STR ANALYSIS OF HUMAN DNA FROM MAGGOTS FED ON DECOMPOSING BODIES: ASSESSMENT ON THE TIME PERIOD FOR SUCCESSFUL ANALYSIS

M.Sc. Thesis By:

Njau Daniel Gachuiri

Reg. No. H56/65991/2010

Department of Biochemistry

A thesis submitted in partial fulfillment of the requirements for the award of a Masters degree of Science in Biochemistry

Department of Biochemistry

School of Medicine, College of Health Science

University of Nairobi

September 2013

DECLARATION

This is my original work and has not been presented for a degree in any other university.

Mr. Njau Daniel Gachuiri, BSc. Biochemistry (UON).

Department of Biochemistry

University of Nairobi

Signature: ……………………………………..Date: ……………………………………..

This Thesis has been submitted with our approval as university supervisors

Dr. E. K. Muge

Department of Biochemistry, University of Nairobi

Signature…………………………………...Date:……………………………………..

Ms Sophie Mukwana

Biotech Forensics

Signature..Date: ………………………………….

Prof. C. O. A. Omwandho

Department of Biochemistry, University of Nairobi

Signature…………………………………..Date: ………………………………….

Prof. P.W. Kinyanjui

Department of Biochemistry, University of Nairobi

Signature…………………………………..Date: ……………………………………..

Chairman

Department of Biochemistry, University of Nairobi

Signature…………………………………...Date: …………………………………..

DEDICATION

This thesis is dedicated to my family members and friends who provided me with moral and

financial support throughout my studies.

ACKNOWLEDGEMENT

I am very grateful to the following individuals and organizations that contributed towards successful completion of this research work.

First and foremost, I would like to thank God for His wisdom and guidance throughout my life and studies.

I express my sincere gratitude to my supervisors Ms Sophie Mukwana (Biotech Forensics-Kenya) and Dr. E. K. Muge, Prof. C. O. A. Omwandho and Prof. P.W. Kinyanjui (Department of Biochemistry-University of Nairobi) for their patience, guidance, suggestions, encouragement, support and excellent advice through the course of this study.

I would like to express my appreciation to Miss Jasveen Kaur Manku for all her support from the beginning of the research to the end.

I gratefully thank the Biotech Forensics for providing all the required instruments, equipment and kits that were required for this research.

I am also grateful to all the teaching and non-teaching staff at the Department of Biochemistry for all the support they provided in the course of this research.

Lastly my acknowledgement goes to the University of Nairobi for my studentship as well as allowing me to do my research within its facilities.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1. STRs   Short tandem repeats

2. Y-STR   Y- Chromosome Short tandem repeats

3. PMI   Post-mortem interval

4. MtDNA   Mitochondrial DNA

5. HVR   Hyper variable Region

6. DNA   Deoxyribonucleic acid

7. AmpFLP   Amplified Fragments Length Polymorphisms

8. PCR   Polymerase chain reaction

9. REF   Reference sample

10. +C   Positive control

11. OL   Off Ladder

12. 2DSM   2 Days Starved Maggots

13. 2DBM   2 Days Beef Fed Maggots

14. 4DBM   4 Days Beef Fed Maggots

ABSTRACT

Forensic entomology is a branch of biology involving study of insects that are present on carrion in order to facilitate identification of the body, and to determine Postmortem Interval (PMI). Its importance becomes inevitable when one comes across some incident where a corpse is unidentifiable and lots of maggots or other insects are present. Frequently, application of forensic entomology is in the use of insect maggots for identification of specimen or human remains. Maggots' crop analysis could be valuable in criminal investigations when maggots are found at a crime scene and a corpse is absent. If live maggots are found at a location without the corpse around, it is likely that a corpse has been moved from the crime scene. If there is a different food source near the scène, maggots may move away from the corpse to the new food source. Human short tandem repeat (STR) profile from this study was used to support the association of maggots to a specific corpse. The aim of this research was to assess the time period for successful STR analyses of human DNA from the contents of crop of third instar maggots (*Protophormia terraenovae*) obtained from decomposing human corpses and investigate the human DNA turnover and degradation in the maggots crop after they are removed from food and/or are fed on a new/different food source. Maggots were collected from human corpse after a specific post-mortem interval. Some maggots were starved while others were fed on beef. Ten maggots were used in each case. The maggot crops were removed and DNA extracted from the reference sample and gut contents. Human DNA was quantified using real time PCR prior to amplification and STRs profiling at 16 human genetic loci. Results showed that amount of human DNA recovered from the maggots decreased with time. For maggots fed on beef, the human DNA could only be recovered up to day two. Since the starved maggots did not have any other food

source after they were collected from the corpse the human DNA could only be recovered up to day four. STR analyses of DNA from maggots' crop content generated profiles that matched that of reference samples although some of the alleles were not amplifiable therefore generating partial profiles. This may be due to nucleases' activity present in the gut of the larvae which may have caused degradation of DNA and consequently reduction in DNA yield. Results of this study will help forensic analysts in determining the window of time during which maggots are useful in criminal investigations involving death of humans.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Forensic entomology is a branch of biology involving study of insects that are present on a carrion in order to facilitate identification of the body, and to determine Postmortem Interval (PMI). Insects are attracted to corpses within a very short time after death by odour of gases from the decomposing bodies due to the breakdown of tissues by bacteria. Insects therefore give the most accurate PMI (Nuorteva, 1997; Anderson and Vanlaerhoven, 1996) due to their predictable developmental rate (Chapman, 1980) and their invasion sequence of carrion. (Pyre, 1965; Anderson and Vanlaerhoven, 1996; Early and Goff, 1986; Dillon and Anderson, 1995).

Several situations have been demonstrated where analysis of crop contents of maggots that feed on carrion would be helpful in forensic investigations (Wells *et al.,* 2001). In maggots, the crop is an organ in the guts anterior end, which is used to store food temporarily. Sequencing of Mitochondrial DNA (mtDNA) obtained from crop contents can be used to find out who or what the larva had been feeding on. In addition, such analysis provides a forensic entomologist with an alternative way of associating a larva with a body when estimating PMI (Linville and Wells, 2002). Insect evidence can also be found in orifices, sores and wounds hence helping investigators determine the events that may have taken place before death (location of injuries and wounds). When insects are used for PMI estimation, the assumption made by entomologist is that the entire insect specimen's development took place on the victim (Linville and Wells, 2002).

A study by Boakye and Chow-Shaffer showed how DNA recovered from insect blood meals could be used for species or individual host identification (Boakye *et al*., 1999; Chow-Shaffer, 2000). Host identity using DNA obtained from excreta and blood meals of crab louse has also been explored as potential forensic utility (Lord *et al*., 1998; Replogle *et al.*, 1994). In addition, in Wells *et al*., (2001) human mtDNA that had been recovered from the gut contents of maggots raised on human tissue was successfully sequenced.

In the amplification of human mitochondrial DNA from crops of maggots, the risk of contamination must always be considered just like in bone or hair samples (Wilson *et al*., 1995). Contamination presents a risk of interference with the analysis of crop contents, however, this could significantly be reduced by simple washing of the maggot's exterior. An appropriate wash method would eliminate exterior DNA contamination without compromising the recovery of DNA from the crop (Wells and LaMonte, 2001). Since eggs of flies are rarely deposited on a corpse before death, an accurate way to analyze forensic cases would involve determination of the age of immature insects collected from a decomposing body (Linville *et al*., 2004).

Studies on effect of specimen preservation technique have shown that PMI determination relies greatly on the forensic entomologist's knowledge and may only take simple microscopic examination (using scanning electron microscope) of insect specimens. Forensic investigators are thus recommended to select a method of preservation which would avoid shrinkage, distortion and discolouration of maggots causing errors in estimating the age of the maggots, estimation of PMI and compromising the recovery of DNA from the crop (Linville *et al*., 2004). A good method will therefore maintain the quality and integrity of identification features until

examination of data (Haskell and William, 1999; Haskell *et al*., 2001). Entomological evidence analysis has gone beyond examination of morphological features due to advances in technology. In cases where identification of specimen by visual inspection is difficult, DNA analysis is currently used to accomplish this task (Sperling *et al*., 1994; Well and Sperling, 2001; Wells *et al*., 2001).

Analysis of carrion DNA from insects has the benefit of being applicable to any developmental stage even with specimens that are immature (Zehner *et al*., 2004b). The high copy number of mtDNA within the cells has made it more helpful in human identification since they can be used even on degraded tissues (Morley *etal*., 1999; Holland &Parsons, 1999). Reports on human mt DNA and STR analyses indicate that within the crop of blowfly larva, digested liquid food is stored temporarily and initial digestion does not take place here due to absence of proteolytic enzymes (Campobasso *et al.*, 2005). It has however been demonstrated that maggot saliva contains enzymes for pre-oral digestion which are re-incorporated with food into the crop (Hobson, 1932). The possibility of DNA degradation within the crop cannot be ruled out and the extent of the degradation is affected by other variables. This in turn will affect the time period for successful STR analysis and while this has not been studied, it can certainly be examined (Zehner *et al*., 2004b).

A study by Zehner demonstrated that when maggots ingest human tissue, the tissue can be extracted from the crop, undergo STR analysis and generate a profile that can be compared against a corpse from which the maggots were feeding (Zehner *et al*., 2004b). During the digestion process, the liquefied host tissue is stored in a specific region of maggot's foregut, the crop. In order to associate maggots to a particular corpse, examination of maggot's crop by STR

3

typing (Butler, 2001) or evaluation of a hyper variable region (HVR) within the mitochondrial d-loop sequence (Lutz *et al.*, 1996; Morley *et al.*, 1999) is advisable. It has been demonstrated that it is possible to obtain mtDNA, amplified fragment length polymorphism (AFLP) and STR data from adult insect blood meals that can lead to individualization of the ingested host blood (Lord *et al.*, 1998; Kreike J and Kampfer S, 1999; Mukabana *et al.*, 2002). Human hyper-variable region analysis using human-bone-fed larvae of beetle is also possible (Dizinno *et al.*, 2002).

Laboratory studies have also revealed that the contents of maggot crop can be used to obtain a human or non-human mtDNA haplotype (Well *et al.*, 2001; Linville and Wells, 2002) or a human Y-STR (STR on the Y-chromosome) profile (Clery, 2001).). Researchers have presented arguments for the potential forensic utility of carrion insect gut analysis. Within a laboratory setting, researchers successfully generate a mtDNA profile from undegraded human tissue. Whether the more sensitive STR analysis can be used in similar fashion but on degraded human tissue has not been explored (Zehner *et al.*, 2004b). This study seeks to investigate the relevance and window of time during which analysis of maggot crop contents could be useful to forensic analysts in cases involving death of humans.

1.2. LITERATURE REVIEW

1.2.1. Forensic Entomology and its applications

Application of entomology in forensic science dates back to 19th century and detailed observations were made by a number of French researchers on the sequence of invertebrate colonization of human corpses in cemeteries and attempts were made to use this knowledge to determine the time since death in murder investigations (Benecke, 2001). Reports indicate that corpses even when burnt still attract blowflies while other reports suggest that there is a decreased likelihood of blowflies to lay eggs on these types of corpses (Avila & Goff, 1998; Catts & Goff, 1992). Less attention has been given to research in forensic entomology in some parts of the world but most countries within the northern hemisphere have embraced entomological evidences unlike the southern hemisphere countries, which some studies indicate that they are still far behind in acknowledging entomological evidences. This is partly due to the fact that until recently, entomological research in these countries had not been geared towards forensic application. Some of the countries from which forensic entomology data is being generated include Columbia (Barreto *et al.*, 2002), Australia (Dadour *et al.*, 2001), Argentina (Centeno *et al.*, 2002), Brazil, and South Africa. Reviews of forensic entomology are available (Catts and Haskell, 1990; Benecke, 2001; Byrd &Castner, 2001), but the focus is on species, such as Diptera that are widely distributed.

Two categories of forensic entomology Medical-Legal and Stored Product Forensic Entomology are of most importance. The former focuses largely on the PMI prediction. Insects have also been used in assessing the possibility that carrion may have been relocated after death (Becker *et al.*, 2007). Detailed studies on PMI estimation have shown that two general methods can be used

5

to predict PMI. The first one is the assessment of the age of insects found on dead body, and from this, the age of the corpse can be determined while the other method is based on colonization sequence of arthropods present on carcass (Wells & LaMotte, 2001). The analysis of human DNA extracted from maggots is another important application of molecular tools in forensic entomology (Wells *et al*., 2001b; Clery 2001). This kind of analysis is important in cases where the source of the maggot's food is disputed, when only maggots but no corpse is found at the suspected scene of possible murder, or where an alternative food source is present at the scene. Through analysis of individual-specific DNA loci such as short tandem repeats (STR) a maggot can be assigned to a specific corpse (Zehner *et al*., 2004a).

STRs used in human identification consist of 4 or 5 nucleotide base pairs. They generally fall within non-coding and flanking sequences but occasionally within coding regions of a human genome (Edwards *et al*., 1991). STRs analysis was first applied in the early 1990s and has since become a major forensics tool applied for identification of humans in mass disasters, paternity disputes as well as in military and country's internal security settings (Edwards *et al*., 1991). Multiplex amplification by the polymerase chain reaction (PCR) and typing of STR loci offers forensic scientists a method of characterizing specimens that is sensitive, has high discriminating power and can be performed rapidly. Studies have reinforced previous findings that multiplex STR typing is sufficiently robust for implementation into forensic laboratories and will be effective for characterizing the vast majority of human biological samples encountered at crime scenes (Moretti *et al*., 2001). A maggot's "last meal," can be identified from the carrion larvae collected during a forensic investigation (Wells *et al*., 2001). Other work has shown that human

DNA retrieved from the gut of a blood feeding insect can through STR analysis, be linked to the original human donor (Wells *et al.*, 2001).

1.2.2. Forensically important insects

For the purposes of forensic entomology, species from the orders Diptera (flies) and Coleoptera (beetles) are the most widely used (Amendt *et al.*, 2004). Necrophagous insects are attracted by decaying corpses and colonize them immediately after death (Fig. 1) (Amendt *et al.,* 2004).

Blowfly species are of great forensic importance since they arrive first at a corpse, with some reports indicating that they arrive within few minutes after death (Arnaldos *et al.*, 2005; Byrd & Castner, 2001; Goff, 2000). Numerous species of blowfly exist and it is not unusual to find several species on a single corpse. Research shows that a decomposing body may contain different Diptera species larvae besides those from family Calliophoridae, an indication that not all blowflies species utilize corpses (Gunn A, 2009). It has been observed that different species of carrion fly differ in preference for habitat type (Norris, 1965). Therefore, it is possible to determine that a corpse was moved following death, if the immature insects on a corpse are not typical of the habitat where the body is found (Amendt *et al.*, 2004). For identification purposes the gut contents of forensically important insects left behind in a crime scene if a corpse removed could potentially identify a victim, and also blood-feeding insects at a scene could link a suspect to that scene if they were found to have fed on the suspect (Campobasso *et al.*, 2005).

Maggot crops are suitable sources of DNA for both the insect and its gut contents identification (Wells *et al.*, 2001). Li *et al* (2011) discovered a headless corpse and the skull at different

locations with a large number of full-grown maggots. Upon STR analysis of 16 loci, the STR profiles that were obtained from the human tissue matched each other, and the profiles obtained from crop of maggots were identical and matched those from the human tissues completely.

Figure 1: Blowfly maggots developing upon a corpse. Mature maggots can be seen over the surface and the discoloration of the skin. (Gunn A, 2009).

1.2.3. Blowfly life cycle

A 'typical blowfly species' life cycle, such as for those found on dead bodies is straight forward (Fig. 2). Studies on life cycle have reported that the gravid female fly lays her eggs on one of the natural openings such as the nose, ears and mouth, eyes, or site of a wound in a corpse. Blowfly eggs are laid in batches that may number up to 180 and hence a female fly lays a total of several thousand eggs over the course of its life. A first instar larva, small and delicate emerges when an egg hatches and rapidly moves to where it can find optimum conditions for survival, optimum temperature and nutrients for growth. These larvae feed using chitinous mouth-hooks that drag

material into their oral cavities consuming both the tissues of the corpse and the microbes that grow on it (Gunn A, 2009).

After approximately 24 - 48 hours, the larva moults to the second instar and after a further 24 - 48 hours, it moults to the third instar. The third instar is a voracious feeder and rapidly increases in size and weight over 3 - 4 days. Development times are strongly influenced by temperature and some blowfly species develop quicker than others. Blowfly larvae tend to develop faster when reared in groups and their development rate increases with larval density (Ireland & Turner, 2006). Once a third instar larva has developed sufficiently, it empties its gut and leaves the corpse in search of somewhere to pupate and emerges as adult flies (Gomes *et al*., 2006).

The blow fly life cycle has six parts: the egg, three larval stages, the pupa, and adult.

130 hours

Pupa

3rd Instar

22 hours

At 70 degrees F, each stage in a blow fly's life takes a known amount of time to complete...

143 hours
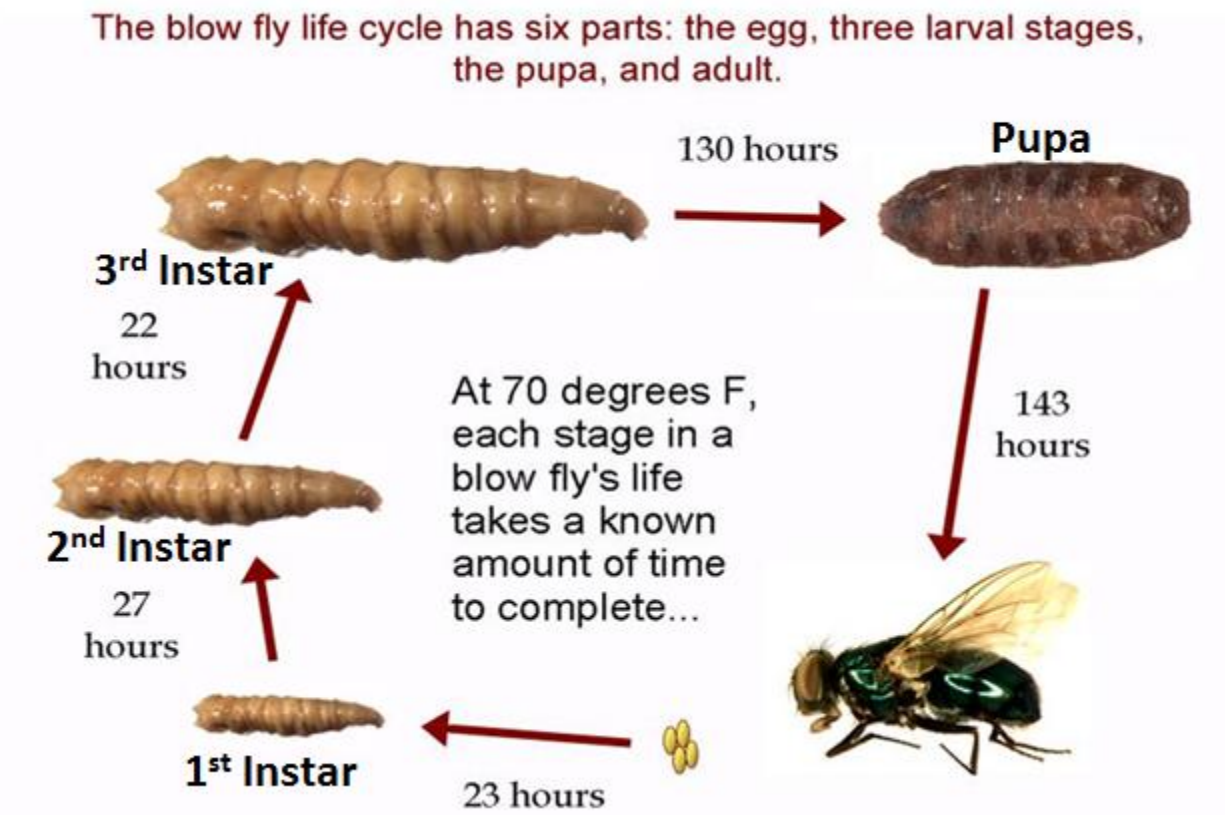
2nd Instar

27 hours

1st Instar

23 hours

Figure 2: The life cycle of a typical blowfly (Extracted from Rognes, 1991).

STR profiling and gut content analysis has proven to be extremely useful in forensic investigation. Campobasso *et al*., (2005) pointed out that the feeding stage is usually needed for the successful isolation of host DNA from the alimentary tract of the larvae. However, Carvalho *et al*., (2005) reported isolation of 197 and 87 base pairs fragments of host sheep DNA (satellite I region) in all immature stages, including 2-days old pupae of *Calliphora dubia*.

Linville *et al* (2004) collected eggs of *Calliphora vicina* flies and reared them on human spleen. After 4 days of development, maggots were collected. STR analysis of human DNA was performed and was successful on all crop extractions. Crop content analysis may be useful in identifying a missing corpse that has been removed from a crime scene, or answering questions about whether a maggot has fed on multiple food sources (Wells *et al*., 2001). Zehner *et al* (2004) collected feeding third instar larvae from 13 corpses during autopsy and STR analysis of human DNA from maggot crop contents was successful in 9 cases. In 4 cases STR typing failed which was attributed to biochemical alterations of digestion process leading to further degradation of DNA or too low amount of DNA (Zehner *et al*., 2004).

1.2.4. Larvae handling

Changes in the cephaloskeleton and morphology of the posterior spiracles between instars provide an indication of the stage of larval development. The collection method and storage may result in the maggot shrinkage, however the amount of shrinkage varies with the method used and the species and size of the maggot. Shrinkage could result in their age being underestimated and also compromising the crop removal as well as the extraction and stability of DNA (Gunn A, 2009). Maggots should therefore be carefully collected and measured before they are placed in a preservative to ensure that subsequent analysis and integrity of DNA is not compromised (Adams and Hall, 2003).

1.2.5. Rearing blowfly maggots

Sometimes it is difficult to identify the eggs and larval stage of many Diptera, therefore a representative sample should be reared to adulthood to confirm identification. Since species may have different digestion rate, this may affect analysis of their gut content (Archer and Ranson, 2005). Maggots should be reared on liver or minced meat surrounded by a layer of dry, friable material like sawdust into which the maggots can move for pupation (Sherman and Tran, 1995).

1.2.6. Killing methods for soft bodied invertebrates

Near-boiling water provides a quick and effective method of killing for all invertebrates (Fig. 3). Once dead, the specimen should be dried and preserved and dried immediately to prevent swelling. Maggots that are killed and left in water after they have died also melanize very quickly, and this may obscure some morphological features and thereby making it difficult for crop removal (Gunn A, 2009). A very important requirement for recovering vertebrate DNA from a live insect is to halt the digestion process as soon as possible by killing and preserving the specimens to avoid further degradation and recover sufficient amount of DNA (Wells and Stevens, 2008).

Figure 3: The size and shape of maggots killed differently. The lower maggot was killed in near-boiling water whilst the upper, contracted, maggot was placed directly into 70% v/v ethanol (Gunn A, 2009).

1.2.7. Preservation of soft-bodied invertebrates

Soft-bodied invertebrates, such as maggots, should be killed before placing them in preservative, otherwise they will shrink and their morphological features become obscured. A typical procedure would be to dehydrate them through increasing alcohol concentrations (30 - 80%) before storing in acetic alcohol (Gunn A, 2009). Preservation of entomological evidence is even more important if DNA analysis of the maggot crop is to be attempted, because some methods of preservation that are suitable for storing maggots for microscopic examination may not be suitable for keeping DNA intact (Linville *et al.*, 2004). Formaldehyde and formalin-fixation preservations have been reported to cause DNA degradation and reduce efficiency of DNA extraction respectively (Toduka *et al.*, 1990; Shedlock *et al.*, 1997).

1.2.8. DNA analysis for human identification

STRs have widely been used in identification of human, mostly in criminal cases (Hagelberg *et al*., 1991; Wiegand *et al*., 1993; Monaghan and Newhall, 1996) and in mass disasters (Olaisen *et al*., 1997; Clayton *et al*., 1995). Substrates for the analysis in such cases may be decomposing bodies (Whitaker *et al*., 1995). Even under extreme conditions of biological samples STR typing has been successful during forensic investigations (Clayton *et al*., 1995; Whitaker *et al*., 1995; Gill *et al*., 1994). Dead bodies and parts collected from the crime scene one week, after the 1996 Spitsbergen aircraft disaster could still be analysed. All bodies which had reference samples were identified and sorting of body parts based on DNA was not hampered (Olaisen *et al*., 1997). It has been demonstrated that human microsatellite DNA extracted from the digestive tract of carrion feeding larvae that fed on decomposing bodies with a PMI of four months can be analyzed, and the STR profiles that were generated from the corpse and the profiles of the corresponding maggots' crop contents matched (Zehner *et al*., 2004b). It was demonstrated that even if a dead body is not present there is possibility of finding maggots, and that maggots may crawl away from a dead body as a result of physical disturbance, upon exhausting bodies' soft tissues, or if mature and enter the post feeding period (Wells *et al* 2001). The list of sources of DNA evidence has also been expanded by showing that analysis of maggot crop contents for STR and mitochondrial DNA may be used in associating a maggot with a specific corpse, even when there has been no observation of physical contact between the corpse and a maggot (Wells *et al*., 2001; Li *et al*.,2011). It was also demonstrated that it is possible to obtain data from maggots found at a crime scene involving death of human and attributes unsuccessful STR analysis to either DNA degradation or limited amounts of targeted templates (Zehner *et al*.,

2004). He thus proposes analysis of human mitochondrial DNA as an alternative given its high copy number.

1.3. PROBLEM STATEMENT

In the current times DNA analysis is applied in forensic science to identify human remains that are found at crime scenes (Jobling *et al*., 2004; Butler, 2005). Although any biological material is a probable source of DNA for analysis, the success rate of DNA typing frequently depends on the nature and quality of the sample. Previous studies have shown that in the past, samples that were used for DNA-typing were those that had high chances of providing relatively large amounts of intact DNA and yielding a full DNA profile. However, in crime scenes trace amount of evidence samples with limited amounts of biological material are increasingly common. Although there has been an increase in success rate for DNA analysis from these trace samples some samples still do not yield adequate DNA amount for analysis, and even if DNA yields appear high, the DNA may be degraded to the point where amplification is impossible (Zehner *et al*., 2004b). Depending on the degree of degradation, analysis of human STR loci from degraded tissue has been shown to result in partial or no DNA profiles (Rerkamnuaychoke *et al*., 2000; Hoff-olsen *et al*., 2001). For forensic purposes, DNA analyses of maggot crop contents provide an alternative way to identify the corpse a maggot has been feeding on. It is still unknown for how long a maggot's gut-content DNA can be viable for analysis if they are removed from the food source and/or are placed on a new source of food.

1.4. JUSTIFICATION

Importance of forensic entomology becomes inevitable when one comes across some incident where a corpse is unidentifiable and alot of maggots or other insects are present. Variety of widely practised molecular genotyping methods helps in forensic investigation, the most frequently used being analysis of specimen DNA, but analysis of insect gut contents is also used. When a human corpse is in an advanced stage of decomposition, analysis of DNA recovered from a degraded tissue and subsequent identification of the individual is a challenge. There are situations when investigators discover maggots but no corpse or maggots are not physically on a corpse, and there is anearby alternative food source. Analysis of maggot crops has to be probative though, otherwise one may spend a lot of time analysing all maggots to determine whether they fed on a human corpse. It would be necessary to carry out additional legal investigation incase of a human corpse. This study seeks to investigate the posibillity of human microsatellites analysis of DNA present in the crop of the maggots that fed on a decomposing body. It also seek to investigate the turnover and degradation of human DNA after removal of maggots from the food source and/or placed on a new source of food.

1.5. OBJECTIVES

1.5.1. General objective

To investigate the viability of using secondary diet in assessing accuracy and window of time for successful analyses of human DNA in maggots crop following removal of corpse.

1.5.2. Specific objectives

i. To invesigate the turnover and degradation of human DNA after the maggots'removal from the primary food source.

ii. To invesigate the turnover and degradation of human DNA after maggots' placement on a secondary(new) food source.

iii. To evaluate the viability of various DNA isalates for STR profilling using specific primers.

2. CHAPTER TWO: MATERIALS AND METHODS

2.1. Maggot and tissue sample collection

Third instar maggots were collected by hand with glooves from three different human corpses at the City Mortuary Nairobi Kenya, prior to disposal of the corpse. The species of maggots were identified using morphological features. Near-boiling water was used to kill the maggots before storage in 70% ethanol at room temperature prior to analysis. Small pieces of finger nails were cut from each human corpse at the initial stage of the experiment and stored at $-20^{o}C$ for extraction of reference DNA sample. Maggots crops were visible from the exterior at collection time and they were close to maximum size. All the three corpses showed marked signs of putrefaction ranging from released rigor mortis to advanced decay.

2.2. Experimental design

Three experimental blocks were designed as follows: Ten maggots from each corpse were immediately killed in near-boiling water and separated. These served as controls. The maggots that remained from each corpse were divided into twelve groups of ten maggots each and placed in separate one litre containers. Six groups were allowed to starve and the remaining six fed on protein rich food (beef) in a rearing jar. One group from each condition were removed for dissection after 2, 4, 6, 8, and10 days, killed in near-boiling water and preserved in 70% ethanol prior to analysis. Twenty maggots were also fed on beef until they emerged into insecs for further identification purposes.

2.3. Maggot dissection and crop extraction

Before dissection, The length of each maggot was measured in centimeters. Each maggot was then rinsed for 20 minutes by storing in a tube containing 1mL of 20% bleach to remove potential external contaminants (Linville and Wells. 2002). Each maggot was then rinsed with 1mL of cold distilled water (Linville *et al*., 2004). Each clean maggot was then dissected according to the method described by Livinille and Well (2002). Briefly posterior segments were cut using an iris scissors followed by a ventral incission to the maggots' anterior. The crops were removed with foreceps. In cases where crop removal was difficult, the entire maggot was homogenized. After every dissection the instuments were cleaned with warm distilled water and then sterilized with a flame. Every dissection was done in new a clean sterilized petri dish, and the crop placed into a separate sterile 1.5mL tube.

2.4. Isolation of total DNA from nail clippings of human corpses.

This was carried out using QIAamp DNA Investigator Kit (Qiagen) according to the manufacturer's instructions. Briefly, 300 µl ATL buffer and 20µl each of proteinase K and 1M DTT were added into a 1.5 ml micro-centrifuge tube. Human nails cut into small pieces, were added and mixed by pulse-vortexing for 10s, incubated at 56^0C with shaking at 900 rpm for 1 hour in a heated orbital incubator. Additional 300 µl of AL buffer was then added to the extraction sample and mixed by pulse-vortexing for 10s. To ensure efficient lyses, the sample and AL buffer were thoroughly mixed to yield a homogeneous solution. A white precipitate was formed when the AL buffer was added to ATL buffer; however this precipitate did not interfere with the QIAamp procedure and dissolved during incubation. The precipitate in the tube was then heated in orbital incubator and incubated at 70^0C with shaking at 900 rpm for 10min.

Absolute ethanol (150µl) was then added to the tube and thoroughly mixed by pulse-vortexing for 15s to ensure efficient DNA binding. The supernatant was transferred to the QIAamp MinElute column and centrifuged for 1 min at 6,000 ×g. QIAamp MinElute column was then placed in a clean 2 ml collection tube and the one containing the flow-through discarded. The column was then opened and 500 µl buffer AWV1 added, lid closed and the mixture centrifuged for 1 minute at 6,000 ×g. Above step was repeated with 700µl of buffer AW2 and 700 µl of absolute ethanol. The membrane was dried completely by placing the column in a clean 2ml collection tube and centrifuging at 20,000 ×g for 3 minutes to remove residual ethanol. QIAamp MinElute column was then placed in 1.5ml micro-centrifuge tube with the lid open and the column incubated at 56^0C for 3 minutes. Fifty microlitres ATE buffer was applied to the center of the membrane. The lid was closed and incubated at room temperature for 1 minute then centrifuged for 1 minute at 20,000 ×g and the elute stored at -20^0C till use.

2.5. Maggot DNA extraction

Maggot DNA was extracted using Zymo Kit (Zymo research, U.S.A) according to manufacturers' instructions. Briefly, maggots were homogenized in a micro centrifuge tube and solution of 95 ul of water, 95ul of 2X digest buffer and 10ul of proteinase K added to the tissue then mixed by vortexing and incubated at 55^0C overnight. Genomic lysis buffer (700 µl) was then added and mixed thoroughly by vortexing then centrifuged for one minute at $10,000 \times g$ to remove insoluble debris. Supernatant was transferred to a Zymo-Spin[TM] IIC column mounted in a collection tube and again centrifuged for one minute at 10,000 ×g. Genomic DNA wash buffer (400 µl) was added to the spin column and contents centrifuged again at 10,000 ×g for one minute. The spin column was again transferred to a clean micro-centrifuge tube and 50µl DNA elution buffer added, incubated at room temperature for five minutes and then centrifuged at

20,000 ×g for 30 seconds to elute DNA. The DNA eluted was quantified and then stored at -20^{0}C.

2.6. Quantitation and PCR Amplification using AmpFlSTR®Identifiler®Plus

Quantitation of DNA was performed using Quantifiler Human DNA Quantification kit (Applied Biosystems) and PCR amplification carried out using 7500 Real Time PCR (Appplied Biosystems) instrument following the manufacturer's protocol. For amplification, the volume AmpFlSTR® Identifiler® Plus master mix used was10ul while that of AmpFlSTR® Identifiler® Plus Primer set used was 5ul per reaction respectively. The two reagent solutions were thawed and vortexed for 3 seconds and centrifuged briefly before opening the tubes. The required volume of the components was pipetted into an appropriately sized polypropylene tube, vortexed for three seconds and centrifuged briefly 15µl of the reaction mix was then dispensed into each reaction well of Microamp® optical 96-well Reaction plate which was then sealed with MicroAmp® clear adhesive film.

For PCR, three DNA sample preparations were made: Negative control containing 10µl of low TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0): Sample template containing a dilute portion of the test DNA sample with low TE buffer so that 1.0 µl of total DNA is in a final volume of 10µl. The diluted sample (10µl) was then added to the reaction mix. Lastly, positive control was prepared containing 10µl of 9947A DNA. The reaction plate was then vortexed for 3 seconds and the tubes centrifuged at 2250×g for 20 seconds. Later the sample was placed in GeneAmp ® PCR system 9700 with the silver 96-well block (Applied Biosystems). Twenty-nine cycles were used to amplify the DNA of the maggot crops content and human reference sample using QIAamp DNA Investigator Kit (Qiagen) according to the manufacturer's instructions. The PCR conditions for amplifications of STRs were as follow: Initial denaturation at 94^{0}C for 5 minutes,

Denaturation at 94^0 C for 1 minute, Annealing at 55^0 C for 1 minute, Extension at 72^0 C for 2 minutes, final extension at 72^0 C for 7 minutes and pause at 4^0 C.

2.7. Electrophoresis

Samples for electrophoresis on the ABI 3130 Genetic analyzer (Applied Biosystems) instrument were prepared immediately before loading. The volume of Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard needed to prepare the samples was then calculated using the table below

Table 1: volume of Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard

Reagent	Volume per reaction
GeneScan™ 500 LIZ® Size Standard	0.3µl
Hi-Di™ Formamide	8.7µl

The required volumes of components were then pipetted into an appropriately sized polypropylene tube, vortexed and centrifuged briefly. Into each well of a MicroAmp® Optical 96-Well Reaction Plate, 9µl of the formamide: size standard mixture and 1µl of PCR product were added. The reaction plate was sealed with appropriate septa, and centrifuged briefly to ensure that the contents of each well were deposited at the bottom. Reaction plate was then heated in a thermal cycler for 3 minutes at 95°C and immediately placed on ice for 3 minutes followed by preparation of the plate assembly on the autosampler and electrophoresis.

2.8. STR Analyses

Analysis of STRs was performed on all extractions of maggot crops content on human corpse and reference samples. Amplification of all samples was performed using Applied Biosystems Identifiler Plus System following manufacturer's protocol. Analysis of data was then performed using Genemapper ID-3.2 Software (Applied Biosystems Foster City CA).

3.0. CHAPTER THREE

RESULTS

3.1. Insect Identification

The maggots were collected from corpses prior to disposal. The length of the 3rd instar larvae was between 1 - 1.7cm (Figure 4A) and the crop was visible as a dark coloration on the larvae body. With the aid of morphological keys (Zhao *et al.*, 2010; Wang *et al.*, 2002a & b), all the 3rd instar larvae and pupae were found to be *Protophormia terraenovae* (Diptera: Calliphoridae). Some of the maggots were allowed to pupate and emerge as insects for further identification (Figure 4C). During rearing in a rearing jar, the maggots colonized a specific location and formed a mass to generate heat for their growth as seen in Figure 4B.

Figure 4: Larval characteristics and insect identification.

(A) Third instar larvae 1.7cm long (B) Maggots feeding on beef, colonized and formed mass to generate heat for their growth, (C) Insects emerged from maggots confirmed to be *Protophormia terraenovae* at day 14.

3.2. DNA Concentration

Quantification of DNA extracts from the crop showed that the recovered amount of DNA decreased over time in all cases. Controls had the highest amount of human DNA, which reduced with time due to DNA degradation by nucleases. Maggots collected from reference one (REF 1) gave the best concentrations and they were used for generating STR profiles. For maggots starved for four days the concentration of human DNA was too low (<0.05ng/ul) but was still amplified.

Table 2: Average Human DNA concentrations from different sample types after amplification in real time PCR

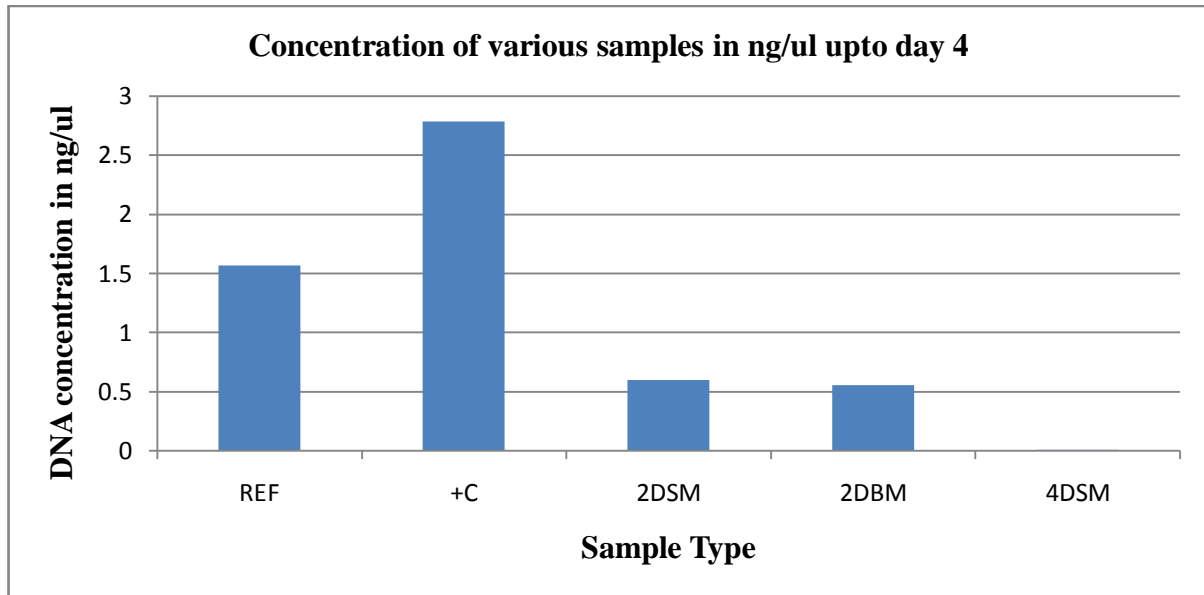| Days	Reference ng/ul	Control ng/ul	Starved ng/ul	Beef Fed ng/ul
0	1.56	2.78	-	-
2	-	-	0.6	0.41
4	-	-	0.01	0
5	-	-	0	0
6	-	-	0	0
8	-	-	0	0
10	-	-	0	0

Figure 5: Mean DNA concentration from maggots gut content at different day intervals
Average concentration of human DNA from human tissue and maggots gut content, the concentration of human DNA decreased with time from their last meal. Amount of human DNA recovered from maggot's crops content decreased from time of collection up to day four when they were starved but decreased up to day two after they were fed on beef. REF=reference sample, +C= control group, 2DSM=2 days starved maggots, 2DBM=2 days beef fed maggots, 4DSM=4 days starved maggots. Relative concentrations show that control had the highest amount of human DNA, while 4DSM had the least amount. In general the amount of extracted human DNA decreased from Day 1 to Day 4. No human DNA was detected in the maggot crops after Day 5 through Day10.

3.3. STR Analyses

STR genotyping of the reference sample maggots' crops content was successful and complete STR profiles (16 loci) were generated from most specimens. Profiles from the maggots' crop contents were the same and matched with the ones from the human nail clippings completely (Fig.6). The amelogenin loci showed that REF 1 was a male and upon genotyping of DNA extracted from nail clipping generated complete STR profiles. The internal control also generated complete profiles (Fig.7). The human DNA extracted from maggots that were sacrificed immediately after collection and maggots that had been starved for two days also generated complete profiles.
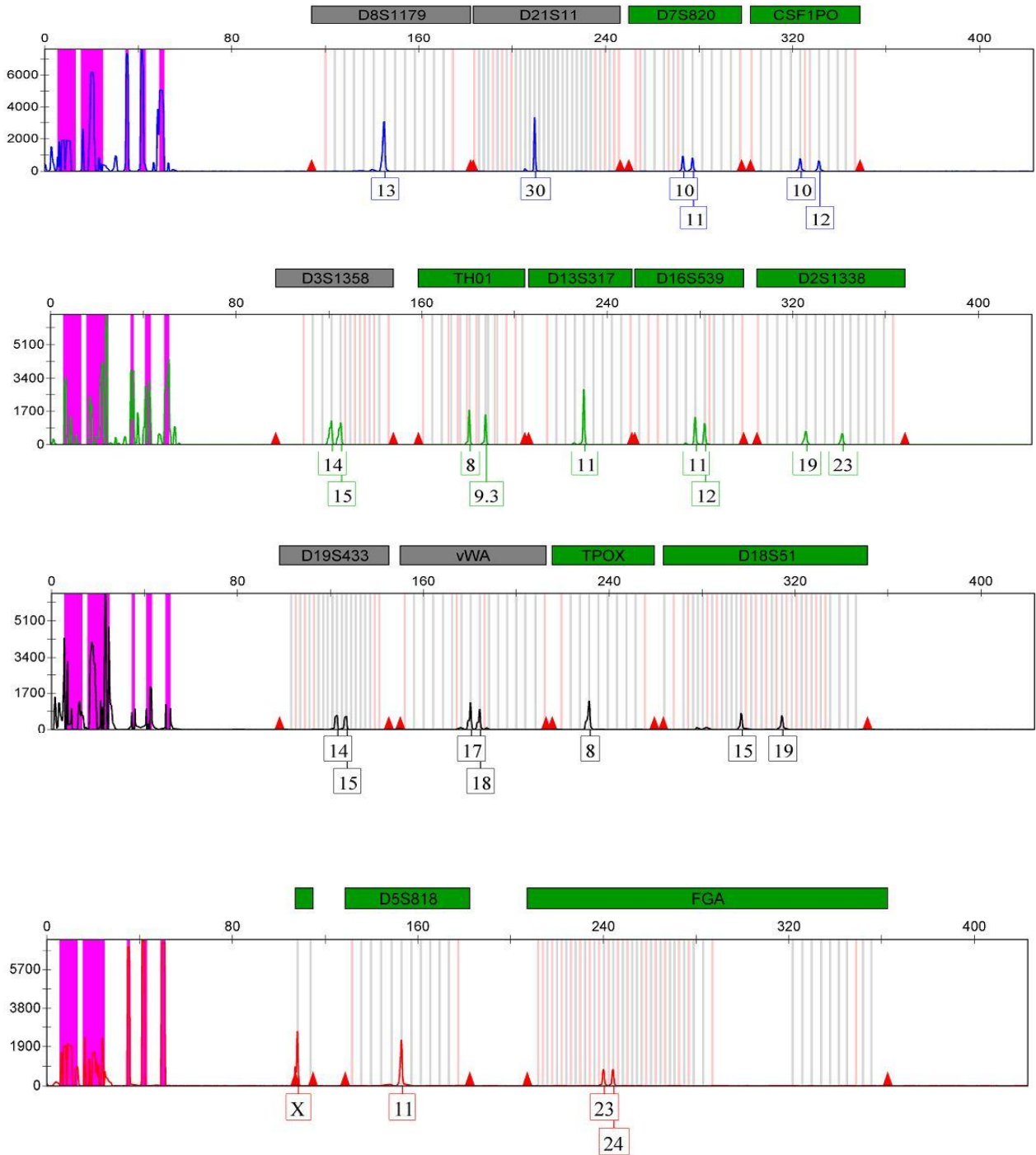
3.4. Figures 6-12 present STR profiles of the various samples

**Figure 6:** STR profiles of human reference 1 sample
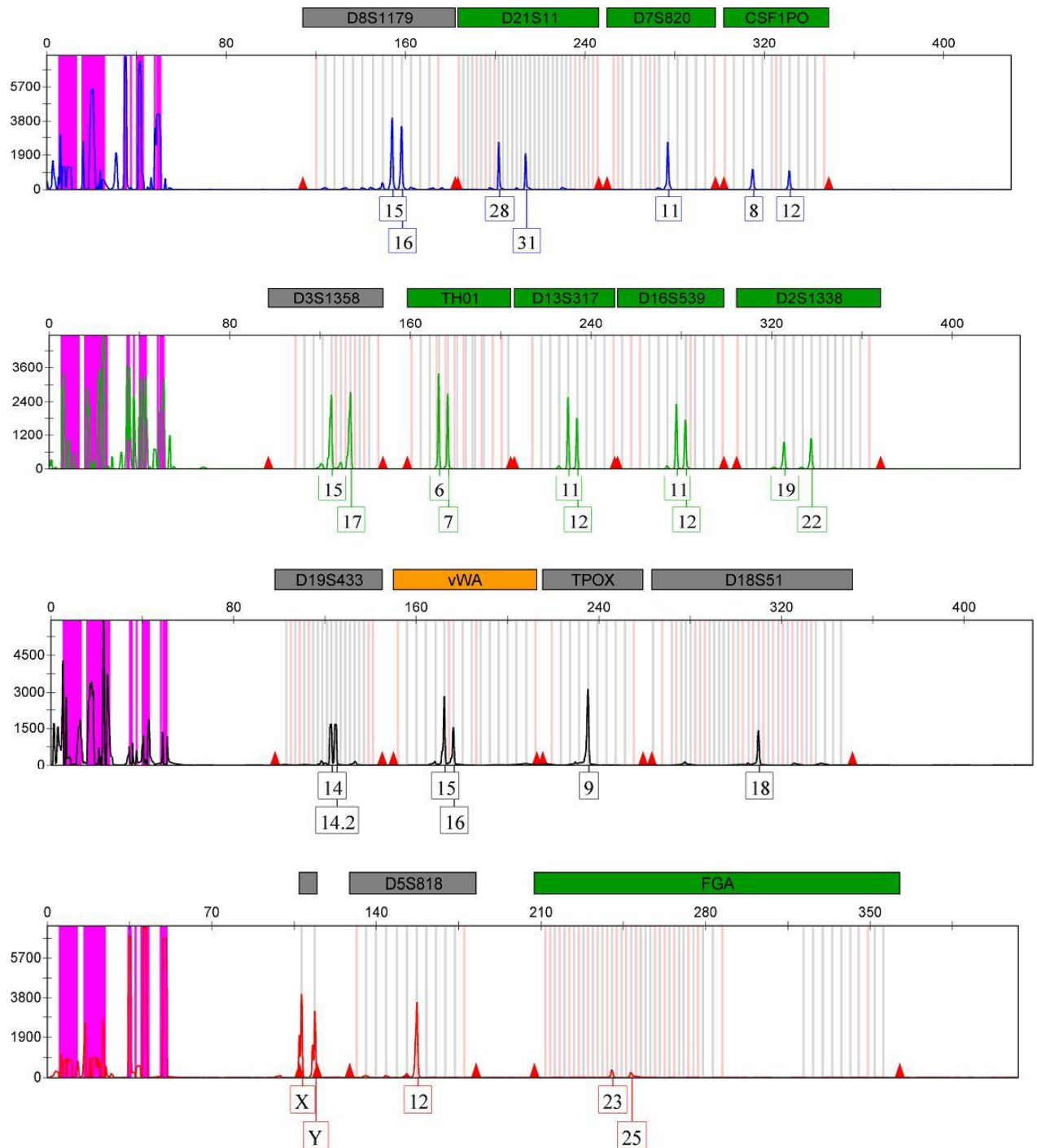
The STR profiles of reference sample DNA using 16 human genetic loci, and genotyping

generated complete profile although at loci FGA peaks were too low.

**Figure 7:** STR profile of internal positive control (9947A) DNA,

The STR profile of internal positive control using 16 human genetic loci, and genotyping

generated complete profile.

**Figure 8:** STR profile of DNA from a negative control
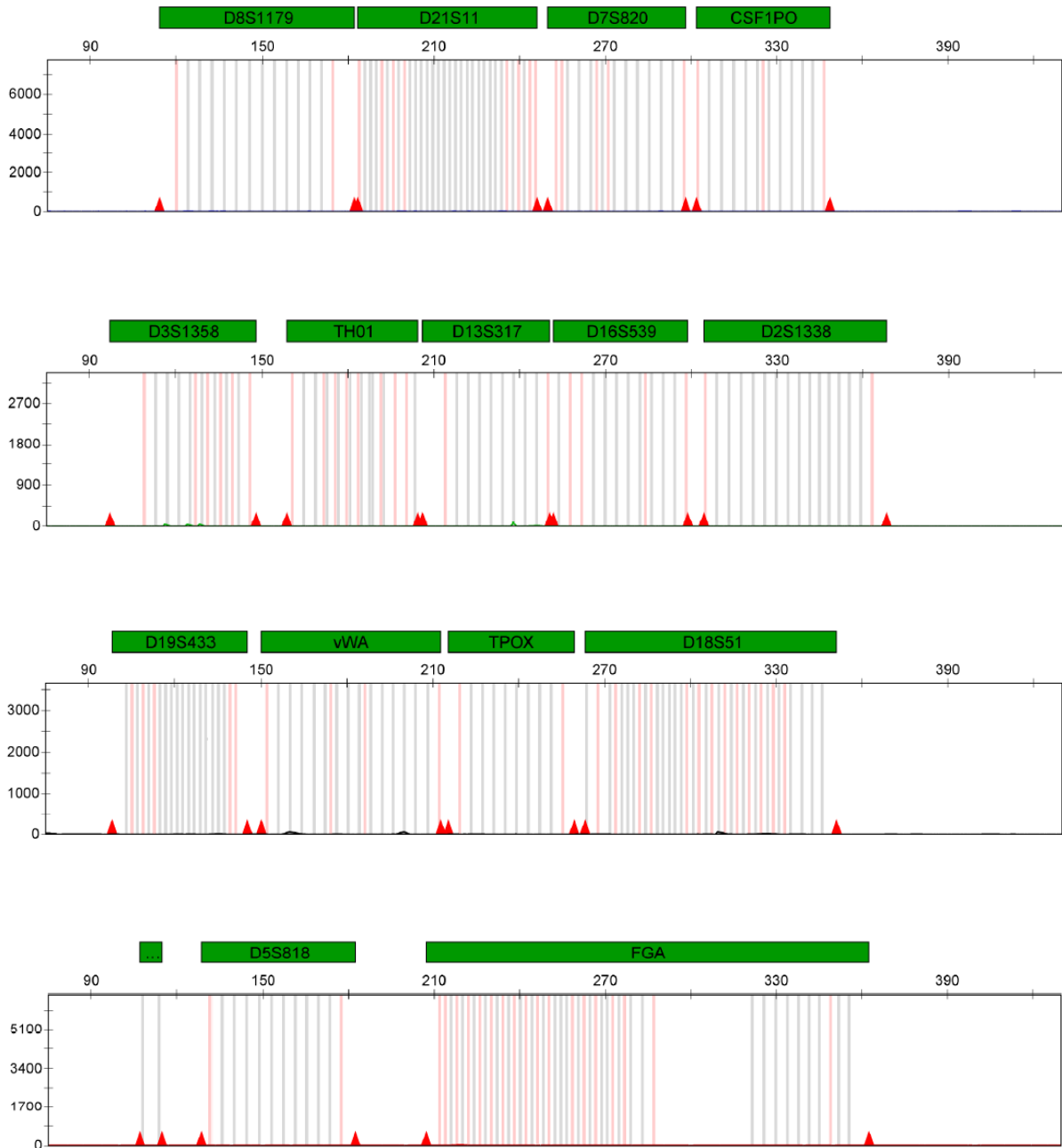
The STR profiles of DNA from negative control using16 human genetic loci.

Figure 9: STR profile of human DNA extracted from gut content of 2 days starved maggots.
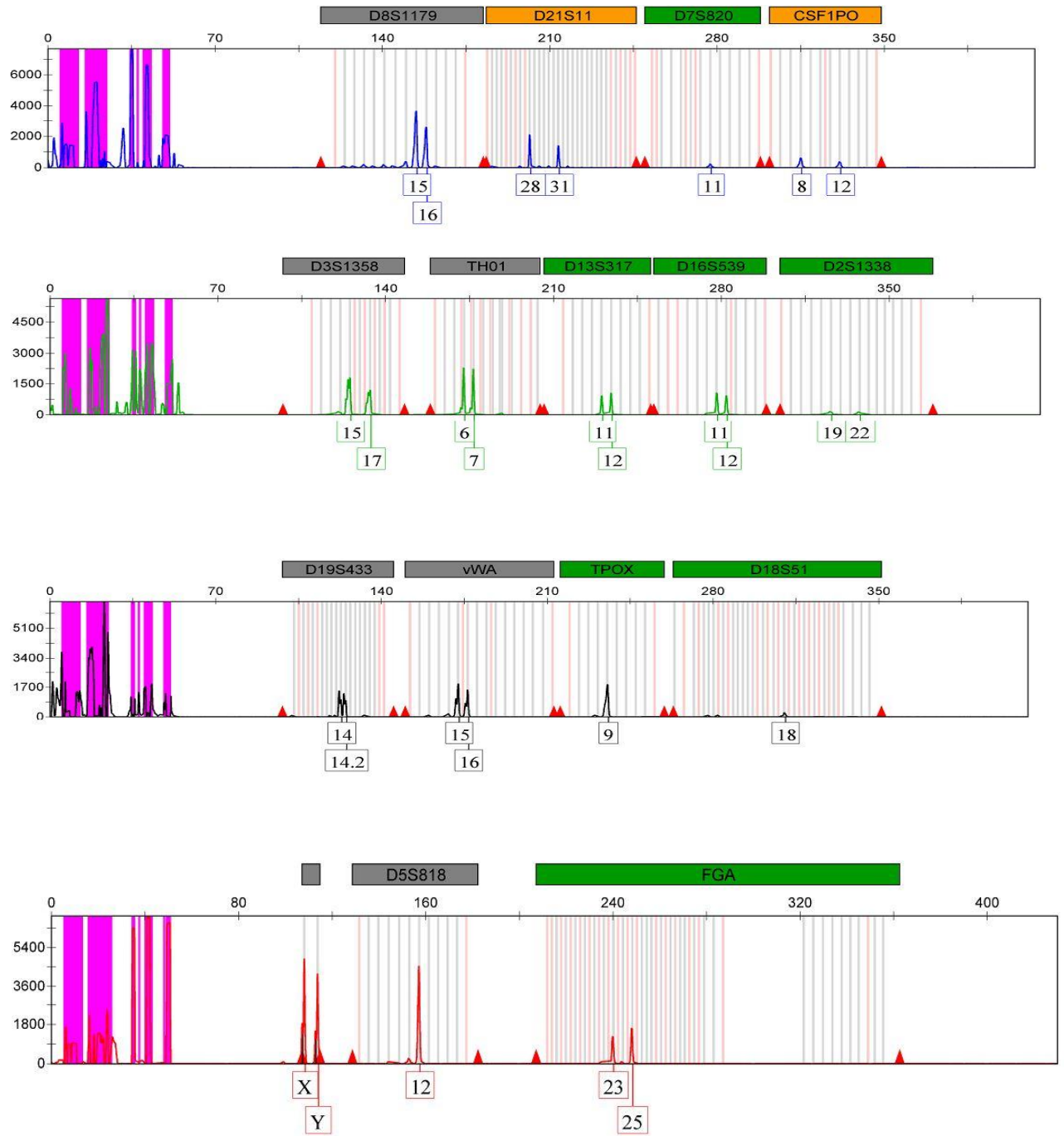
The STR profile of human DNA extracted from gut contents of 2 days starved maggots using 16 human genetic loci. The genotyping generated a complete profile.

Figure 10: STR profile of human DNA extracted from gut content of 2 days beef fed maggots
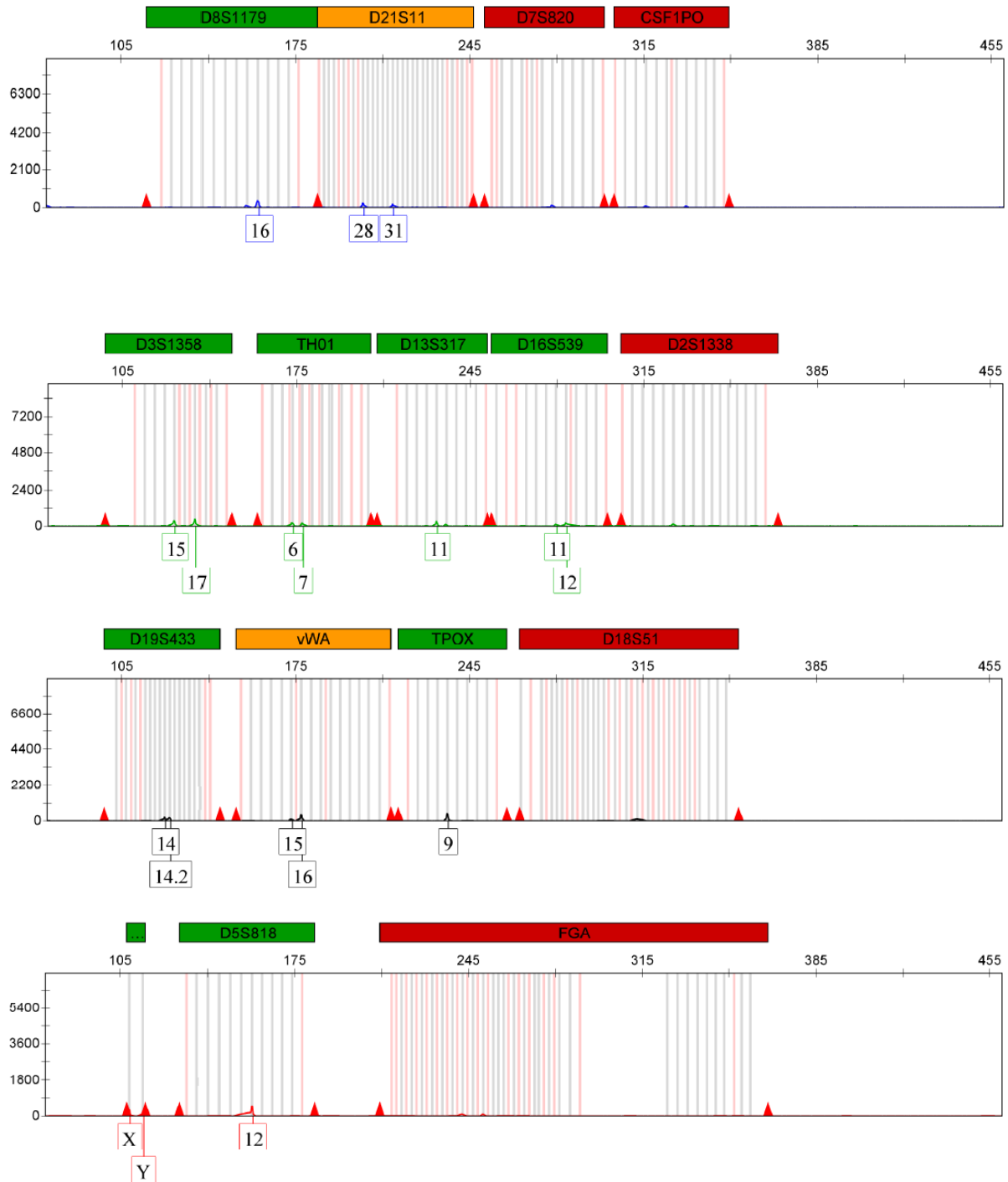
The STR profile of human DNA extracted from gut contents of 2 days beef fed maggots using 16 human genetic loci. Genotyping generated incomplete profile most loci generated very low peaks but they still matched those of reference sample.

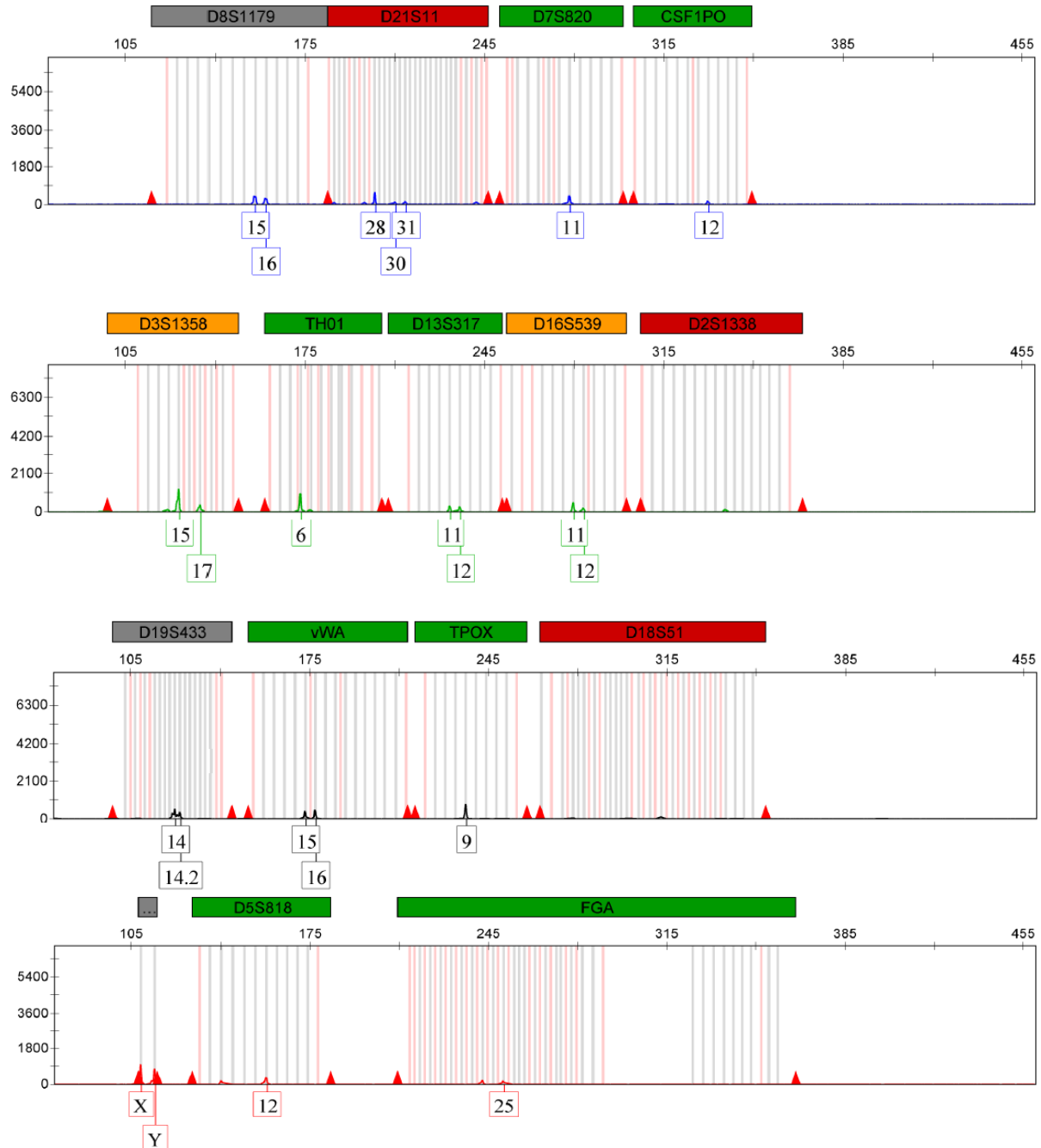**Figure 11: STR profile of human DNA extracted from gut content of 4 days starved maggots.**
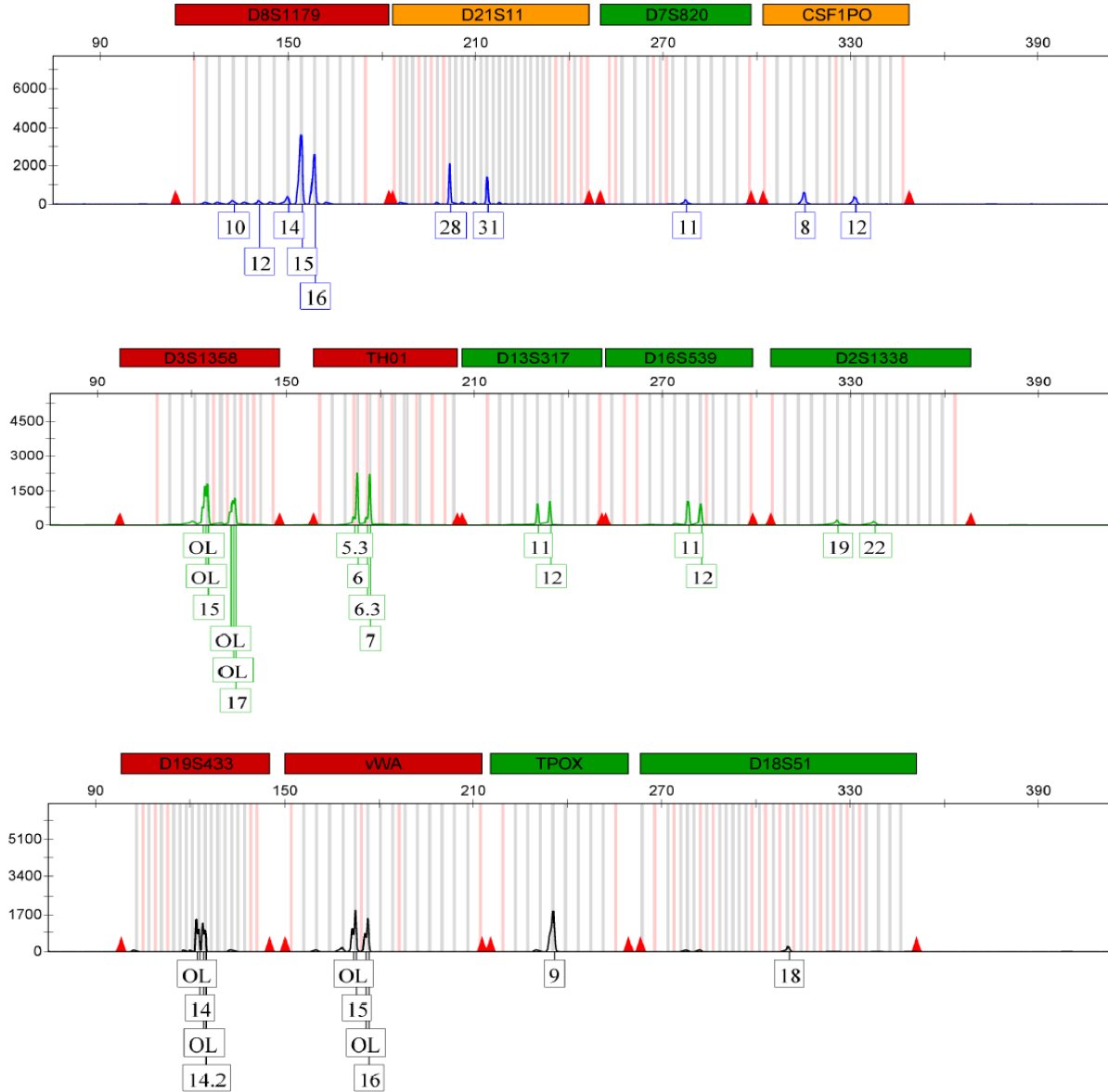

The STR profile of human DNA extracted from gut contents of 4 days starved maggots using 16 human genetic loci. Genotyping generated incomplete profile most loci generated very low peaks but they still matched those of reference sample.

Figure 12: STR profile of human DNA extracted from gut content of maggots killed immediately after collection.
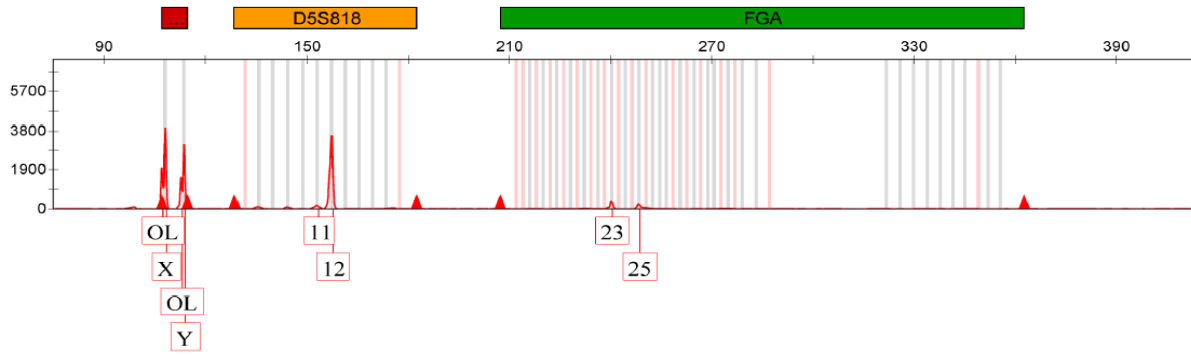
The STR profile of human DNA extracted from gut content of maggots collected and immediately killed to serve as control. The profiles were generated using 16 human genetic loci. Genotyping generated few off ladder (OL) and mixed peaks in 6 of the loci: D8S1178, D3S1358, TH01, D19S433, vWA and Amelogenin.

4. CHAPTER FOUR: DISCUSSION

Although a lot of research has been done on the value of molecular genotyping techniques, for forensic specimen identification purposes, information on identification and characterization of population genetic structure of important insects in forensics and gut contents of insects need to be expanded (Wells & Stevens, 2008). It has been demonstrated that analysis of insect gut contents, including those of the lice (Mumcuoglu *et al*., 2004) mosquito, (Mukabana *et al*., 2002), and carrion feeding flies (Zehner *et al*., 2004a) have a genetic potential for use in forensics. The maggots' gut contents analysis has proven to be suitable for all of the genetic procedures in a typical human identity process (Wells &Stevens, 2008; Well *et al*., 2001; Linville *et al*., 2004; Zehner *et al*., 2004a), since gut contents provide similar material for associating with its last food (Wells *et al*., 2001).The current study was therefore motivated by the fact that the amount of time that a maggot's gut-content DNA of *Protophormia terraenovae* can be viable for analysis after it is removed from the food source and/or is placed on alternative/new food type is still unknown.

Although variety of taxa represented in gut content literature is limited mostly blowfly species have been examined (Linville *et al*., 2004; Linville and Wells, 2002; Zehner *et al*., 2004b). Accurate identification of species of an insect specimen is usually a crucial first step in a forensic entomological analysis and has been reported that insect larvae differ in growth rates and biology. Larvae of *Lucilia sericata*, for instance, grow faster at 25^0 C than larvae of *Calliphora vicina* (Amendt *et al*., 2004). Other studies have shown that the host *Protophormia terraenovae* needs about 9 days at the above temperature to reach the stage appropriate for the parasitoid's oviposition (Marchenko 2001; Grassberger and Reiter 2002a).

The maggots collected for the study were identified as those from the northern blowfly (*Protophormia terraenovae*) using their morphological features. Confirmation was however achieved by the identity of the emerging insect upon the maggots' pupation as seen in Figure 4C. During rearing, the maggots were seen to colonize a particular location forming a mass (Fig. 4B), which is essential for generation of heat for their growth. After a few days they moved away from the food source to nearby sawdust, placed for pupation. Maggots collected were then preserved in 70% ethanol and human DNA isolated from their gut contents.

Ethanol has the capacity to dehydrate specimens and denature nuclease enzymes, hence often recommended as a preservative (Dessauer *et al.*, 1996). DNA recovery from the crops did not seem to be greatly affected by the duration of time (2 weeks) at which maggots were stored in ethanol, as evidenced in both quantification method and genotyping profiles. Although there was moderate success in amplification of DNA throughout this study, the actual dissection and crop removal from maggots that were preserved in 70% ethanol was challenging and for that reason it was necessary to homogenize the whole maggot. However, the recovery of DNA did not appear to directly be affected by the slight variations in the sample preparation processes.

From this study, it was hypothesized that DNA present in the gut contents of maggots would generate human DNA profiles upon analysis. This was thought to be true even after the maggot moved from its primary food source. STR analysis of DNA obtained from contents of maggot crop may potentially be used in associating maggots with a particular corpse. The data presented in this work demonstrates that by use of standard DNA profiling protocols and amplification conditions, DNA can be detected in crop contents of maggots resulting in STR profiles which

when compared to human STR profiles can be used in successful identification of a decomposing human body. Body 1 was more decomposed as compared to bodies 2 and 3, however, DNA content was higher from Maggots collected from it than the other two, this can be attributed to the larger sized maggots obtained from the body which provided for larger crop content hence more DNA thereby improving the chances of successful STR typing.

From the data (Table 2) obtained in this study, DNA was successfully extracted from starved maggot crops up to the fourth day and only up to the second day for the group of maggots that were fed on beef, a new food source.

Subjecting the extracted DNA from the maggots in the various groups to STR analysis generated various profiles. These STR profiles were compared with reference sample profiles so as to determine the extent to which STR analysis can be used to identify a decomposing body. In the starved group of maggots, the DNA that was detected in their guts up to the fourth day gave STR profiles similar to those of the control (Figs. 9 & 11). This indicated that maggots collected from a decomposing body are useful for purposes of forensic criminal investigation within a particular time frame. Since DNA obtained from their crop contents can be used in the development of STR profiles that match the DNA profile from the decomposing body, they are therefore of great value in identification of bodies if the generated profiles can be compared to a known reference. Studies have reviewed the potential application of gut content analysis and reported that the source of the gut content of forensically important insects could potentially identify a victim from a maggot left behind when a corpse was moved and a blood-feeding insect at a scene could connect a suspect to that scene if it were found to have fed on the suspect (Campobasso *et al*., 2005).

On the other hand, DNA extracted from the gut contents of the group of maggots obtained from decomposing body, which were subsequently fed on beef could only be detected up to the second day. Such extracted DNA also gave profiles that were similar to the controls for the first two days when DNA could be detected (Fig. 10). This indicates that if maggots from a decomposing body are introduced or move to alternative food source, their usefulness is only limited to two days in identification of bodies. This can be attributed to the fact that the feeding process forces the gut contents to move faster along the alimentary canal away from the gut and since the DNA containing materials used is only localized in the gut, they are moved from the crop to the other parts of the alimentary canal. In general feeding maggots move food contents away from the crop. Other studies have reported that crop length can vary, depending on larval development, as well as on the species; for example, the crop of *Protophormia sericata* empties rapidly during the first day after peak feeding, while the crop of *Calliphora rufifacies* empties gradually and it is believe that the crop size is the main factor affecting the ability to analyze host DNA in maggots and the success of the genetic analysis (Amendt *et al*., 2004).

Some DNA profiles were obtained which were not present in the corresponding reference DNA profiles (Fig. 12: loci D8S1179 and TH01Fig. 11: loci D21S11), the presence of secondary and Off Ladder profiles is however more likely to be due to contamination of DNA in the process of extraction, stutter products, or maggots may also have fed on different bodies given that the three bodies were stored a few centimeters from each other.

The corpse nail clipping and controls yielded complete profiles (Fig. 6 and 7). In most cases, STR typing of the maggot crops content (approximately 75%) was successful. In control cases and two days starved maggots complete STR profiles (i.e., 16 loci) were generated. In two cases,

two days beef fed maggots and four days starved maggots, incomplete STR profiles (<16 loci and/or allelic drop-out) were generated (Fig. 10 & 11) but they still matched those of the reference sample. From figure 10 the following loci did not generate peak; D7S820, CSF1P0, D2S1338, D18S51 and FGA, and from figure 11 D2S1338 and D18S51. Although some of the peak heights were low due to low concentration and degradation of DNA, they still matched those of the reference sample. STR profiles obtained from the crop content matched the profile of the corresponding corpse in all cases. STR analysis is the standard for the determination of human nuclear DNA profile and countries around the world have adopted a somewhat overlapping set of standard STR loci for human identity testing (Butler, 2005), and recent gut content studies have used STR loci as markers (Zehner *et al*., 2004a). In general, profiles from the maggots' crop contents were the same and matched with the ones from the human nail clipping completely although some of the samples generated partial profiles. Studies have reported that STR analysis was successful for maggots 2.5– 4.5 days (fully third-instar larvae) while young and post feeding individuals failed to produce a STR genotype at any locus. It was believed that the cause of the failed STR attempts was essentially due to the amount of food stored inside the gut content, as clearly expressed by the crop size (Campobasso *et al*., 2005). In this study there was some dropout in some alleles from sample profiles, which could be attributed to degradation of human DNA isolated from their gut by nucleases.

5. CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS

The results presented in this study demonstrate conclusively that STR analysis of human DNA from maggots can be used in identification of corpse from which maggots has been feeding on. Starved maggots were the best in identification of decomposing bodies and are only useful in body identification up to the 4th day if they are removed from the food source and up to second day if they are placed on a new food source. This study recommends that more research be done on analysis of DNA from the crops of other carrion feeding insect maggots.

Since proteolytic enzymes are not found within the foregut area of the maggot, the site for primary digestion is therefore not within the crop, it mainly acts as a food container. However, saliva of the maggot has enzymes for pre-oral digestion and they are re-incorporated with the food into the crop (Hobson, 1932). Degradation of DNA may therefore occur even within the crop, but the extent of degradation may be limited and vary from species to species. In this study the amount of human DNA recovered from maggots crop decreased with time.

Because different species of carrion feeding insects have different rates of digestion after cessation of ingestion followed by crop emptying, further investigations from other necrophagous insects are necessary to determine the time period during which STR typing of crop extracts can be useful after the maggot had left or had been removed from the corpse. Further study should also be undertaken with a number of maggots greater than 10 in each group, to investigate whether full profiles could be generated. Due to limited number of maggot samples only 10 maggots in each group were used in this study. Other reports have demonstrated that, for

identification of both the insect species and its gut contents, the maggots crops could be an ideal source of DNA and the list of sources of DNA evidence has further been expanded by the evidence that analysis of mtDNA may potentially also be used in associating a maggot with a human corpse, even in the absence of any physical contact between them. To date, there are no documented reports about the application of forensic entomology in legal investigations in Kenya; therefore it is necessary that our courts of law and law enforcement agents start considering entomological data as evidence applicable in solving criminal cases.

Human mtDNA sequences analysis is an alternative method and can also be evaluated. Maggots and insects are common in a crime scene, therefore their careful collection and identification is very useful in management of a crime scene involving death of human beings.

6. REFERENCES

Adams, Z.J.O. and Hall, M.J.R. (2003). Methods used for the killing and preservation of blowfly larvae, and their effect on post-mortem larval length. *Forensic Science International.* 138: 50–61.

Amendt, J., Krettek, R., Zehner, R. (2004). Forensic entomology. Naturwissen schaften 91:51–65.

Anderson, G.S. (2000). Minimum and maximum development rates of some forensically important Calliphoridae (Diptera). *Journal of Forensic Sciences.* 45: 824–832.

Anderson, G.S and Vanlaerhoven S.L. (1996). Initial Study on Insects Succession on Carrion in South-Western British Columbia, *Journal of Forensic Science.* 41:617-625.

Archer, M.S. and Ranson, D.L. (2005). Potential contamination of forensic entomology samples collected in the mortuary: a case report. *Medicine Science and the Law.* 45: 89–91.

Arnaldos, M.I. *et al.* (2005). Estimation of postmortem interval in real cases based onexperimentally obtained entomological evidence. *Forensic Science International* 149:57–65.

Avila, F.W. and Goff, M.L. (1998). Arthropod succession patterns onto burnt carrion intwo contrasting habitats in the Hawaiian Islands. *Journal of Forensic Sciences* 43:581 – 586.

Barreto, M. Burbano, M. A. and Barreto, P. (2002). Flies (Calliphoridae, Muscidae) and beetles (Silphidae) from human cadavers in Cali, Columbia. *Memoires of the Institute of Oswaldo Cruz,* 97 (1): 137 – 138.

Becker, A., Maraun, M., Zehner, R. and Amendt, J. (2007). Evidence of post-mortal relocation of a carcass by analysing its insect infestation – dream or reality? *Proceedings of the Fifth Meeting of the European Association for Forensic Entomology.2nd – 5th May 2007, Brussels, Belgium.*

Benecke, M. (2001). A brief history of forensic entomology. *Forensic Science International,* 120: 2 – 14.

Boakye, D.A., Tang, J., Truc, P., Merriweather, A. andUnnasch, T.R. (1999). Identification of bloodmeals in haematophagousDiptera by cytochrome B heteroduplex analysis. *Medical Veterinary Entomology.* 13: 282-287.

Butler, J. M. (2001). Forensic DNA typing. San Diego: *Academic Press.*

Butler, J.M. (2005). Forensic DNA Typing.Biology and Technology behind STR Marker, second ed*., Academic Press, San Diego USA*, 322.

Byrd, J. H. and Castner, J. L. (2001). Insects of forensic importance. In: Byrd, J. H. and Castner, J. L., (Eds), *Forensic Entomology: The utility of arthropods in legal investigations.* CRC Press. Boca Raton, pgs 43 – 80.

Campobasso, C.P., Linville, J.G., Wells, J.D. andIntrona, F. (2005). Forensic genetic analysis on insect gut contents. *The American Journal of Forensic Medicine and Pathology*. 26: 161–165.

Campobasso, C.P., Linville, J.G., Wells, J.D. & Introna, F. (2005). Forensic Genetic Analysis of Insect Gut Contents. *The American Journal of Forensic Medicine and Pathology.* 26: 161-165.

Catts, E.P. and Goff, M.L. (1992). Forensic entomology in criminal investigations. *Annual Review of Entomology.* 37: 253 – 272.

Carvalho, F, Dadour, I.R., Groth, D.M. & Harvey, M.L. (2005) Isolation and detection of ingested DNA from the immature stages of *Calliphora dubia* (Diptera: Calliphoridae): a forensically important blowfly. *Journal of Forensic Science Medicine and Pathology.* 1: 261–265.

Catts, E. P. and Haskell, N. H. (1990). *Entomology and death: A procedural guide.* Joyce's Print Shop, Clemson, South Carolina, pgs 1 – 180.

Centeno, N., Maldonado, M., and Olivia, A. (2002). Seasonal patterns of arthropods occurring on sheltered and unsheltered pig carcasses in Buenos Aires Province (Argentina). *Forensic Science International*. 126: 63 – 70.

Chapman, R.F. (1980). The Insects Structure and Function. London, Hodder and Stoughton.

Chow-Shaffer, E., Sina B., Hawley, W.A., De Benedictis, J. and Scott, T.W. (2000). Laboratory and field evaluation of polymerase chain reaction-based forensic DNA profiling for use in identification of human blood meal sources of Aedesaegypti (Diptera: Culicidae). *Journal of Medical Entomology.* 37 (4): 492-502.

Chung-Yen Pai., Ming-Cheng Jien., Li-Hong Li., Yen-Yuh Cheng. & Chiou-Herr Yang1. (2007). Application of forensic entomology to postmortem interval determination of a burned human corpse: A homicide case report from southern Taiwa, *Journal of Formos Medical Association.* 106 (9): 792-798.

Clayton,T.M., Whitaker J. P., Maguire C. N. (1995). Identification of bodies from the scene of a mass disaster using DNA amplification of short tandem repeat (STR) loci. *Forensic Science International.* 76: 7–15.

Clery, J.M. (2001). Stability of prostate specific antigen (PSA) and subsequent Y-STR typing of *Lucilia* (*Phaenicia*) *sericata* (Meigen) (Diptera: Calliphoridae) maggots reared from a simulated postmortem sexual assault.*Forensic Science International*. 120: 72–6.

Dadour, I. E., Cook, D. F., Fissioli, J. N., and Bailey, W. J. (2001). Forensic entomology: Application, education and research in Western Australia. *Forensic Science International*. 120: 48 – 52.

Dessauer, H.C., Cole, C.J. and Hafner, M.S. (1996).Collection and storage of tissues.In: Molecular systematics. Hillis DM, Moritz C, Mable BK, editors.Sunderland, MA: Sinauer Associates, Inc 29–50.

Dillon, L. C. and G. S. Anderson. (1995). Forensic entomology: The use of insects in death investigations to determine elapsed time since death, Canadian Police Research Centre, Technical Report TR-05-95, Ottawa, Ontario. Insects Associated with the body

Dillon, N., Austin, A.D. and Bartowsky, E. (1996). Comparison of preservation techniques for DNA extraction from hymenopterous insects. *Insect Molecular Biology*. 5: 21–4.

Dizinno, J.A., Lord W.D., Collins-Morton, M.B., Wilson, M.R. and Goff, M.L. (2002). Mitochondrial DNA sequencing of beetle larvae (*Nitidulidae: Omosita*) recovered from human bone. *Journal Forensic Science*. 47 (6): 1337–9.

Early, M. and M.L. Goff. (1986). Arthropod Succession Pattern in Exposed Carrion in the Island of O'hu,Hawai'I," *Journal of Medical Entomology*. 23: 520-531.

Edwards, A., Civitello, A., Hammond, H.A. and Caskey, C.T. (1991). DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*. 49: 746-56.

Gill, P., Ivanov P. L., Kimpton C., Piercy R.,, Benson N.,, Tully G.,, Evett I.,, Hagelberg E.,, and Sullivan K. (1994). Identification of the remains of the Romanov family by DNA analysis, *Nature Genetics*. 6 (2): 130–135.

Gill P., Whitaker J., Flaxman C., Brown N, and Buckleton J. (2000b). An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Science International.* 112: 17-40.

Goff, M.L. (2000). *A Fly for the Prosecution: How Insect Evidence Helps Solve Crimes*. Harvard University Press.

Gomes, L. *et al*. (2006). A review of post feeding larval dispersal in blowflies: implications for forensic entomology. *Naturwissenschaften.* 93: 207 – 215.

Grassberger M. and Reiter C. (2002a) Effect of temperature on development of the forensically important holarctic blowfly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae). *Forensic Science International*. 128:177–182

Gunn, A. (2009). Essential Forensic Biology, Second Edition, Liverpool John Moores University, Liverpool, UK, p 11-279.

Guo, Y.D., Li, X., Cai, J.F., Xiong, F., Zhang, L., Feng, H., Meng, F.M., Fu, Y., Li, J.B. and Chen, Y.Q. (2011). Mitochondrial DNA and STR analyses for human DNA from maggots crop contents: A forensic entomology case from central-southern China. *Tropical Biomedicine.* 28 (2): 333–338.

Hagelberg, E., Gray I.C. and Jeffreys, J.A. (1991). Identification of the skeletal remains of a murder victim by DNA analysis. *Nature.* 352: 427–429.

Haskell, N.H., Lord, W.D. and Byrd, J.H. (2001). Collection of entomological evidence during death investigations. In: Forensic entomology: the utility of arthropods in legal investigations. Byrd JH, Castner JL, editors. Boca Raton: CRC Press.

Haskell, N.H. and Williams, R.E. (1999). Collection of entomological evidence at the death scene. In: Entomology and death: a procedural guide. Catts EP, Haskell NH, editors. Clemson, SC: Joyce's Print Shop, Inc,

Hobson, R.P. (1932a). Studies on the nutrition of blow-fly larvae II. *Journal of Experimental Biology*. 9: 128–38.

Hobson, RP. (1932b). Studies on the nutrition of blow-fly larvae III. *Journal of Experimental Biology*. 9: 359–65.

Hoff-Olsen, P, *et al.* (2001). Microsatellite stability in human post-mortem tissues, *Forensic Science International*. 119 (3): 273–278.

Holland, M.M. and Parsons, T.J. (1999). Mitochondrial DNA sequence analysis: validation and use for forensic casework. *Forensic Science Review.* 11: 21-50.

Ireland, S. and Turner, B. (2006). The effects of larval crowding and food type on the size and development of the blowfly *Calliphora vomitoria*. *Forensic Science International.* 159: 175–181.

Jobling, M. A., Gill, P. (2004). Encoded evidence: DNA in forensic analysis, *Nature Genetics Review.* 5:739–751.

Kocher, T.D., Thomas, WK, Meyer, A., Edwards, S.V., Pˈaˈabo, S., Villablance, F.X. and Wilson AC. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceeding Nature Academic Science* USA. 86: 6196–200.

Kreike, J. and Kampfer, S. (1999). Isolation and characterization of human DNA from mosquitoes (Culicidae). *International Journal of Legal Medicine*. 112: 380–2.

Li, X., *et al.* (2011).Mitochondrial DNA and STR analyses for human DNA from maggots' crop contents: A forensic entomology case from central-southern China. *Tropical Biomedicine.* 28 (2): 333-338

Linville, J.G. and Wells, J.D. (2002). Surface sterilization of a maggot using bleach does not interfere with mitochondrial DNA analysis of crop contents. *Journal of Forensic Science.* 47 (5): 1055–9.

Linville, J.G., Hayes, J. and Wells, J.D. (2004). Mitochondrial DNA and STR analyses of maggot crop contents: Effect of specimen preservation technique. *Journal of Forensic Sciences.* 49: 341-344.

Lord, W.D., DiZinno, J.A., Wilson, M.R., Budowle, B., Taplin, D. and Meinking T.L. (1998). Isolation, amplification, and sequencing of human mitochondrial DNA obtained from human crab louse, Pthirus Pubis (L.), blood meals. *Journal of Forensic Science.* 43 (5): 1097-1100.

Lutz, S., Weisser, H.J., Heizmann, J. and Pollak, S. (1998). Location and frequency of polymorphic positions in the mtDNA control region of individuals from Germany. *International Journal of Legal Medicine.* 111: 67-77.

Lutz, S., Weisser, H.J., Heizmann, J. and Pollak, S. (1996). mtDNA as a tool for identification of human remains, Identification using mtDNA. *International Journal Legal Medicine.* 109: 205–9.

Marchenko M. J. (2001) Medicolegal relevance of cadaver entomofauna for the determination of time since death. Forensic Science International. 120:89–109

Monaghan, B., Newhall, P. (1996). STR results for both epithelial and spermatozoal fractions obtained from 13 year old microscope slides of vaginal swab material, Can. Soc. *Forensic Scince Journal.* 29: 13–19.

Moretti, T.R., Baumstark, A.L., Defenbaugh, D.A., Keys, K.M., Smerick, J.B. andBudowle, B. (2001). Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. *Journal of Forensic Science.* 46 (3): 647–660.

Morley, J.M., Bark, J.E., Evans, C.E., Perry, J.G., Hewitt, C.A. & Tully, G. (1999).Validation of mitochondrial DNA minisequencing for forensic casework. *International Journal of Legal Medicine.* 112: 241-248.

Mukabana, W.R., Takken, W., Seda, P., Killeen, G.F., Hawley, WA. and Knols, B.G. (2002). Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae). *Bull Entomology Research.* 92: 233–9.

Mumcuoglu, K.Y., Gallili, N., Reshef, A., Brauner, P. and Grant, H. (2004).Use of human lice in forensic entomology. *Journal of Medical Entomology.* 41: 803-806.

Norris, K.R. (1965). The bionomics of blow flies. *Annual Review Entomology*. 10: 47–68.

Nuorteva, P. (1997). Sarcosaprophagous Insect as Forensic Indicators. Forensic Medicine:A study in Trauma and Enviromental Hazard .Tedeschi, C.G,Esker, W.G., and Tedeschi, L.G. philadephia,W.B. Saunder Co. II: 1072-1094.

Olaisen, B. M. Stenersen, B. Mev•ag. (1997). Identification by DNA analyses of the victims of the august 1996 Spitsbergen civil aircraft disaster, *Nature Genetics.* 15: 402–405.

Pyre, J.A. (1965) "A Summer carrion study of the baby pig *Susscrofa* Linnaeus"Ecology 46: 592-602

Replogle, J., Lord, W.D., Budowle, B., Meinking, T.L. andTaplin, D. (1994). Identification of host DNA by amplified fragment length polymorphism analysis: Preliminary analysis of human crab louse (Anoplura: Pediculidae) excreta. *Journal of Medical Entomology*. 31 (5): 686-690.

Rerkamnuaychoke, *et al*. (2000). Comparison of DNA extraction from blood stain and decomposed muscle in STR polymorphism analysis. *Journal of Medical Association*. Thai. 83 (Suppl. 1) S82–S88.

Rognes, K. (1991). Blowflies (Diptera, Calliphoridae) of Fennoscandia and Denmark. Fauna Entomologica Scandinavica 24 , E.J. Brill/Scandinavian Science Press Ltd , Leiden .

Shedlock, A.M, Haygood, M.G., Pietsch, T.W. andBentzen. P. (1997). Enhanced DNA extraction and PCR amplification of mitochondrial genes from formalin fixed museum specimens. 22(3):394–400.

Sherman, R.A. and Tran, J.M.T. (1995). A simple, sterile food source for rearing the larvae of *Lucilia sericata* (Diptera: Calliphoridae). *Medical and Veterinary Entomology*. 9: 393–398.

Sperling, F.A.H., Anderson, G.A. and Hickey, D.A. (1994). A DNA-based approach to the identification of insect species used for postmortem interval estimation. *Journal of Forensic Science*. 39 (2): 418–27.

Tokuda, Y., Nakamura, T., Satonaka, K., Maeda, S., Doi, K., Baba, S., et al. (1990). Fundamental study on the mechanism of DNA degradation in tissues fixed in formaldehyde. *Journal of Clinical Pathology*. 43: 748–51.

Wang, J.F., Hu, C., Hen, Y.C., Min, J.X. & Li, J.T.(2002a). Chronometrical morphology of *Aldrichina grahami* and its application in the determination of postmortem interval. *Acta Entomologica Sinica.* 45: 265- 270.

Wang, J.F., Hu, C., Chen, Y.C., Min, J.X. and Li, J.T. (2002b). Application of the pupal morphogenesis of *Aldrichina graham* (Aldrich) to the deduction of postmortem interval. *Acta Entomologica Sinica.* 45:696-699.

Wells, J.D., Introna, F.Jr, Di Vella, G., Campobasso, C.P., Hayes J. and Sperling, F.A. (2001b) Human and insect mitochondrial DNA analysis from maggots. *Journal of Forensic Science.* 46: 685–687.

Wells, J.D., Pape, T. and Sperling, F.A.H. (2001). DNA-based identification and molecular systematics of forensically important Sarcophagidae (Diptera). *Journal of Forensic Science.* 46 (5): 1098–102.

Wells, J.D. and Sperling F.A.H. (2001). DNA-based identification of forensically important Chrysomyinae (Diptera: Calliphoridae). *Forensic Science International.* 120: 110–5.

Wells, J.D. & Stevens, J.R. (2008). Application of DNA-based methods in forensic entomology. *Annual Review of Entomolog.y* 53: 103-120.

Wells, J. D., and LaMotte, L. R. (2001). Estimating the post-mortem interval. In: Byrd, J. H., and Castner, J. L., (Eds), *Forensic Entomology: The utility of arthropods in legal investigations.* CRC Press. Boca Raton, pgs 263 – 286.

Whitaker, J.P, Clayton, T.M., Urquhart, A.J., Millican, A.J., Downes, T.J., Kimpton, C.P. and Gill, P. (1995). Short tandem repeat typing of bodies from a mass disaster: high success and characteristic amplification patterns in highly degraded samples, Biotechniques 18: 670–677.

Wiegand, P., Bajanowski, T. and Brinkmann, B. (1993). DNA typing of debris from fingernails, *International Journal of Legal Medicine.* 106: 81–83.

Wilson, M.R., DiZinno, J.A., Polanskey, D., Replogle, J. and Budowle, B. (1995). Validation of mitochondrial DNA sequencing for forensic casework analysis. *International Journal of Legal Medicine.* 108: 68-74.

Zehner, R., Amendt, J. and Krettek, R. (2004a). STR Typing of human DNA from fly farvae fed on decomposing bodies. *Journal of Forensic Sciences.* 49: 337-340.

Zehner, R., Amendt, J., Schütt, S., Sauer, J., Krettek, R. and Povolný, D. (2004b). Genetic identication of forensically important flesh flies (Diptera: Sarcophagidae). *International Journal of Legal* Medicine. 118: 245-247.

Zhao, B., Wang, H., Wang, L., Wang, W.J. & Li, Z.M. (2010). Chronometrically morphological change of larvae of two species of necrophagous flies and its implication in forensic medicine. *Chinese Bulletin of Entomology.* 47: 360-367.