male and female at this time point were more similar to each other than to their other previous time points (T0, T2).
Discreet enquiries revealed, unlike the preceding time points, the couple in question had engaged in sexual intercourse prior to the collection of T5 hair samples. It is noteworthy that intercourse had taken place 18hr prior to the collection of pubic hairs and both individuals had showered in the interim period.

Figure 5.2.1. Principal Coordinate Plots (PCoA). Clustering of microbial taxa from each individual at each collection time point. The lilac circle represents post SI bacterial sequences, whilst the pale blue and yellow circles represent non-SI bacterial sequences - both circles relate solely to the co-habiting couple. Panel A represents pubic hair microbial taxa from male (orange) and female (red) participants. Panel B represents scalp hair microbial taxa from male (green) and female (blue) participants. Panel C represents microbial taxa present in male and female scalp and pubic hair samples.

Cross-transference of bacteria during intercourse may account for the variation in taxa observed. Cross transference, or shedding of skin micro flora, is not uncommon for individuals sharing living or communal spaces (Hospodsky, Qian et al. 2012) or during contact sports in which Meadow et al. (Meadow, Bateman et al. 2013) observe ‘Our results are consistent with the hypothesis that the human skin microbiome shifts in composition during activities involving human to human contact’. The results we present here suggest that the pubic hair microbiome might be quite stable, even during cohabitation, but it might be shifted dramatically during sexual intercourse for some time. This present study is the first to suggest cross-transference of pubic/genital microbial taxa as a result.
of intercourse. Although further analyses need to be conducted, this initial finding bodes well for future forensic applications involving sexual crimes.

An additional advantage is that compared to other body areas such as the skin, gastro-intestinal tract (GIT) and mouth, fewer bacterial species seem to comprise the vaginal microbiome (Fettweis, Serrano et al. 2012). The advantage of simpler communities and fewer taxa in the vaginal microbiome is one that may facilitate forensic investigations by providing results in a timely manner.

The clear microbial distinctions between pubic hairs from the sexes may largely be attributable to the prevalence of Lactobacillus spp in the female pubic hair samples and the absence of these bacteria in the male samples (excepting the cohabiting male at T5) (Figure 5.2.1). Additionally, male pubic hair microbial taxa were clustered along axis PC2 suggesting that these taxa (OTUs) were common to the male microbiota. The elongation of data along PC1 may be attributable to females harboring different lactobacilli species (Tables 5.2.2 and 5.2.3). However, the concomitant elongation of data along axis PC2 suggests the presence of secondary differences; differences that may be due to the presence of personalized taxa (Table 5.2.3).

The PCoA plot of male and female scalp hair microbiota over the 5 month time period did not demonstrate any significant clustering (Figure 5.2.1). This is most likely attributable to male and female scalp hairs harboring similar bacterial taxa. However, some of the female taxa are slightly spread out along axis PC1 suggesting that there may be some variation in microbial taxa in the hairs of these individuals. The distribution and composition of the microbial communities colonising scalp and pubic hair is discussed in further detail below.
Table 5.2.2. Shared taxa from pairwise comparisons of all data located in scalp (Sc) and/or Pubic (Pu) hairs.
The cohabiting couple (bold) share more taxa, including multiple lactobacilli species, than other individuals. (IND: Individual, F: Female, M: Male)

<table>
<thead>
<tr>
<th>IND.</th>
<th>1 (F)</th>
<th>2 (M)</th>
<th>3 ( )</th>
<th>4 (F)</th>
<th>5 (F)</th>
<th>6 (F)</th>
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<td></td>
<td></td>
<td>Dialister spp (Pu)</td>
<td>L. iners (Sc/Pu)</td>
<td>Prevotella spp (Pu); Peptinophilus spp (Sc/Pu)</td>
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</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td>Beta Proteobacteria (Sc/Pu); Neisseriaceae (Sc); Corynebacteriaceae (Pu); Lactobacillus spp. X4</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>Bifidobacterium (Pu/Sc); Anaerococcus (Pu)</td>
<td>0</td>
<td>Actinomycetales (Pu); Neisseriaceae (Sc)</td>
<td>Dietziac (Sc.) Knoellia subterrana (Sc/Pu)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactobacillus spp. X2 (Pu); Rhodobacteriaceae (Sc)</td>
<td>Limnohabitans spp (Sc)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Corynebacterium spp. (Sc/Pu); Aggregibacter (Pu)</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5.2.3 Personalised (unique) bacterial taxa colonising male and female scalp and pubic hair and their natural habitats. Data shows sex and somatic origin of hairs that harboured personalised bacterial taxa, as well as the natural habitats of the taxa.

<table>
<thead>
<tr>
<th>Somatic origin</th>
<th>Bacterial Taxa</th>
<th>Natural Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scalp/Pubic (F.)</td>
<td><em>Lactobacillus</em> spp. x1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Most prevalent genera in human vagina; also present in GIT (Wilson 2005)</td>
</tr>
<tr>
<td>Scalp (F. Ind.1)</td>
<td><em>Neisseriaceae</em></td>
<td>Normal human flora of oro-nasopharynx (Wilson 2005)</td>
</tr>
<tr>
<td>Scalp (M. Ind.2)</td>
<td><em>Nocardioidaceae</em></td>
<td>Soil and aquatic habitats (Sensabaugh 2009)</td>
</tr>
<tr>
<td>Scalp (M. Ind.2)</td>
<td><em>Streptococcus sobrinus</em></td>
<td>Implicated in dental caries (Wilson 2005)</td>
</tr>
<tr>
<td>Pubic (M. Ind.3)</td>
<td><em>Corynebacterium</em> x2 spp&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>Major inhabitants of skin flora (Wilson 2005)</td>
</tr>
<tr>
<td>Pubic (M. Ind.3)</td>
<td><em>Tissierellaceae</em> fam.nov. (Formerly Peptostreptococcaceae)</td>
<td>Intestine, vagina, oral cavity; Some general environmental</td>
</tr>
<tr>
<td>Pubic (M. Ind.3)</td>
<td><em>Anaerococcus</em> spp&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Human nasal cavity, skin and vagina (Ezaki, Kawamura &lt;i&gt;et al&lt;/i&gt;. 2001)</td>
</tr>
<tr>
<td>Location (F. Ind.)</td>
<td>Family</td>
<td>Species/Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------</td>
<td>-------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Pubic (F. Ind.4)</td>
<td><em>Campylobacteraceae</em></td>
<td>Human oral cavity, intestinal tracts and environmental (Gillespie 2006)</td>
</tr>
<tr>
<td>Scalp (F. Ind.5)</td>
<td><em>Rhodocyclaceae</em></td>
<td>Activated sludge and waste water (Loy, Schulz et al. 2005)</td>
</tr>
<tr>
<td>Scalp (F. Ind.5)</td>
<td><em>Micrococcales</em></td>
<td>Widely distributed in environment and commensal on human and oral cavity (Wilson)</td>
</tr>
<tr>
<td>Pubic (F. Ind.5)</td>
<td><em>Lactobacillus spp.</em></td>
<td><em>Lactobacillus</em> most prevalent and abundant in human vagina; also present in GIT (Wilson 2005)</td>
</tr>
<tr>
<td>Pubic (F. Ind.6)</td>
<td><em>Gardnerella spp.</em></td>
<td><em>Gardnerella vaginalis</em> implicated in bacterial vaginosis (BV) pathology (Wilson 2005)</td>
</tr>
<tr>
<td>Pubic (F. Ind.6)</td>
<td><em>Enterobacteriaceae</em></td>
<td>Many species harmless symbionts of the human gastro-intestinal tract (Wilson 2005)</td>
</tr>
<tr>
<td>Pubic (F. Ind.6)</td>
<td><em>Methylobacteriaceae</em></td>
<td>Ubiquitous in nature (Fanci 2010)</td>
</tr>
<tr>
<td>Pubic (F. Ind.6)</td>
<td><em>Pasteurellaceae</em></td>
<td>Respiratory, alimentary and reproductive tracts (Naushad and Gupta 2012)</td>
</tr>
<tr>
<td>Pubic (F. Ind.6)</td>
<td><em>Pseudomonas spp.</em></td>
<td>Transients on human skin (Wilson) also present in water purification systems (Wilson )</td>
</tr>
</tbody>
</table>

*Superscript note:* a indicates a particular species or subspecies.
<table>
<thead>
<tr>
<th>Location</th>
<th>Genus/Species</th>
<th>Environment/Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubic (M. Ind.7)</td>
<td><em>Aurantimonadaceae</em></td>
<td>Marine environment (Valme Jurado 2006)</td>
</tr>
<tr>
<td>Scalp (M. Ind.7)</td>
<td><em>Brachybacterium spp.</em>&lt;sup&gt;a&lt;/sup&gt; (Dermabacteraceae)</td>
<td>Variety of environments e.g. deep poultry litter, salt fermented sea food (Lo, Lang <em>et al.</em> 2013)</td>
</tr>
<tr>
<td>Scalp</td>
<td><em>Gordoniaceae</em></td>
<td>Majority environmental (Natalia Ivanova 2010) but some human pathogens (Aoyama, Kang <em>et al.</em> 2009)</td>
</tr>
<tr>
<td>Scalp/Pubic (M. Ind.7)</td>
<td><em>Rhodobacteriaceae</em></td>
<td>Aquatic habitats (Allers, Gomez-Consarnau <em>et al.</em> 2007)</td>
</tr>
<tr>
<td>Scalp/Pubic</td>
<td><em>Sphingomonadaceae</em></td>
<td>Widespread in nature; present in water (McFeters, Broadaway <em>et al.</em> 1993, Srinivasan 2008)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bacteria identified to the genus or species level are regarded as the lowest taxonomic levels or the most discriminatory (personalised) data, which may be identified. As such, the presence of these taxa may in forensic investigations, excluded or not excluded individuals as the source of these bacteria.
5.2.4.2 Hair microbiota

Bacteria colonising male and female scalp and pubic hair samples are classed as either ‘core’ or transient (Tr) bacteria (Figure 5.2.2, see methods). In relation to the number of OTUs extracted from scalp and pubic hair microbiomes, far less bacterial sequences were lost post control filtering for pubic microbiomes in comparison to scalp hair. Pubic hairs in general contained more OTUs than scalp hair (approximately 50 male OTUs/55 female for scalp hairs c.f. approximately 73/76 for pubic hairs). Therefore, in general, pubic hair microbiomes appear to be less influenced by environmental bacteria than scalp hairs and possible harbor more niche specific bacteria. Zhou et al. (Zhou et al 2013) support this premise, by demonstrating that (in comparison to other areas of the body) vaginal microbiota consisted of less stable bacteria (i.e. more transient bacteria) and showed lower alpha-diversity (i.e. low species richness), supporting the premise of pubic hair harboring niche specific bacteria.
5.2.4.2.1 Pubic hair microbiota

Male pubic hairs could be readily distinguished from female pubic hairs on the basis of their respective microbiota. *Lactobacillus* was the most prevalent taxon that clearly differentiated male and female pubic hair microbiota (Figure 5.2.2). Whilst the prevalence of *Lactobacillus* spp in the vagina and vaginal secretions is well established (Wilson 2005, Witkin, Linhares *et al.* 2007, Verstraelen, Verhelst *et al.* 2009), this present study is the first to discuss these bacteria.

**Figure 5.2.2. Microbial data extracted from scalp and pubic hairs.** Diagram illustrating core and transient (Tr) bacterial taxa on male and female scalp and pubic hair samples.
colonising pubic hairs, and general pubic area, in the context of probative value in forensic investigations. Fleming and Harbison (Fleming and Harbison 2010) suggested the presence of two *Lactobacillus* spp (*L. crispatus* and *L. gasseri*) as suitable forensic markers to identify vaginal secretions. However, microbial data garnered in this present study suggest that a NGS metagenomic approach may be preferable to those that target specific species. The variety of *Lactobacillus* spp detected in pubic hairs from the female cohort consisted of 11 OTUs (taxa) in total; three *Lactobacillus* spp were unique to Female 5, one *Lactobacillus* spp occurred in Female 1, and four *Lactobacillus* spp were uniquely between the cohabiting couple. In addition, two *Lactobacillus* spp were uniquely shared between F4 and F5, and one OTU was uniquely shared between F4 and F1 (Tables 5.2.2 and 5.2.3).

Compared to male pubic hairs, female pubic hairs harboured fewer transient bacteria (Figure 5.2.2); the number of bacterial sequences comprising transient bacteria of female pubic hairs was approximately half the number of those found in male pubic hair, (Table 5.2.4). This disparity may be attributable to lactobacilli conferring ‘antimicrobial protection’ to the vagina by preventing colonisation by other microorganisms (Verstraelen, Verhelst et al. 2009). Li et al. (Li, Bihan et al. 2013) also found that in comparison to other body areas, the vaginal microbiome is less transient (i.e. more stable). This stability was apparent in the differences between the number of OTUs detected in the scalp and pubic hair controls; there were significantly less OTUs present in controls from the pubic hairs in comparison to the scalp hairs. Post-control filtering for FSH and MSH samples there were 33% and 43% (respectively) of sequences left. In comparison, for FPH and MPH there were 70% and 72% (respectively) sequences left, post filtering. The disparity between the two somatic origins suggests that the bacterial taxa in scalp hair extracts had a high proportion of environmental bacteria that readily appear in controls.
Table 5.2.4 Number of bacterial 16S sequences found in core microbiomes compared with transient number of sequences for each sex/somatic origin sampled. Female pubic hair harbored less transient bacteria, but more core bacteria than male pubic hair.

<table>
<thead>
<tr>
<th>Sex/Somatic Origin</th>
<th>Core Bacteria</th>
<th>Transient Bacteria</th>
<th>Total Bacteri</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female scalp hair</td>
<td>3123</td>
<td>2162</td>
<td>5285</td>
</tr>
<tr>
<td>Female pubic hair</td>
<td>16019</td>
<td>1524</td>
<td>17543</td>
</tr>
<tr>
<td>Male scalp hair</td>
<td>4838</td>
<td>1220</td>
<td>6058</td>
</tr>
<tr>
<td>Male pubic hair</td>
<td>8109</td>
<td>2819</td>
<td>10928</td>
</tr>
</tbody>
</table>

*The total number of sequences found to remain once sequences found in controls and low abundant clusters were removed.

5.2.4.2.2 Scalp hair microbiota

In contrast to the pubic hairs, scalp hair microbiota showed no correlation with the sex of the donor (Figure 5.2.2). Male and female scalp hair bacterial taxa consisted of normal human skin commensals e.g. *Anaerococcus* spp, and environmentally derived taxa e.g. *Knoellia subterranea* many of which occurred in both male and female samples (Table 5.2.5). In the present study, the most significant difference observed in male and female scalp hairs was the disparate proportions of the transient bacterial taxa (Figure 5.2.2). Almost twice as many transient bacterial taxa were present in female scalp hair compared to males (Table 5.2.4). This may be due to the greater frequency of females grooming and/or washing and/or dyeing or bleaching their hair in comparison to males. Such grooming practices may prevent establishment of more stable bacterial colonies in favor of less stable (transient) bacterial colonies. Irrespective of the cause of this disparity, this observation cannot be regarded significant in relation to forensic investigations.
Costello et al. (Costello, Lauber et al. 2009) identified two dominant 16S sequences from scalp swabs; *Propionibacterinae* in which members are predominant bacteria in hair follicles and other sebaceous sites (Wilson 2005), and *Streptophyta* (a plant phylum). In contrast, the predominant bacterial taxa from hair shafts in this study were *Corynebacteriaceae* and *Tissierellaceae fam.nov* (‘new family’) (Figure 5.2.2). The difference may be attributable to either environmental differences (i.e. different study sites) or the collection technique employed by Costello et al. (Costello, Lauber et al. 2009) where swabbing the top of the head might have favored the removal of scalp/follicular bacteria (i.e. propionibacteria rather than hair shaft bacteria).
Table 5.2.5 Natural habitats of bacterial taxa shared between individuals. These taxa relate to those contained within Table 5.2.2.

<table>
<thead>
<tr>
<th>Shared Bacterial Taxa</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aggregibacter segnis</em> (Pasteurellaceae)</td>
<td>Normal human oral flora (Naushad and Gupta 2012)</td>
</tr>
<tr>
<td><em>Anaerococcus</em> spp. (<em>Tissierella</em> nov.fam.)</td>
<td>Commensal human flora, opportunistic pathogens also environmental (Alauzet, Marchandin <em>et al.</em> 2014) (Ezaki, Kawamura <em>et al.</em> 2001)</td>
</tr>
<tr>
<td><strong>Beta-Proteobacteria</strong></td>
<td>High order taxon (Class). Members largely environmental, also include human pathogens and commensals</td>
</tr>
<tr>
<td><em>Bifidobacterium</em> spp. (<em>Bifidobacteriaceae</em>)</td>
<td>Normal intestinal flora (Wilson 2005)</td>
</tr>
<tr>
<td><em>Corynebacterium</em> spp. (<em>Corynebacteriaceae</em>)</td>
<td>Key members associated as part of skin flora (Wilson 2005)</td>
</tr>
<tr>
<td><em>Dialister</em> spp. (<em>Veillonaceae</em>)</td>
<td>Implicated in oral cavity disease (Wilson 2005)</td>
</tr>
<tr>
<td><em>Dietziaceae</em></td>
<td>Environmental and implications as an emerging human pathogen</td>
</tr>
<tr>
<td><em>Knoellia subteranea</em> (<em>Intrasporangiaceae</em>)</td>
<td>Environmental (Groth, Schumann <em>et al.</em> 2002)</td>
</tr>
<tr>
<td><em>Lactobacillus</em> iners and <em>Lactobacillus</em> spp. X6 (<em>Lactobacillaceae</em>)</td>
<td>Part of a suite of lactobacilli that inhabit human female genital-urinary area (Witkin, Linhares <em>et al.</em> 2007)</td>
</tr>
<tr>
<td><em>Mycoplasma</em> spp. (<em>Caulobacteraceae</em>)</td>
<td>Environmental (Urakami, Oyanagi <em>et al.</em> 1990)</td>
</tr>
<tr>
<td><em>Neisseriaceae</em></td>
<td>Members may be human commensals (of the mouth) or</td>
</tr>
<tr>
<td><em>Paracoccus</em> (<em>Rhodobacteriaceae</em>)</td>
<td>Environmental (Loy, Schulz <em>et al.</em> 2005)</td>
</tr>
<tr>
<td><em>Peptinophilus</em> spp. (<em>Tissierella</em> fam.nov.)</td>
<td>Pathogenic (Ezaki, Kawamura <em>et al.</em> 2001)</td>
</tr>
<tr>
<td><em>Prevotella</em> spp. (<em>Prevotellaceae</em>)</td>
<td>Oral, vaginal and gastro-intestinal tract commensals</td>
</tr>
<tr>
<td><em>Xanthomonadaceae</em></td>
<td>Plant pathogen (opportunistic human pathogen) (Mhedbi-Hajri, Jacques <em>et al.</em> 2011)</td>
</tr>
</tbody>
</table>

Forensic investigations seek to establish ‘common origin’ or ‘source attribution’ of evidence, that is, to establish with reasonable scientific certainty that a particular individual is the source of an evidentiary sample. In relation to biological evidence, this question may be addressed through the detection of...
individualising biological characteristics for example a human DNA profile; characteristics which excludes other individuals as being the source. Ideally, these characteristics should not commonly occur within in the general population, or one that is solely found in males or females.

Inside the confines of the 16S V4 region, with the exception of one male (cohabiting male at T5), all individuals harbored unique taxa on their pubic hairs (Figure 5.2.3). In addition to personalised bacteria that were part of the normal skin flora e.g. *Corynebacteriaceae*, pubic hairs were also colonised by environmentally derived bacterial taxa e.g. *Methylobacteriaceae* (Table 5.2.3).

Hairs from scalp and pubic regions, for both sexes, included shared taxa that are common inhabitants of human skin or scalp e.g. *Corynebacteria*, or were environmental in origin e.g. *Rhodobacteriaceae* (Tables 5.2.2 and 5.2.5). At first glance, the commonality of these bacteria may appear to be of minimal probative value; as discussed in a preceding section, personalised features should be uncommon traits or features. However, common bacteria may harbor single nucleotide polymorphisms (SNPs) within their genome, which may further discriminate between individuals.

Among all mammals the microbiota composition is extensively conserved at the high taxonomic levels such as phylum or class. At these taxonomic levels humans are very similar to each other (and other mammals) but variation

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**Figure 5.2.3 Personalised microbial data.**

Diagrammatic summary of unique bacterial taxa found in male and female scalp and pubic hair samples. Male individuals left to right: Individuals M2 (cohabiting male), M3 and M7. Female individuals left to right: Individuals F1, F4, F5 (cohabiting female) and F6.
increases progressively at the lower taxonomic levels. Personalised taxa, which allow discrimination between individuals (the goal of forensic applications), are likely to be detected at these lower taxonomic rankings. Personalised taxa may be present in high or low abundance; detection of low abundance taxa may only be detected by ultra-deep sequencing of the extracted bacterial DNA. In this regard, higher depth of coverage afforded by NGS platforms such as IonTorrent or Illumina may be more informative than the 454 data presented here. As Ursell et al. (Ursell, Metcalf et al. 2012) noted ‘it is important to realize that sampling depth may be critical for distinguishing taxa that are absent from those that are merely rare’. Under these circumstances it is critical to discount bacterial taxa present in all control samples in order for the results to be not only robust but also scientifically accurate and capable of withstanding scientific and legal scrutiny.

Temporal stability data garnered in this study broadly suggest that bacteria on scalp hairs may be more prone to fluctuations in comparison to pubic hairs (in addition to being more prone to environmental contaminants). The data show that, on average and post-filtering, approximately 17% (range 6%-25%) of pubic hair bacterial OTUs were temporally stable across all time points; whilst, on average, scalp hair harbor approximately 5% (range 0-13%) of bacterial OTUs (Table 5.2.6).
Table 5.2.6. Temporal stability of bacterial taxa (OTUs).

Bacterial taxa present in scalp and pubic hairs sampled at three time points over a period of five months

These preliminary findings suggest that pubic hair bacteria may be more temporally stable than scalp hair bacteria and therefore potentially of more probative value than scalp hair bacteria. Although temporal stability of an individual’s bacterial taxa may appear to be an important prerequisite for metagenomics to have forensic value, the most relevant attributes will mostly likely be transference of bacteria (during contact), persistence of bacteria post transfer and storage conditions. Consider a case of unlawful sexual intercourse (of an adult female), the most relevant microbial data will be taxa available for transfer at the time of the assault (rather than what it was weeks, months or days before or after) and the persistence of the victim’s bacteria on the offender’s genitals/pubic area (and vice-versa). This, of course, is reliant upon collection of evidence from the victim and suspect(s) within several hours of the time of the assault rather than several days. Microbial data from the cohabiting couple, albeit preliminary, are encouraging, in supporting the suggestion of bacterial transfer and persistence following sexual intercourse.

5.2.5 Conclusion

Despite the modest sample size, we believe that the data in this qualitative assessment of metagenomic analyses of hairs are sufficient to warrant further development of this approach. For this approach to gain traction there is a need to refine molecular targets – the broad-brush approach of the 16S V4 region looked at here is a good starting point. Additional analyses may provide further information in relation to the microbial composition of ‘core’ microbiomes and
their potential value in forensic investigations. However there is ultimately a need to develop a more focused approach that targets, for example, population level differences within Lactobacillus spp or even more variable genomic sections of common commensals that might contain probative information at a population level.

It is suggested that microbial data gathered from hairs may provide independent data to augment other forensic results, such as mtDNA or YSTR (when DNA yields are sufficient), and possibly provide association between victims of sexual assault and offender, which is currently not possible in the absence of hairs, fibres or seminal fluid. Importantly, conducting metagenomic analyses on hairs does not preclude conducting traditional molecular analyses on the DNA extract. Despite the complexity of microbial forensic investigations, a substrate such as hair is arguably much simpler to profile than soil a gram of which may contain up to 50,000 different microbial species (Sensabaugh 2009) or skin, which exhibits high taxonomic divergence and numbers distributed across multiple niches (Grice and Segre 2011); on the basis of our qualitative assessment, hairs harbor more modest numbers of bacterial diversity. In comparison to scalp hair, pubic hair is somewhat insulated from the environment being colonised with niche specific bacteria. With perseverance, metagenomic analyses of hairs might develop into a useful component of the forensic toolkit to augment existing forensic techniques.

5.2.6 Abbreviations

**BLAST**: Basic local assignment search tool; **BLASTn**: Programs search nucleotide databases using a nucleotide query; **Bp**: base pair; **emPCR**: emulsion-based clonal amplification; **FSH**: Female scalp hair; **FPH**: female pubic hair; **GIT**: Gastro intestinal tract; **HTS**: High throughput sequencing; **MSH**: Male scalp hair; **MPH**: Male pubic hair; **MID**: Multiplex identifier or ‘barcode’; **NGS**: Next-generation sequencing; **qPCR**: Quantitative polymerase chain reaction; **SI**: sexual intercourse.
5.2.7 Competing Interests

The authors declare that they have no competing interests.

5.2.8 Acknowledgements

The authors would like to thank the volunteers for their participation, diligence and patience. We thank the Murdoch University Centre for comparative genomics (M. Bellgard) and iVEC computing resource. MB was supported in this research by an ARC future fellowship FT0991741.

5.2.9 References


pregnancy suggests that L. crispatus promotes the stability of the normal vaginal microflora and that L. gasseri and/or L. iners are more conducive to the occurrence of abnormal vaginal microflora." *BMC Microbiol* 9: 116.


### 5.2.10 Chapter Summary

Although modest in scale, this chapter provides tantalising glimpses into the nature of bacteria that colonise two disparate areas of the human body and addresses aims 1 and 2 of the study. Fulfillment of aim 3, which involved assessing the temporal stability of bacteria, did not yield conclusive results; this may be due to the small sample size, inadequate number of collection time points or is a reflection of the naturally transient or dynamic nature of bacteria on the human body. With regard to aim 4 there is certainly evidence herein to warrant further investigation of this method as a tool in forensic science.

One of the most significant outcomes of this chapter is one which unequivocally shows that the sexes may be differentiated on the basis of their respective microbiomes; female pubic hair, unlike male pubic hair, harbor indigenous, niche specific bacteria.
by the study conducted by Mann (REF). This paucity of pubic hair transfer, coupled with an increased use of condoms by offenders, there may be a lack of human biological material present after heterosexual, vaginal rape. The suggestion of bacterial transfer during sexual intercourse is therefore significant and has the potential to provide molecular data to associate offender and victim through bacterial, not human, DNA.

Within the present study group, pubic hair microbiomes of each study participant harboured a bacterium that was unique within the group (with the exception of the co-habiting male) However, in relation to scalp hair bacteria, fewer study participants harbored personalised bacteria, this may be a reflection of the commonality of bacterial taxa found in scalp hairs. Furthermore, this study suggests that scalp hairs, in contrast to pubic hairs, are mainly colonised by environmental bacteria which is not a surprising result.

Several bacterial taxa were shared between individuals, which consisted of common human bacterial flora or bacteria that are implicated in causing disease. However, the most significant results were pubic hair bacteria that were shared between the cohabiting couple, which not only consisted of common skin commensals, but also consisted of niche-specific bacteria unique to female pubic hairs.

These results, despite being preliminary, suggest that pubic hairs are more likely to be of greater probative value in forensic investigations in comparison to scalp hairs. Although the current data set is too small to be statistically viable or provide conclusive data, they are sufficiently provocative to merit further, more in-depth and focused future studies. The future directions section of this thesis (6.2.4) explores what might be done to further advance this field of study.
Chapter Six:
General discussion and future directions for the examination of mammalian hair

‘Now this is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning’

Winston Churchill 10 November 1942

6.1 General Discussion

The work presented in this thesis has advanced scholarly knowledge in the interpretation of microscopic features exhibited by hairs. It is the first body of work that specifically addresses present and future challenges in relation to the examination of hair derived from extinct species (e.g. Chapters Three and Four) and ancient peoples (Appendix Seven). Some of the manuscripts are provocative in nature in challenging well-established paradigms (e.g. chapters Two and Four) whilst others, introduce novel concepts in relation to future analyses (e.g. Chapter Five).

This body of work provides novel insights into the microscopical examination and analyses of hairs that not only originate from different species but also those which originate from different epochs. In doing so, previously unrecorded structural features were discovered that, together with other adaptations, may have contributed to survival of now extinct megafauna. Throughout this thesis two inextricably central themes emerge: the necessity of practitioners to have appropriate competencies and expertise in order to accurately interpret microscopic characteristics of human and animal hairs, and the value of multi-disciplinary approaches to hair examination and analyses.

Furthermore, this thesis is the first to introduce the concept of using bacterial ‘signatures’ on human hairs for forensic investigations. Despite the preliminary nature of this work (see future directions in 6.2.4) this study reinforces the value of a multi-disciplinary approach to the use of hair in forensic applications.
This general discussion presents advancements made in regards to the interpretation and significance that can be ascribed to microscopical hair structures and novel uses that molecular analyses of hairs can be put to. Furthermore it also advocates future directions that this research could take.

6.1.1 Microscopical characterisation of human and animal hair morphological structures

Superficially, mammalian hair appears to be a simple structure that differs in individuals solely in the amount and colour. However, this naive overview belies a complex structure whose anatomy varies within and between individuals, irrespective of species. Chapter Two is the first publication to openly address consequences of ill trained /untrained examiners attempting to interpret microscopic structures present in human and animal hairs. The tenets discussed in Chapter Two are directly relevant to the following chapters in which microscopical hair features are discussed.

The majority of misinterpretations of hair structures appear to be more prevalent in relation to those exhibited by animal hairs rather than human hairs; this may be due to the fact that all human hairs share the same genetically determined morphological characteristics, differing only in colour, length, pathology and environmental/treatment history. Another reason may be due to the fact that human hairs have significantly fewer microscopic characteristics to recognise and interpret in comparison to those exhibited by some 5,000 animal species that inhabit the Earth (Wund M 2005), each of which exhibit numerous and varied taxon-specific microscopic structures.

A critical aspect of the taxon identification process is reliant on microscopical comparison between the features exhibited by the hair in question, with reference hairs taken from vouchered specimens. These specimens are necessary to ensure that the identity of organisms studied in the field or laboratory can be verified. Mis-identification of animal hairs may implicate an individual as the offender of a heinous crime or may waste scarce resources relating to conservation studies or
misdirect them. For example, if a mis-identification fails to identify a rare or endangered species or, conversely mis-identifies a common species as one that is rare; one may lead to extinction whilst the other may lead to mis-allocation of finite funds and/or unreliable results regarding to faunal distribution. It is hoped that the morphological identification of animal hair manuscript will raise awareness of the serious forensic and wildlife conservation consequences of mis-identification of animal hairs.

6.1.2 Biodegradation of mammalian hairs-cause and effect

Chapter Three of this thesis provides the first comprehensive, visual catalogue of morphological signatures produced by biological agents and prolonged exposure to the environment. It also highlights the consequences of misinterpreting signatures caused by these agents as genetically acquired ones.

This chapter is also the first to dispel previously held views that a number of effects can take place after the death (post-mortem) of the organism by presenting data that indicates that only one agent of biodegradation occurs post mortem. This finding is of significance in forensic investigations and in scenarios of searching for missing persons. In relation to paleontological and ancient hairs the presence of biodegradation may provide insights into the environment in which the mammals perished. Furthermore, Chapter Three demonstrates the value of judicious selection of microscopes to best articulate and demonstrate the feature(s) of interest.

The microscopic effects of biodegradation, evident in ancient and palaeontological hair samples, challenge historic and current views in relation to the true colouration of hairs from ancient peoples and extinct megafauna.

The sentiments expressed in Chapter Two of this thesis are echoed in Chapter Three regarding competencies and expertise to interpret microscopic hair features. The inability to recognise genetically determined features from those that are ‘acquired’, could negatively impact on the health of animals kept in captivity, compromise forensic investigations or result in erroneous faunal audits.
in wildlife conservation and faunal ecological studies. Chapter three exemplifies the consequences of these mis-interpretations, which are promulgated through widely distributed electronic and written resources.

6.1.3 Multi-disciplinary approaches to the examination of extinct megafauna hairs

The ability to conduct a variety of independent analyses and examinations on mammalian hairs is a powerful approach in gleaning as much data as possible from hairs; particularly those that are rarely encountered, such as prehistoric hairs. Chapter Four represents the first body of work to introduce the concept of a multi-disciplinary protocol to the examination of hairs; furthermore, it is the first to demonstrate the value of such an approach. The benefits derived from microscopical audits of extinct megafauna hairs prior to destructive analyses are evident from the morphological audits conducted in Chapter Four. These audits revealed unique characteristics that may have been critical to the animals’ survival. Furthermore, the results of these audits challenged and dispelled myths surrounding the colour of extinct megafauna pelages. For the first time, microscopical assessments revealed that hairs from extinct megafauna were equally susceptible to agents of biodegradation and hair ‘nits’ as are modern mammals; a finding that illustrates the adaptation and evolution of niche-specific organisms.

Chapter Four presents compelling evidence to support the value of conducting microscopical evaluation of hairs prior to their consumption for destructive molecular analyses. The demonstrable value of a multi-disciplinary approach to the examination hairs is further supported from studies conducted on 4,000 year old permafrost-preserved scalp hairs from a male individual, which significantly contributed knowledge about now-extinct cultures (Rasmussen, Bertalan et al. 2010) (Co-author, Appendix B.1); similarly molecular and microscopical analyses reveal disparate data from a 100 year old lock of hair from an Australian aboriginal, (Rasmussen, Lohmueller et al. 2011) (Co-Author, Appendix B.2).
6.1.4 Future potential of bacterial DNA profiles in forensic investigations

Chapter Five debuts an avant-garde concept for future forensic molecular analyses of hairs. It is one that utilises Next-generation Sequencing (NGS), or massive parallel sequencing, technology to generate microbial, not human, DNA profiles to potentially associate victim and offender. The modest sample size used in the inaugural study is one that supports a proof of principle, qualitative evaluation of a novel forensic technique. However, that notwithstanding, the results provides tantalising evidence to support the premise that a metagenomic approach to future examination of human hairs is one that is worth pursuing. The preliminary results are encouraging and warrant more detailed and focused analyses (see future directions 6.2.4). The study suggests that pubic hair bacteria are most likely to provide greater probative value than scalp hair bacteria in forensic contexts.

6.2 Scope for further study and research

In all areas of research and challenges to established procedures, many questions remain unanswered and further avenues of investigation remain unexplored; this thesis is no different. On the basis of the results obtained in Chapters 2 through to 5, I believe that future efforts and research should be focused in the following four areas (6.2.1 to 6.2.4). This list is meant to represent what I regard as the four most pressing/interesting issues that are raised as the result of this thesis research.

6.2.1 Developing guidelines into microscopical characterisation of hair structures

The future of microscopical analyses of animal hairs is poised for a renaissance through the promulgation of the inaugural ‘Standards and Guidelines for Wildlife Forensic Practitioners’ (by SWGWILD), to which I contributed. These guidelines may result in future service recipients, such as those in government and members of the legal profession, to scrutinise competencies and expertise of service providers in greater detail than is currently possible. Furthermore, these guidelines have the potential to provide a credible foundation upon which future animal hair microscopists may build their expertise.
Future compliance with SWGWILD Standards and Guidelines will not only underpin the ability of hair morphologists to correctly identify and interpret microscopic hair structures but also provide a measure of credibility and accountability.

Chapters Three and Four showcase hair images resulting from use of a variety of microscopes in order to best articulate the feature of interest. It is hoped that this may encourage future hair examiners (where possible) not to limit their examinations to one type of microscope or technique. Furthermore, it is hoped that this thesis will encourage future examiners to become proficient in the identification and recording of hair structures using light microscopy before they are irrevocably compromised in preparation for electron microscopy.

6.2.2 Continued microscopical studies of hairs from ancient and palaeontological hair samples and multi-disciplinary approaches

Morphological data gleaned from extinct megafauna hairs in Chapter Four were ‘not in context’ in as much that their somatic origins were largely unknown. Future studies in which the somatic origin of hairs are known, may provide a morphological framework within which a more accurate picture of the composition of pelages of extinct megafauna. This approach may provide additional information regarding form and function of extinct woolly mammoth hairs that may lead to greater insights into the natural history of these charismatic species. Future morphological studies would benefit from hair samples taken from additional woolly rhino remains and/or carcasses. Based on the findings of hair nits on hairs from woolly rhino (illustrated in Chapter Four) hair samples taken from other woolly rhino remains may further our knowledge in Pleistocene parasitism. Future microscopical studies of extinct megafauna hairs may benefit greatly if, in addition to known somatic origin of hairs, the sex and age (at the time of death) of the animal is known.

The value of a multi-disciplinary approach to the examination of ancient hair is exemplified by Chapter Four and from past studies ((Rasmussen, Bertalan et al. 2010) (Rasmussen, Lohmueller et al. 2011) and Appendix B).
As a field, it is imperative that such multi-disciplinary approaches continue to be developed as in my opinion it holds great promise for innovation, especially in the forensic sector. Forensic examinations that result in associative evidence derived from a number of disparate analyses and examinations may result in more reliable verdicts and fewer mis-carriages of justice.

6.2.3 Improving the ability to differentiate between genetically determined hair structures from those that are ‘acquired’

Throughout the tenure of this thesis, the promulgation of misinformation in the literature due to misinterpretations of genetically determined microscopic hair structures has been widely debated. The same tenet applies to the misinterpretation of ‘acquired’ hair structures as a result of pre- or post mortem insults as discussed in Chapter Three of this thesis.

To enable future generations of hair microscopists to be able to correctly differentiate between genetically determined hair features from those that are ‘acquired,’ future training should incorporate familiarisation and recognition of pre- and post mortem artifacts on hairs. This approach would greatly increase reliability of results and encourage promulgation of ‘best practice’ in the recognition and interpretation of hair artifacts.

Another area of future studies may be directed towards the identification of fungi that have developed specialisations that enable them to derive nutrients from the degradation of keratin. In doing so this would not only enhance our knowledge of these fungi, but would further promote multi-disciplinary approaches to the examination of hairs between hair analysts and mycologists.

6.2.4 Continued assessment of metagenomic analyses of hairs as a novel molecular tool in forensic investigations

Chapter Five suggests a potential avenue of research on human hair samples focusing on bacterial DNA rather than human DNA. The paucity of pubic hair transfer during rape, coupled with the increased use of condoms, result in a lack of biological fluids being transferred and thereby negating opportunities for human DNA profiling. However, in order to fully realize the forensic potential of
metagenomic analyses, considerably more in-depth and focused analyses are clearly required. Future bacterial studies would benefit from substantially larger data sets (multiple 16S regions, greater depth of sequence), together with more frequent sample collections, in order to fully explore personalised, core and temporal microbiomes.

The evaluation of the forensic potential of the cross-transference of bacteria during heterosexual intercourse would require a large group of participants. Future research should address post coital sampling techniques, which may include the collection of pubic hairs and also the efficacy of swabbing pubic areas (relevant to individuals that regularly remove their pubic hair). Swabbing the pubic area (irrespective if hairs are present or absent) is of additional interest because it may be a less invasive manner in which forensic samples are taken from traumatised rape victims. Factors that may affect or alter bacterial populations on hair shafts, such as the use of antibiotics should also be explored in future analyses.

Forensic techniques are designed to exploit differences between individuals in order to personalise the evidence. The presence of common and ubiquitous bacteria present in each individual of the present study suggests that bacterial assemblages alone lack the required discriminatory potential. However, NGS has the capability to detect single nucleotide polymorphisms (SNPs), or single base changes, in the genomes of bacteria. Future studies in this area will need to investigate the discriminatory potential resulting from SNPs present in bacterial genomes of different individuals. An example of this approach is the use of a multiplex PCR assay that targets highly variable regions within *Lactobacillus* spp. that are indigenous to female uro-genital areas. Ultimately, if the forensic community adopts metagenomic analyses, a significant future direction should incorporate global standardisation of terms, definitions and sample processing in order for inter-laboratory comparisons of data to be viable.
6.3 Concluding Remarks

The research described in this thesis has provided advancements and recommendations in relation to microscopical approaches to the examination of ancient and modern mammalian hairs. Furthermore, it has also provided molecular glimpses into the potential advancements in the field of forensic science regarding human hair analyses.

In Australia and elsewhere, stakeholders such as law enforcement personnel, or other government bodies, have an expectation that service providers are subject matter experts in the interpretation of microscopic hair structures. The research conducted in this present study has, I believe, raised the awareness of the value of microscopical examination of hairs being performed by appropriately qualified personnel; it has also provided a provocative glimpse into the role that bacteria and fungi might play in future forensic investigations.

In achieving each of the aims listed at the start of this thesis (1.5), additional questions and avenues of exploration were revealed. Although these avenues were beyond the scope of this thesis, it is hoped that the foundations laid herein are sufficiently robust for future endeavors to be built upon. In doing so they may further advance forensic investigations and the study of ancient peoples and animals; in doing so, they secure the credibility of future interpretations of microscopic hair morphologies and their molecular analyses of animals and humans, both past and present.

References


Appendix A: Published Manuscripts

The citations for the published manuscripts arising from this thesis research appear below; due to copyright laws, published manuscripts 1-3 and publications cited in Appendices B-D, which I have co-authored, have been omitted.


APPENDIX B: Ancient human genome sequence of an extinct Palaeo-Eskimo

The following citation resulted from research that is of relevance to this doctoral thesis and was published during my candidature. In the context of this multi-author publication I conducted the morphological analysis of the hair sample.


‘Supplementary Information’ may be viewed at:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3951495/
APPENDIX C: An Aboriginal Australian genome reveals separate human dispersals into Asia.

The following citation resulted from research that is of relevance to this doctoral thesis, which was published during my candidature. In the context of this multi-author publication I conducted the morphological analysis of the hair sample.


Supplementary Online Material (SOM) (which contains the microscopical results of the hairs) may be viewed at: http://www.sciencemag.org/content/334/6052/94/suppl/DC1
APPENDIX D: Tracing the dynamic life story of a Bronze Age Female

The following citation resulted from research that is of relevance to this doctoral thesis and was published during my candidature. In the context of this multi-author publication I conducted the morphological analysis of the hair sample.


The supplementary information (which contains microscopical hair images) may be viewed at http://www.nature.com/srep, pp 16-17, Figures S4, S5.