University of HUDDERSFIELD

DNA characterisation from gut content of larvae of *Megaselia scalaris* (Diptera, Phoridae) for forensic investigations

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16s rRNA: 16 subunit of ribosomal RNA

A: Adenosine

ABI: Applied Biosystems

AFLP: Amplified Fragment Length Polymorphism

AGE: Agarose Gel Electrophoresis

AL: Allelic Ladder

ANOVA: Analysis of Variation

bp: base pair

C: Cytosine

CODIS: Combined DNA Index System

COI+II: Cytochrome C oxidase subunits I and II

COX 1: Cytochrome C oxidase

C_T: Cycle Threshold

Cyt *b*: Cytochrome *b*

dATP: Deoxyadenosine Triphosphate

dCTP: Deoxycytidine Triphosphate

dGTP: Deoxyguanosine Triphosphate

DNA: Deoxyribonucleic Acid

dNTP's- Deoxyribonucleotide Triphosphate

dsDNA: Double strand DNA

DTT: Dithiothreitol

dTTP: Deoxythymidine Triphosphate

EDTA: Ethylenediaminetetraacetic Acid

EtOH: Ethyl alcohol/ethanol

FLEA: Forensic Laboratory for Entomology and Archaeology

F.O: First Officer

FSS: Forensic Science Services

FTA: Flinder Technology Agreement

G: Guanine

HCL: Hydrochloric acid

HGP: Human Genome Project

HLA: Human Leucocyte Antigen

HS: High Sensitivity

M. scalaris: *Megaselia scalaris*

$MgCl_2$: Magnesium Chloride

mtDNA: Mitochondrial DNA

N-PCR: Nested Polymerase Chain Reaction

ng/µl: Nanograms per microlitre

nuDNA: nuclear DNA

p-value: Significance value

PAGE: Polyacrylamide Agarose Gel Electrophoresis

PCR: Polymerase Chain Reaction

Pm: Population match probability

PPE: Personal Protective Equipment

RBCs: Red Blood Cells

RFU: Relative Fluorescence Unit

RLFP: Restriction Length Fragment Polymorphism

RPM: Revolutions Per Minute

rRNA: Ribosomal Ribonucleic Acid

SD: Standard Deviation

SDS: Sodium Dodecyl Sulfate

SGM: Second Generation Multiplex

SNP: Single Nucleotide Polymorphism

STR: Short Tandem Repeat

T: Thymine

TBE: Tris/Borate/EDTA

Tm: Melting Temperature

V: Volts

VAP: Vaginal Acid Phosphatase

VNTR: Variable Number of Tandem Repeats

W/V: weight over volume

°C: Degrees Celsius

Abstract

The role of DNA in crime scene investigation over the last couple of decades has been immense. DNA materials as evidence are routinely collected from conventional sources (body fluids) from a wide range of crime scenes. In the absence of conventional sources, DNA evidence can be obtained from non-conventional sources, like touch DNA and gut contents of Dipteran larvae found on or near the body. While most studies about insects and their larval stages obtained from crime scenes have been done for PMI estimation, the use of gut contents from *Megaselia scalaris* (Diptera, Phoridae) larvae for human identification has not been yet investigated. The larvae's ability to crawl through tight spaces make them an important species for both indoor crime scenes and also in the cases of buried corpses. In the present study, a comprehensive framework has been developed to extract non-insect DNA from the gut contents of larvae of *M. scalaris* (Diptera, Phoridae), fed on *Sus scrofa* tissue, and use it for STR analysis, making a tool for human identification, aiding forensic investigations. The larvae were fixed using 5 different protocols: (a) suspending the larvae in hot water (>80°C); (b) larvae kept at -20°C; (c) larvae kept in EtOH (98%) and stored at -20°C; (d) larvae kept at -20°C for 4hrs and later kept in EtOH; (e) larvae first suspended in hot water (>80°C) and kept in EtOH (98%) -20°C. Despite the small size of the larvae (2.0 ± 0.5 mm) and low amount of gut content (0.2-0.5 mg), DNA extraction of the gut contents of larvae was undertaken successfully using the Qiagen® Investigator Extraction Kit. The extracted samples were quantified and the maximum quantification was obtained from the larvae fixed by freezing at -20°C, with an average of 3.67 ± 0.05 ng/µl per sample, followed by larvae fixed with EtOH at -20°C with 2.55 ± 0.06 ng/µl per sample. A positive PCR amplification result was obtained from the mitochondrial gene cytochrome *b* (149bp) and ribosomal gene 16s rRNA (138bp), which was confirmed by analysis through BlastN, showing a positive result of *Sus scrofa* DNA sequence. STR analysis of the samples was done using Multiplex PCR test kit with 11 autosomal markers and 1 gender specific marker for *Sus scrofa*. A complete STR profile was obtained from the samples (minimum 1 crop) with a match on all loci when compared to the control sample. The results obtained from this study are significant, since *M. scalaris* is an important fly of forensic interest with a cosmopolitan distribution, generally encountered by investigators in crime scenes. The results obtained also show that preservation of larvae with EtOH (-20°C) and only freezing (-20°C) help in proper DNA typing, which is helpful for

investigators as it is a more practical and easy method for proper collection and preservation of the larvae.

1. Introduction

In a wide-ranging definition, forensic science is a combination of different branches of sciences that can provide information to a court of law, enforced by investigating agencies in the criminal justice system all around the globe. Through the ages, as society grows more complex, stringent rules and laws are required to regulate the actions of its members. Forensic science, and its application of knowledge and technology, can be used by investigators for the enforcement of such laws (Saferstein, 2013).

Forensic entomology is a specialised tributary of forensic science, where arthropod science interacts with the judicial system (Lord *et al.*, 1986). Insects are generally found colonising decomposing corpses, and this colonisation can be used to estimate the post-mortem interval (PMI) of the corpse (Amendt *et al.*, 2004). The application of forensic entomology is not only restricted to the morphological identification of insects, but also to molecular level analysis based on DNA extraction and sequencing of specific nucleotide regions of both adult insects and larval stages (maggots) (Tuccia *et al.*, 2016).

DNA has acted as a multifaceted tool of crime scene investigation over the last couple of decades. DNA materials as evidence are routinely collected from a wide range of crime scenes. Most of the DNA material is gathered from conventional sources, like body fluids and body tissue. However, in situations where none of these can be recovered, the investigators can shift to non-conventional sources, such as touch DNA and gut contents of larvae. The DNA material recovered can be analysed further after a performing an extraction from the collected sample using specific protocols.

DNA extracted from gut content of larvae (non-conventional sources) is often present in very low amounts and utmost care is required for the proper preservation of the same. Short Tandem Repeat (STR) profiling of the extracted sample can establish a relation between the questioned sample and the reference sample (Butler, 2005). A positive multi-locus STR profile is a gold standard in terms of individualisation and establishment of relationship between samples (Lynch, 2003). In this study, an attempt to extract DNA from the gut content of *M. scalaris* (Diptera, Phoridae) larvae, a non-conventional source of DNA evidence was made, assessing its reliability as a tool for human DNA identification in the forensic context.

1.1 The advent of forensic science

The history of the discipline of forensic science dates back to many centuries (since 275BC). During the initial period, the primary tool of investigation was limited to careful observation and interpretation of the physical evidence (Eckert, 1997). But, with the advancement of science over the years, careful observation has been joined by much more precise and accurate scientific methodology for evidence examination and its understanding (Fig. 1.1).

1.1.1 Early development

The birth of this discipline dates back to 275BC, where ancient forensics were mainly practiced by Romans and ancient Greeks. The famous case of the golden crown of King Syracuse, and the application of science to solve it, is probably the first recorded instance of the use of science in the criminal justice system (Williams, 2015). In this case, investigators measured the exact weight of the gold used to make the crown which was weighted in water against the golden crown of King Syracuse. The difference in the water level due to presence of different light material led to the prosecution of the criminal. Similarly, in the 3rd century AD in modern day China, the manuscript titled as *'Yi Yu Ji'* (Collection of Criminal Cases) reported a case involving a woman suspected of killing her husband and burning the corpse. When asked by the investigators, she said her husband had died due to an accidental fire. During the investigation, the coroner noticed that there was no deposit of ashes around area of the mouth of the deceased. He performed an experiment by burning a dead pig and an alive pig and noticed that there was no ash deposition on the pig that was dead before the experimentation. He concluded that the husband was dead before burning. Upon confrontation, the lady admitted her crime (Saferstein, 2013).

1.1.2 Initial scientific progression

During the late 18th and the early 19th century AD, the breakthroughs in chemical science facilitated forensic science to take substantial strides forward. In 1775, Carl W. Scheele, a Swedish chemist, devised a test for successful detection of arsenic from deceased bodies. A significant contribution in 1814 was made by a Spaniard, Mathieu Orfila, when he published a scientific paper based on detection of poison and its effects on animals (Davies, 1986). Later, in 1830, a British chemist, James Marsh, described a test, known as 'Marsh Test', to detect

very low amount of arsenic (Gerber & Saferstein, 1997). The developments made by Orfila and Marsh were applied to a case in 1840, where Mr. Lafarge was found dead under mysterious circumstances. Upon analysis, Orfila concluded that the death was due to arsenic poisoning present in the food. Subsequently, Mrs. Lafarge admitted guilt on confrontation (Wilson & Wilson, 2003). A spate of other chemical discoveries were made at this time which had practical application in forensic science. These included microcrystalline test for haemoglobin in 1853 and presumptive test of blood in 1863 (Saferstein, 2013).

1.1.3 The need of personal identification and modern scientific breakthrough

By late 19th century AD, investigators began to use science to study crime. With the increasing number of criminal activities, the major hurdle investigators faced was personal identification (Moenssens *et al.*, 2007). French scientist, Alphonse Bertillon, in 1879, devised the first method of personal identification calling it anthropometry. However this method of body measurement was soon outdated and was replaced by the use of fingerprints, introduced by the Englishman Francis H. Galton, which was used later successfully by an Argentine scientist, Juan Vucetich, in 1892 (Ruggiero, 2001). After the discovery of blood groups by Karl Landsteiner in 1901, and successful grouping of dry blood by Dr. Leone Lattes in 1915, the process of identification by blood groups began to be used in criminal investigation. Around the 1920s, a pioneer of forensic science, Edmond Locard (1877-1966), declared the exchange principle, stating that 'every contact leaves a trace'. The mid-twentieth century saw the use of microscopes for different purposes and, in parallel, there was a revolution in computer technology. However, the most significant contribution was made in 1986, when DNA fingerprinting was introduced for the first time by Sir Alec Jeffreys (Jeffreys *et al.*, 1985b). Later, with the development in biotechnology and the use of PCR, DNA techniques became the most powerful tool for forensic investigators around the world. Further, the development of PCR by Kary Mullis (1986), RT-PCR by Higuchi (1992) and the start of the Human Genome Project (HGP) in 1990, have changed the modern criminal investigation (Cantor & Smith, 1999). Development of STR kits, like Powerplex 16, has also aided personal identification.

1.2 Role of forensic science in criminal investigation

In most criminal investigations, the role of forensic science is pivotal (Kirk, 1963). The process of complete examination of evidence is demarcated into three phases (Fig. 1.1). The first phase begins at the crime scene, which is the opening point of the forensic investigation and acts as a foundation around which all the resulting analysis revolves (Houck *et al.*, 2015). The proper identification of the items (physical evidence) at the scene is very important. Moreover, successful recovery of the items is critical as it dictates the terms of subsequent analysis (Jackson & Jackson, 2011). During the second phase, the evidence collected is examined and analysed in the forensic laboratory. The analysis of the evidence is undertaken to establish a relation between it and the scene of the crime and any suspect. The data obtained upon examination will corroborate with the prepositions put forward by the prosecution or by the defence (Siegel, 2010).

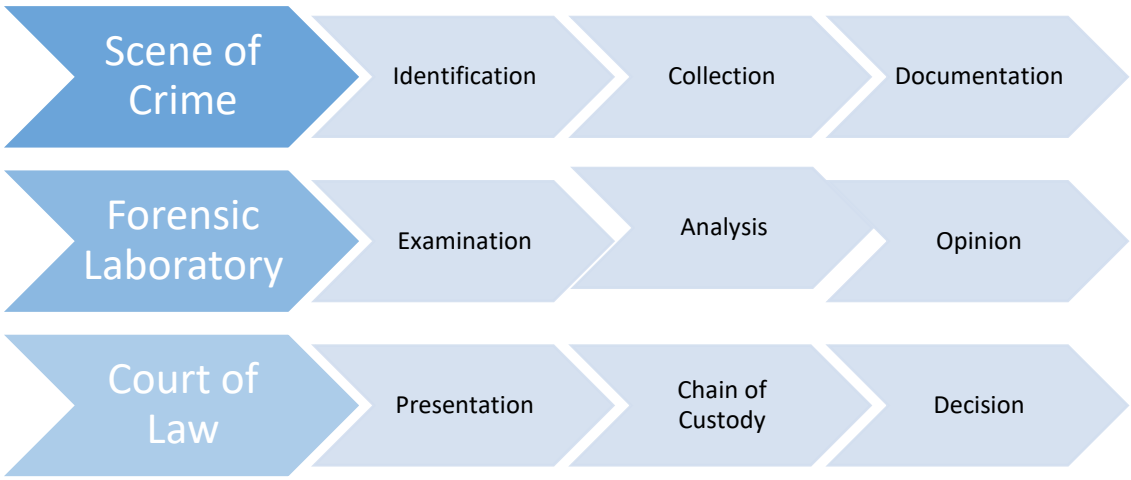


Fig 1.1: The process of forensic examination and legal examination of evidence. It is important to note that every sample analysed in the forensic lab is generally not presented as an exhibit in the court of law for judicial analysis. In routine, the most suitable sample is presented. However, as samples are not destroyed completely, the court, on its discretion, can ask for this work to also be presented. (Jackson & Jackson, 2011).

In the last and the final phase, a report is prepared based on the data obtained by the forensic scientist involved in the analysis and examination of the evidence, which is presented in the court of law for judicial review. Generally, the report is enough for judicial review, but sometimes the court can also ask the scientist to be present in the court to give an expert testimony (Jackson & Jackson, 2011).

1.3 Crime scene investigation

As already presented in section 1.2, the scene of crime holds all the keys to an investigation. Improper examination of the scene can have far-reaching negative effects on the outcome of a case. It is a scientific analysis, which needs to be systematic, logical and methodical (Lee & Pagliaro, 2013). The importance of collection of different types of evidence and proper analysis can also be not neglected (Miller, 2009; Saferstein, 2013). The process of securing the crime scene, collection and analysis of different types of evidence are further discussed in the following sections.

1.3.1 Securing the scene

The first officer (FO), or the first responder, to the scene is responsible for securing the place. Though first priority is always given to any injured person for medical assistance, cordoning off and securing the area is also significant (Fisher, 2000). A note of the immediate surroundings should be made in an attempt to preserve the scene in its original form with minimum disturbance. The FO uses police tape to seal off the area and set boundaries from the rest of the environment (FBI, 1999).

1.3.2 Searching the scene: identification and collection of evidence

After securing the scene, the next step is proper searching of the crime scene in order to identify and collect evidence. The process of searching for evidence at a crime scene is similar to that of any archaeological search, in which artefacts are unearthed in order to have information about the ancient historic site (Siegel, 2016). Before starting the search, it is important to know that contaminating the crime scene is very easy, as people walking in and out of a scene can introduce a lot of contaminants, like soil from the shoes, fibres from clothes and hair strands. These contaminates can be mistaken for evidence, hence, minimum human interaction is kept during the search and Personal Protective Equipment (PPE) is also worn (Fisher, 2000). After restriction of the personnel, a search method is established by the officer in-charge. The method of search varies from crime scene to crime scene (Fig. 1.2).

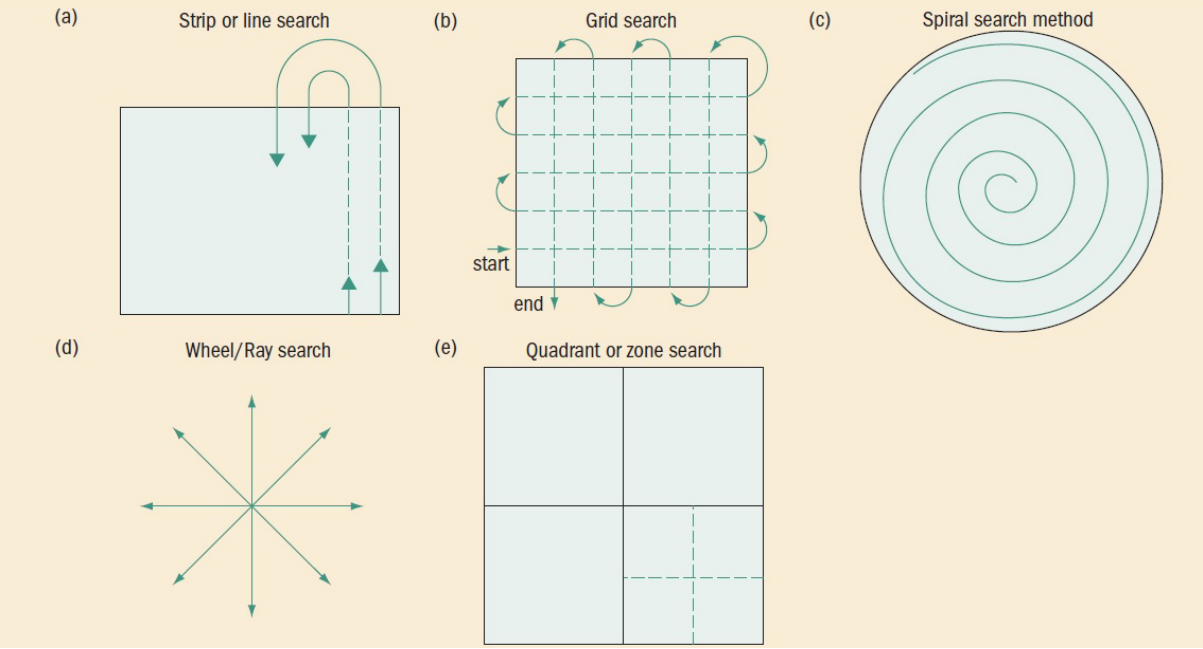


Fig. 1.2: Different types of search methods (Picture retrieved from Saferstein, 2013): *(a)* Strip/Line search method is used by investigators in an indoor environment where they walk in a straight path across the scene; *(b)* Grid search, where two people start the search perpendicular to each other covering the whole boundary forming a grid. This is very useful in indoor conditions; *(c)* Spiral search method employs one individual moving inward or outward from the boundary set. Generally, the movement should from region of less evidence to a region of high evidence; *(d)* Wheel or Ray search is used in an outdoor crime scene, where investigators start from the centre and move outwards; *(e)* Quadrant method is used in a large crime scene (indoor) where each quadrant is divided into smaller zones searched by separate individuals to prevent cross contamination.

The search begins with the establishment of the possible point of entry and exit by the culprit (Hawthorne, 1999). A permanent reference point datum is setup, and with the help of photography, the scene is documented. The position of all the items found is noted in reference to the datum. A crude sketch (not to scale) is also made. Once the evidence is located and numbered, it is carefully collected making sure that no damage or cross contamination takes place (Fisher, 2002). A plethora of evidence maybe encountered at a crime scene and, for ease of further analysis, they are classified on the basis of their origin (Table 1.1). After collection, a chain of custody is prepared to ensure that no evidence is left behind. All the evidence collected with prospective forensic significance is submitted to the forensic lab for analysis (Lee & Pagliaro, 2013).

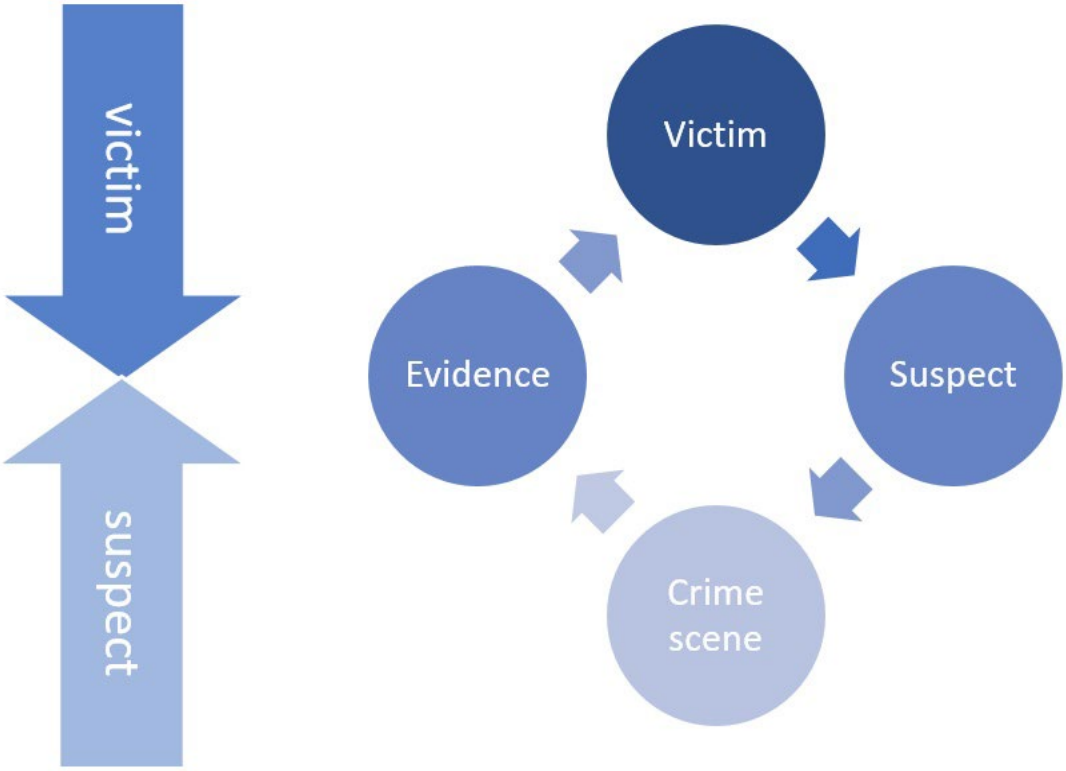
Non-Biological Evidence (origin : non-living)	Biological Evidence (origin : living sources)
Fingerprints	Dry stains of body fluids
Impressions- tire marks, shoeprints, tracks, bite marks	Wet stains of body fluids
Fibers	Hair
Paint	Bone
Petroleum products (e.g. gas residues, grease or oil)	Plant material
Building Materials	Fingernails and scrapings
Glass particles or fragments	Organ samples collected at autopsy
Firearms	Soil
Computers and electronic devices	Entomological Evidence
Questioned documents	
Explosives - explosive charge material and residues	
Tool marks (object having imprint of another object)	

Table 1.1: Commonly found physical evidence in a crime scene. These evidence are classified as either biological or non- biological on the basis of their origin.

1.3.3 Nature of evidence

Evidence is the basic unit of a criminal investigation (Siegel, 2016). It can exist in various forms, such as physical evidence, oral or written testimony, or an eye-witness account. Anything that imparts information to aid the investigation can be called evidence (Lee & Harris, 2011). In recent times, physical evidence has become an imperative aid in criminal investigation. Physical evidence, like fingerprints and DNA, can objectively link the suspect to the scene of crime (Lee *et al.*, 1994). The basis of physical evidence is the transfer theory put forward by Edmond Locard, who stated that '*Every contact leaves a trace*' (Fig. 1.3). So, in theory, physical evidence should be present if there was a contact between the suspect and the victim. The

purpose of analysis of evidence is undertaken to set a link between the evidence, the suspect and the scene of the crime.



Locard Exchange 4-Way Linkage

Fig. 1.3: The basis of physical evidence is the transfer theory of forensic science (Lee & Pagliaro, 2013). In 1934, Edmond Locard stated the underlying principle of physical evidence that every contact leaves a trace. This principle governs the persistence and utility of the physical evidence.

Physical evidence recovered from a crime scene can be classified into biological and non-biological evidence on the basis of origin, as already illustrated in Table 1.1. The accurate scientific analysis of this evidence is very important, as the corroborative value of physical evidence in criminal investigations hinges on it (Coyle, 2012). Evidence establishing the victim-suspect relationship is generally given the highest priority during the collection and analysis process (Li, 2015). Although, non-biological evidence is very useful for establishing class characteristics, it generally fails to individualise the evidence and link to the perpetrator (Saferstein, 2013). To overcome this problem, biological evidence is mostly used for accurate individualisation. Biological evidence, like body fluids, has a high degree of discriminating

factor. Scientific analysis from this kind of evidence, like extraction of DNA, helps to determine its source. That is, if non-biological evidence gives an indication about the *modus operandi*, biological evidence indicates the *corpus delicti* (Saferstein, 2003; Li, 2015). The correct collection, preservation and storage of biological evidence is extremely necessary for accurate analysis (Table 1.2).

Type of Exhibit	Collection	Storage
Hair	Minimum of 10 plucked hairs with visible roots	Store frozen in a polythene bag
Post – Mortem samples (Cadaver)	Tissue samples such as psoas muscle and bone marrow should be taken. Deep muscle tissue or bone marrow may yield DNA in badly decomposed bodies.	Tissue samples should be frozen in appropriate, sealed, sterile plastic containers. Tissue samples should not be stored in a fixative or preservative.
Blood on movable items (e.g. clothing, bedding etc.)	If present as liquid, allow the stain to dry before collection. Care should be taken to avoid contamination. Wherever possible, whole items should be submitted. When stain is present as dried, if possible whole items should be submitted.	After drying, items should be submitted individually packaged in paper bags or sacks. Fold twice at the top and seal. Store in a cool, dry environment. If wet items cannot be dried or submitted immediately to the laboratory, they should be stored frozen in polythene bags.
Liquid blood on immovable exhibits	Collect the stain on a dry, sterile swab. A control swab should also always be taken from the unstained surrounding area	Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible. Polythene bags should be avoided for storage.

Type of Exhibit	Collection	Storage
Dry blood in large amount on immovable exhibits	Using a sterile disposable blade, the surface bearing the bloodstain (such as wallpaper, plywood, fabric, etc.) should be cut away. An unstained area, approx. 2-3cm around the stain, should be left. A non-stained piece of the surface material should be taken as a control. Dry blood can be scraped on to a sheet of paper using a sterile disposable blade.	Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. Each individual item must be stored in a separate, suitable, properly sealed container such as a cardboard box. It should then be frozen as soon as possible. Polythene bags should be avoided for storage.
Dry blood in small amount on immovable exhibits	Tip of sterile swab should be lightly moistened with sterile water. The stain should be swabbed as much as possible by concentrating much of the stain on to a small area of the swab. Small swabs should be used for small stains.	Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible.
Semen stains	It possible the whole item should be submitted to the laboratory. If the stains are still wet, allow them to dry naturally before packaging.	Dry items should be packaged separately in sealed paper sacks. Use of polythene bags should be avoided unless the item is frozen. Clothing and other bagged items should be kept in a cool dry environment.

Type of Exhibit	Collection	Storage
Liquid semen	Liquid stains should be collected in a sterile container or using a sterile swab. If necessary, swab should be moistened with sterile water. Condoms should be sealed with a clip and handled with forceps. It may be possible to recover cellular material from the complainant from the outside of a condom, e.g. saliva, vaginal material, blood, faeces. It may also be possible to recover fingerprints from the outer surface of a condom. If a condom is immersed in water, e.g. a toilet bowl, it can be retrieved using forceps. Liquid should not be decanted , contents should be secured using freezer clip and should be placed in rigid container.	Freeze as soon as possible in a sterile rigid container.
Vaginal, anal or oral Swabs	To maximise recovery of semen, multiple sterile swabs should be taken from each area, with the exception of anal canal and the rectum. Swabs should be labelled as to the order taken and the exact location of sampling.	Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible.
Penile swabs	Sterile swabs should be moistened with sterile water before sampling. Label swabs as to order taken and exact location of sampling	Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible.
Liquid saliva (for reference)	Samples should be collected into a sterile 25ml wide-mouthed universal bottle with screw cap. Glass should not be used. Donor's name should be labelled with date and time.	Samples should be frozen as soon as possible.

Type of Exhibit	Collection	Storage
Saliva stains	Whole items should be submitted where possible. If stains are present on the body (i.e. kissing or biting), the area should be swabbed with a dry, sterile swab if the stain is visibly wet. If the area is dry double swabbing technique should be used (gently apply moist swab in a circular motion on the area followed by a dry swab applied in the same way). Double swabbing should also be used on grip marks, injuries etc. in alleged stranger assaults. Label swabs as to order taken and exact location of sampling.	Small items should be stored frozen. Larger items should be placed separately into paper sacks and stored in a cool, dry environment. Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible.
Cigarette ends	If transfer of the cigarette end to a property/vehicle is likely to be questioned (e.g. walked in on the bottom of shoe), it should be stored in a rigid container	Completely dry cigarette ends should be separately packaged in a polythene bag, paper bag or envelope and stored in a cool, dry environment. Wet or recently smoked cigarette ends should be placed in a sealed bag and frozen.
Envelopes/stamps	Stamps, envelope seals or any other potential DNA evidence should be removed using a sterile disposable scalpel.	Package separately package in polythene bag, paper bag or envelope, and store in cool dry environment.
Foodstuffs (e.g. chewing gum)	Collect using sterile forceps /tweezers	Store in a sterile plastic container and freeze.

Type of Exhibit	Collection	Storage
Faeces	If possible, the whole stool should be submitted, although smears on items and clothing can also give results. Stains on immovable items should be cut from the surface with a sterile disposable blade, leaving an unstained area approximately 2-3cm around the stain. Large stains should be scraped using a sterile disposable blade. Otherwise stains can be swabbed with a sterile swab. Sterile water should be used if the swab needs to be moistened. In drugs cases, the entire wrapping should be submitted.	Faecal stools or scrapings should be placed in a suitable sterile rigid plastic container. Swabs should be returned immediately to appropriate swab sleeve/tube and be sealed. They should then be frozen as soon as possible. Place wrappings that have passed through the body in a plastic sterile container. All faeces, swabs and wrappings should be frozen as soon as possible.
Bones/teeth	If possible at least one piece of bone two teeth must be submitted.	Items should be sealed in suitable sterile rigid plastic containers and should be frozen as soon as possible.
Fingernail Debris	Due to the sensitivity of current DNA techniques, clippings are preferred to scrapings. If nails are too short, fine pointed swabs can be used to swab for debris.	Use sealed nail clipper over prefolded paper packet. Seal in tamper evident bag or sterile plastic container.

Table 1.2: Collection and preservation techniques used for conventional sources of DNA (Biological evidence) encountered at crime scenes (Lord & Burger, 1983; Forensic Science Service, Scene safe Evidence Recovery System, 2004).

1.3.4 Analysis of evidence

The analysis of samples can provide some important answers, like establishment of commission of crime, linking a suspect to a crime scene. The examination of physical evidence is done by two processes; identification and comparison (Lee & Harris, 2011).

1.3.4.1 Identification

Every sample is unique in its own space and time (Houck & Seigel, 2006). As evidence recovered from the scene comes in many forms, proper identification is essential (Butler, 2009). The process of identification involves the examination of physical and chemical properties of the sample recovered. Initially, the object recovered is classified as a member of a class. Two objects sharing a class indicate their origin from a common source, while objects of different class indicate as different source (Saferstein, 2004). For example, a group of hair recovered from the scene can be classified as either human or non-human hair. These characters are called as 'class characters' and they help to preliminarily segregate evidence (Siegel, 2016). Biological samples are analysed carefully before making an attempt to individualise the source of it origin. Presumptive tests are conducted in order to classify them into particular classes. These tests are easy, simple in procedure and cheap (Butler, 2009). Generally, a small amount of material is used for the analysis. Presumptive tests for biological sample not only help to identify appropriate material for individualisation but also help in species identification. The presumptive test is followed by confirmative test to ascertain the source of origin of the biological sample (Table 1.3).

Body Fluids	Test
Blood (Hatch, 1993; Sutton, 1999; Spalding, 2005)	*Presumptive test*: Kastle-Meyer test; Leucomalachite green Assay; Luminol; Fluroscein *Confirmatory test:* Takayma crystal asaay; Heaman/Teichmann crystal assay
Semen (Elliott *et al.*, 2003)	*Presumptive test:* Visual examination using alternate light sources; Acid Phosphate assay *Confirmatory test:* Microscopic examination of spermatozoa using Christmas tree stain; Laser capture microdissection
Vaginal Fluid (Ablett, 1983; Gaensslen,1983)	*Presumptive test:* Visual examination using alternate light sources; Lugol's iodine assay. *Confirmatory test:* Microscopic examination and electrophoresis of Vaginal Acid Phosphatase (VAP)
Saliva (Greenfield and Sloan, 2005)	*Presumptive test:* Visual examination; Starch-Iodine assay; Phadebas test of Amylase *Confirmatory test:* Immunochromatic assay, using kits like RSID® Saliva (Independent Forensics)
Urine (Nickolls, 1956)	*Presumptive test:* Alternate light source for locating stains *Confirmatory test:* Microscopic crystal analysis for Urea crystals
Sweat (Sagawa *et al.*, 2003)	*Presumptive test:* Alternate light source for locating the stains *Confirmatory test:* Immunological assays with monoclonal antibody

Table 1.3 Presumptive and confirmative tests of body fluids generally recovered from crime scenes.

Later, after classifying an object into a class, the next step is to individualise it. If two pieces of evidence are unique to one another, but different from the members of its class, then they are said to be individualised. The uniqueness of one piece of evidence to another allows identification and individualisation of the source of its origin. All physical evidence recovered cannot be individualised due to limitations of space and time, but classification of recovered

items to the class level is often possible and used in evaluation of evidentiary implication (Thornton, 1986) (Table 1.4).

Evidence useful for class characters	Evidence useful for individualisation
Small paint marks and paint chips	Tool marks and bullets
Dyes and inks	Handwriting and fingerprints
Soil	Blood and other body fluids
Individual fibre	Bite marks

*Hair evidence can be used for both class characters and individualization. If root of the hair is present, DNA extraction is possible, whereas without the root, the hair can be used for class characterisation

Table 1.4: Individual and class characteristics. Evidence recovered from a crime scene can sometimes be useful for class characterisation, and sometimes for individualisation. Due to the presence of some common characters, evidence is categorised into a single class. Unique characters of a particular evidence infers individuality to it (Siegel, 2016).

1.3.4.2 Comparison

The second process in analysis of evidence is comparison. A comparison is undertaken between the questioned sample (sample recovered from the scene) and the reference sample (sample collected from the suspect). It is a two-stage analysis (Saferstein, 2013). In the first stage, a fixed number of properties are used to distinguish their source of origin (Kaye, 2009). The number of properties is fixed by the testing laboratory and it can vary from sample to sample. After the comparison, scientists analyse the data and infer a conclusion about the origin of the samples. This conclusion is made after interpreting a likelihood ratio of the samples. The frequency of the independent properties to occur in the given sample is calculated using the product rule. The opinion, and the drawing of conclusion about the origin of the source, is the second stage of the process (Biedermann *et al.*, 2007).

The forensic scientist involved in the analysis can give three types of opinion. First, positive, where the questioned and the reference sample match. Second, negative, where the questioned and the reference sample do not match. Third, no opinion, when there is not enough evidence to disprove the hypothesis of match or no-match.

1.4 Insects and humans

From time imperative, insects and humans have been closely linked to one another. The interaction concerning insects and humans has been documented in some of the earliest forms of writings and symbolism. Their source can be traced back to ancient Greece and Egypt, and also to Mayan hieroglyphics (Berenbaum, 1995). The mention of lice and locusts during the great plagues of Egypt is in the Bible (Rivers & Dahlem, 2014).

Although the interaction between insects and humans is very extensive, the evolution of the use of insects to criminal proceedings has increased since the famous incident that took place in 13th century AD in China. A suspect was convicted of a murder after a careful examination of the murder weapon, whereby the activity and growth of flies on it proved to be decisive (Tomberlin & Benbow, 2015). After the assessment of insect succession on cadavers by Megnin, the science of forensic entomology was established in the 18th century AD (Amendt *et al.*, 2004). The foundation was strengthened in the first half of the 20th century AD, when taxonomists began to identify insects of medico-legal importance. Later in the late 20th century AD, molecular analysis was introduced to identify insects of forensic interest (Sperling *et al.*, 1994).

1.4.1 Forensic entomology

The use of insects as evidence to aid legal investigation, mostly related to violent cases of crime, is called forensic entomology (Hall, 1990). It is not only limited to medico-legal cases but also has its relevance in urban entomology, along with stored products entomology (Lord & Stevenson, 1986). Urban entomology contemplates the complications involving cockroaches, termites and other insects in a human environment (Byrd *et al.*, 2010). Stored product entomology deals with the presence of insects parts in food items, like maggots in vegetable salad or insect debris in food cans (Anderson and Huitson, 2004).

The primary approach used in medico-legal cases is the application of the temperature-dependent growth of insects, especially flies, in estimating the post mortem interval (PMI) of the deceased. The colonisation of insects on a decomposing body takes place in a chronological order (Fig 1.4), and the estimation of the minimum PMI can be calculated, keeping in mind the appropriate field conditions, as the development time of a fly varies according to the conditions of the immediate environment (Amendt *et al.*, 2011).

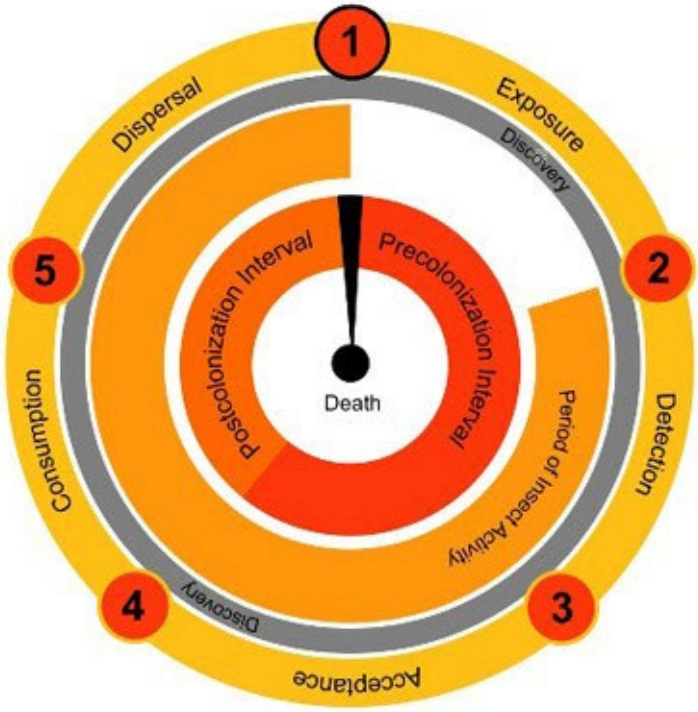


Fig 1.4: Entomological episodes of carrion decomposition. Insects progress through an anticipated sequence of stages on the decomposing vertebrate remains (adapted from Mondor *et al*. 2012): *(i) Exposure phase*: The insect is unable to detect the corpse at this stage; *(ii) Detection phase*: The insects use chemosensory apparatuses to detect decaying corpse; *(iii) Acceptance phase*: Insect and the corpse interact, and insect exploration for oviposition sites begins; *(iv) Consumption phase*: Oviposition by insect commences and faunal succession begins; *(v) Dispersal phase*: Mature flies leave leaving the corpse to be colonised by other insects (Tomberlin *et al.*, 2011).

1.4.2 Body decomposition and colonisation by insects

After death, body cells start to die due to autolysis, marking the beginning of the process of body decomposition (LeBlanc & Logan, 2010). The process of body decomposition is continuous, and is characterised by distinct sequential phases. Although phases are the same and predictable, the manner of decomposition of any two organisms are distinct (Vass, 2001). Even though it is a continuum, the process of decomposition can be divided into five main phases (Fig. 1.5).

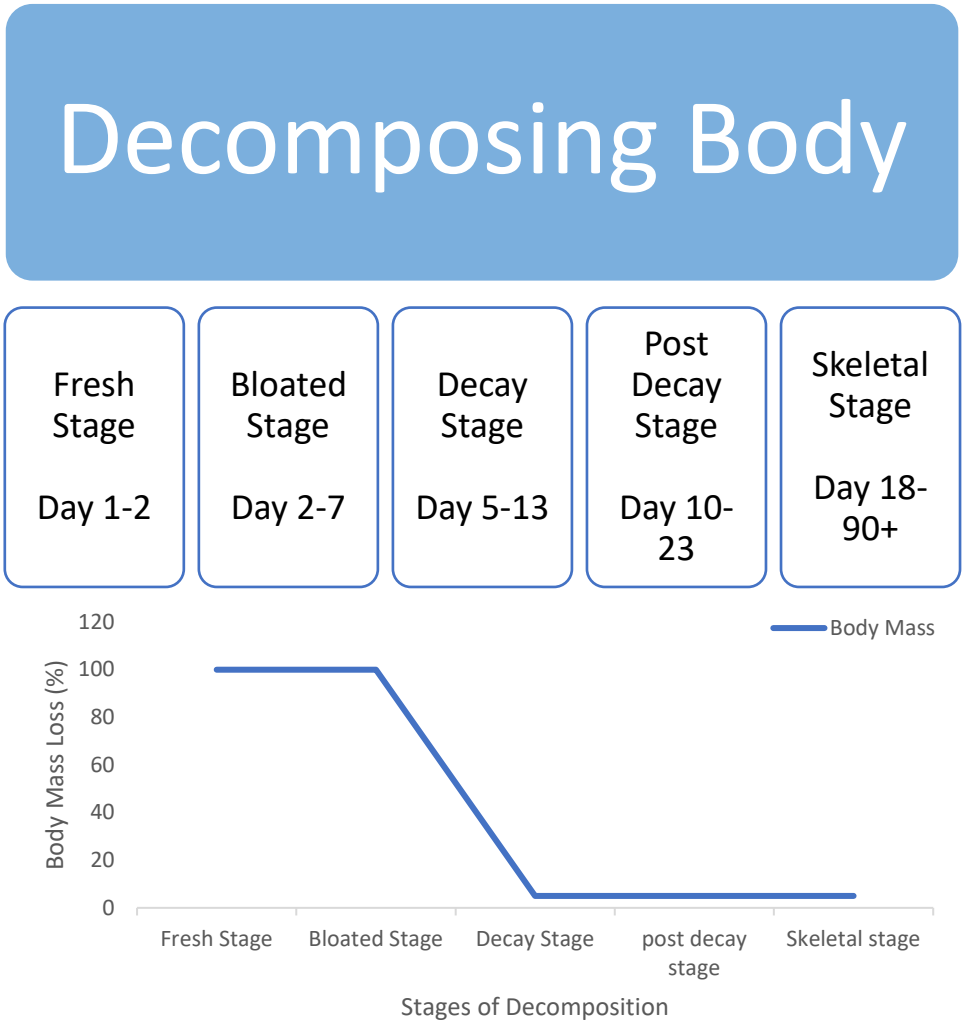


Fig 1.5: Different stages of body decomposition. The stages are based on the physical appearance of the cadaver (Tullis & Goff, 1987; Joseph *et al.*, 2011). The body mass decreases over a period of time as the body decomposition proceeds (Carter *et al.*, 2007; Goff, 2010).

Decomposition begins with the fresh stage and continues until there is no noticeable bloating. During the fresh stage, a series of physical changes are observed, which includes skin discoloration, tache noire[1] and lividity (livor mortis) (Goff, 2010). Microorganism interaction produces odour leading to insect colonisation, which starts under normal circumstances with Calliphoridae and Sarcophagidae (Diptera) (Kreitlow, 2010). The insects use a combination of

[1] Tache noire is the dark, red-brown stripe that develops horizontally across the eyes when the eyelids are not closed after death.

olfactory and visual indicators to locate the decomposing body (Wall & Fisher, 2001). Colonisation begins around natural openings of the corpse, such as the ears, eyes and genitalia. Gravid females arrive and start searching for appropriate places for oviposition or larviposition (Goff, 2010).

Bacteria, present inside the gut and elsewhere on the body, start to destroy the soft tissue, producing liquids and volatile gases (Joseph *et al.*, 2011). Production of these gases leads to swelling of the body with a balloon-like formation, marking the start of the second stage of decomposition, bloating. The internal body temperature increases significantly (>50°C) at this time, due to the combined metabolism of bacteria and maggots. An internal pressure is created due to the gases produced, releasing internal fluids to the surroundings from the openings of the body creating an individual ecology independent of the near-by environment (Goff, 2010). Masses of maggots of Calliphoridae are seen around the natural openings and also within the path of initial invasion. The loss of fluids changes the pH of the soil, making it alkaline. This results in dispersal of the natural fauna and beginning of colonisation by organisms related to the corpse as shown in Fig. 1.6 (Byrd & Castner, 2009).

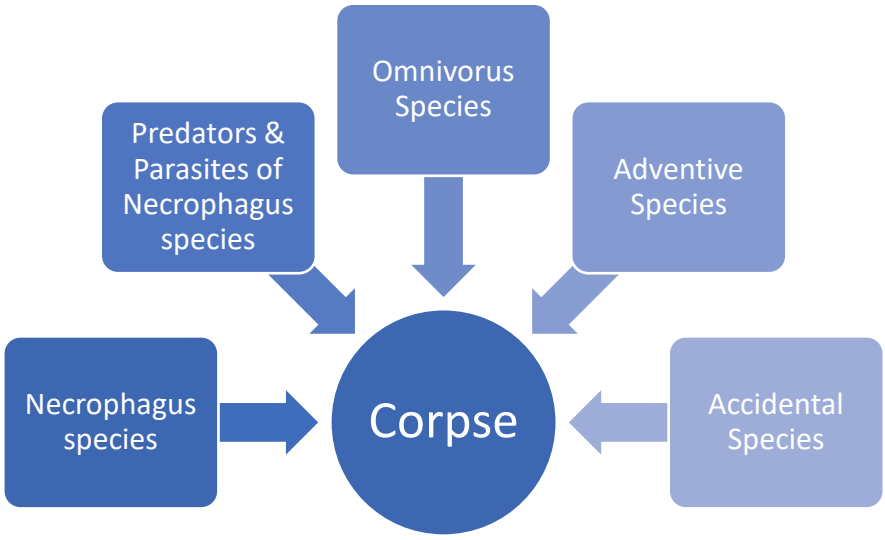


Fig. 1.6: Diverse categories of species are related to the corpse: (i) necrophagus species comprise of Diptera (Calliphoridae and Sarcophagidae) and Coleoptera (Silphidae and Dermestidae); (ii) predators & parasites of Necrophagus species include some Diptera (Calliphoridae, Muscidae and Stratiomyidae), Coleptera (Staphylinidae and Silphidae) and Hymenoptera; (iii) omnivorus species are mainly Hymenoptera (Vespidae and Formicidae) and Coleoptera; (iv) adventive species comprise generally spiders and centipedes; (v) accidental species can be any species found with the corpse from the surrounding vegetation (adapted from Goff, 2010).

The decay phase is marked by deflation of the body, due to the escape of gases from the abdomen. The skin of the abdomen is cracked open due to the combined activity of putrefying bacteria and feeding maggots (Tomberlin *et al.*, 2011). Peak feed activity of maggots leads to highest level of assimilation of the body tissue (Rivers & Dahlem, 2014). Predators like rove beetles (Staphylinidae) are also seen present in large numbers, along with some Histeridae. By the end of this phase, most of the first colonisers (Calliphoridae and Sarcophagidae) have completed their development and pupate in the neighbouring soil. Dipteran larvae remove most of the flesh leaving the body with only skin, bones and cartilage (Goff, 1993).

As most of the soft tissue is removed, the nutritional content of the body is reduced, making the conditions highly mesophytic[2] and xerophytic[3]. In these conditions, the necrophagus species are replaced by Coleoptera, mainly the Dermestidae. With the dispersal of the necrophagus species, the phase of post-decay begins (Greenberg, 1991). Dermestidae adults feed on the dried tissue and cartilage, leaving the bone with a polished finish (Goff, 1991). During this stage, a large diversity of taxa is seen dwelling around the decomposed body.

The last phase, the skeletal phase is characterised by the presence of hair and bone only. Carrion-feeding taxa are not seen in this stage, whereas some adventive[4] species, like mites (Acarina) and springtails (Collembola) are visible. This stage has no definite end point. With the passage of time, the soil pH returns to normal, leading the normal soil fauna to return (Rodriguez & Bass, 1983).

1.4.3 Factors affecting corpse decomposition and insect succession

Body decomposition, along with insect succession, varies among different environments. It also in among different corpses (Knight, 1991). In the presence of certain factors, as discussed later, insect succession and body decomposition can be accelerated, whereas, in other conditions, it may reduce considerably (Goff & Lord, 2010).

The rate of body decomposition depends on intrinsic factors (internal factors) and extrinsic factors (external environment) of the deceased (Table 1.5). Intrinsic factors include age and body constituent of the corpse, the integrity of the body, and the cause of death (Campobasso

[2] Mesophytic is a moderately moist environment

[3] Xerophtic is an extremely dry environment

[4] Adventive species occur in a region/environment in which they are not native or typical and in which they can be present for a short or a long period

et al., 2001). Extrinsic factors include the ambient temperature, humidity, and presence of clothing on the body. Some external factors, like presence of predators, may also affect the body decomposition (Rodriguez, 1997).

Factors	Effect on Body Decomposition
Intrinsic (Internal)	*Age:* Reduced rate in foetus and children. *Body Constitution:* Accelerated rate in obese. *Cause of Death:* Rapid putrefaction is seen in individuals who died due to asphyxia and septic infections. *Integrity of Corpse:* Cuts and damaged skin accelerate decomposition.
Extrinsic (External)	*Ambient Temperature:* Temperature range of 25-35°C is optimum for bacterial growth, making decomposition faster. *Ventilation and Humidity:* Windy and dry conditions dehydrate the corpse, triggering mummification. *Predators:* Dogs and foxes can disintegrate the corpse, leading to a higher rate of decomposition.

Table 1.5: intrinsic and extrinsic factors that affect the rate of body decomposition. Some extrinsic factors directly affect some intrinsic factors, like predators can affect the integrity of the corpse (adapted from Campobasso *et al.*, 2001; Viero, 2018).

As body decomposition depends on various factors, insect succession on the decomposing body also varies (Table 1.6). Geographical zones, defining the vegetation, habitat and metrological conditions, have a major impact on insect succession (MacGregor, 1999). A difference of arrival time can be observed within species at different locations. Generally, species of Calliphoridae and Sarcophagidae are the first colonisers but species vary from place to place. In tropical places, *Lucilia cuprina* and *Chrysomya megacephala,* along with (*Sarcophaga* sp.), are the first colonisers, while, in sub-tropical regions, *Lucilia coeruleiviridis* and *Phormia regina* are seen as frequent first colonisers (Reed, 1958; Early & Goff, 1986). The exposure of the decomposing body to the sun also plays a role in faunal succession. *Calliphora vomitoria* is a shade species, while *Lucilia illustris* prefer direct sunlight (Shean *et al.*, 1993).

Despite the high variety of fly species, some of them show high habitat specificity (Byrd & Castner, 2009). In caseworks from British Columbia and southern Europe, *Protophormia terraenovae* and *Calliphora vomitaria* were discovered in the rural areas, while *Lucilia sericata* was solely found in urban areas (Anderson, 1995; Vanin *et al.*, 2008; Vanin & Huchet, 2017).

Factor	Effect on the rate of colonisation	Effect on the composition of the insect community
Environment	Bodies found in open spaces are colonised faster compared to body in confined environments (e.g. caves, indoor). Altitude can also delay the insect arrival on the body	Because of environmental specificity, the composition of the fauna differs in different environment (e.g *Lucilia ampullacea* in forest environment, *Lucillia sericata* mainly in urban environments, *Lucillia illustris* and *Lucillia Caesar* mainly along rivers and rural environments)
Temperature	In the optimum interval, the higher the temperature, the faster the colonisation. Too low and too high temperature stops or delays the colonisation.	Depending on the temperature, frigophilous* or xerophilous** species will colonise the body.
Indoors/outdoors	Indoor environments delay the rate of colonisation.	Domestic species (e.g. *Musca domestica*) and small species (e.g. *Megaselia scalaris*) are observed as the first colonisers
Body concealment	Burial of the Body and covering delay the colonisation. Burying of the body does not affect the speed of colonisation after the body reaches a thermal equilibrium with the environment.	Only a few and specific species colonise buried remains Burying can create conditions for colonisation of several communities (waves) at the same time, depending of the water content of different body tissues.

*Frigophilous: species adapted to cold environments.
**Xerophilous: species adapted to very dry environments.

Table 1.6: Multiple factors that affect the rate of succession and composition of flies on a corpse. Some factors, like clothing, delay colonisation, and injuries hasten colonisation, but do not have much effect on the composition of the insect community (adapted from Vanin & Huchet, 2017).

The succession of insects varies greatly from outdoor to indoor environments. Calliphoridae and Sarcophagidae have not been found colonising bodies during early stages of decomposition in indoor cases (Vanin & Huchet, 2017). Burying the body is a common method of disposal. Physical barriers due to burial not only alter insect succession but also reduce the rate of body decomposition (Lundt, 1964; Payne *et al.*, 1968). Due to the time-consuming nature of digging, perpetrators generally dispose of a body in a shallow grave. Burial limits the presence of Calliphoridae, and also delays succession on the decomposing body. Due to their high penetrating ability from small spaces and openings, *M. scalaris* (Diptera, Phoridae) is usually seen as the first coloniser in cases of both burial and indoor situations of body decomposition (Campobasso *et al.*, 2004).

1.4.4 *Megaselia scalaris* (Loew, 1866) - a fly of forensic importance

Megaselia is the largest genus of the Phoridae family, with over 1300 species worldwide. Though *M. scalaris* prefers a warmer environment, the species has been dispersed by humans through trade and travel (Disney, 2008).

Megaselia scalaris is a small (2-3 mm) blackish-yellow humpbacked fly with a tiny, flattened head (Fig. 1.7a). Their appearance is similar to genus *Droshophila*, but they have a characteristic wing venation pattern, typical to family Phoridae (Fig. 1.7b). These flies move in an inconsistent way, moving for a short distance and rapidly changing direction to move in another direction, hence the their common name the "scuttle fly" (Loew, 1861; Greenberg, 1991).

Fig1.7a: *Megaselia scalaris*: The thorax is yellowish brown while abdomen is yellowish black with brown bands. The legs are yellow, and well developed, with a stout, expanded, laterally condensed hind femur (scale bar 400 µm).

Fig 1.7b: Phoridae wing: The wing venation is highly reduced, with an absence of cross veins (scale bar 400µm).

The lifecycle of *M. scalaris* consists of four separate stages, which includes egg, larva, pupa and adult (Fig. 1.8). The adults lay eggs on a wide range of organic materials, including decomposing corpses. The embryos develop into small whitish, spindle-shaped larvae. The length of the larvae ranges from 0.5-6mm depending upon the instar stage. The development of the larva takes about 7 days to complete at optimal temperature of 25-28°C, with the third instar taking the longest time (about 4 days), and also being the stage of maximum feeding

(Fig. 1.9). After the completion of development, the larva pupates, from which, later, as adult emerges (Disney, 2012).

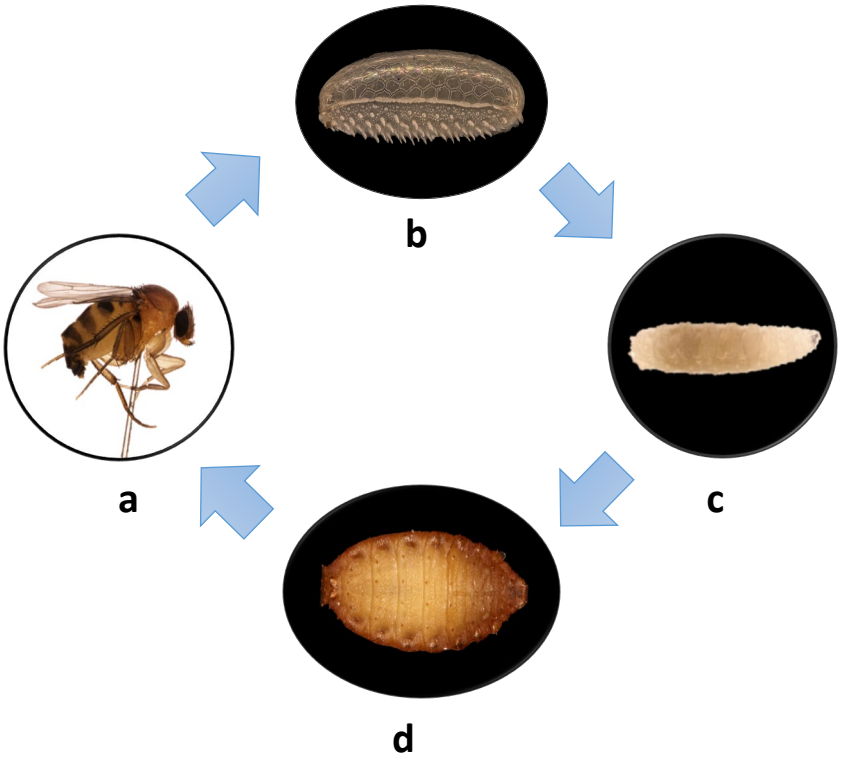


Fig. 1.8: Lifecycle of *M. scalaris:* (a) Adult; (b) Egg; (c) Larva; (d) Pupa. The development of *M. scalaris* is seen throughout the whole year, which helps in accurate PMI estimation, even when activity of other insects is reduced (Schroeder *et al*., 2003).

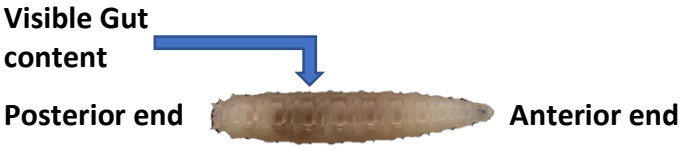


Fig. 1.9: Elongated and tubular shaped 3rd instar larva of *M. scalaris* with visible gut content. The gut content can be used to extract non-insect DNA, useful for human identification (Wells *et al.*, 2001a,b).

As discussed above, *M. scalaris* has a cosmopolitan distribution. It is a dominating species, acting as the first coloniser in the absence of blow flies (Bugelli *et al.*, 2015). Due to their small size, they can be found habituating buried corpses, which gives them the name of "coffin fly" (Fig. 1.10). This ability is very useful in PMI estimation where there is reduced

activity of blow flies. In a study conducted by Zuha *et al.* (2015) in Malaysia, PMI of a corpse found indoors was calculated according to the development stage of *M. scalaris*. Likewise, during a study conducted by Pastula & Merritt (2013), *M. scalaris* was found colonising pig carcases at a depth of up to 60 cm. No visible insect activity coud be found at 90 cm, so depths of 30 cm and 60 cm were chosen. In a similar study conducted by Bugelli *et al.* (2015) in central Italy, *M. scalaris* were found in 37.5% of indoor cases, confirming their ability to colonise indoors. Similarly, in cases where the body had been moved from an indoor condition by the perpetrators, abundant presence of *Megaselia* and absence of blow-flies can give an indication about the movement/transfer of the body (Gunn, 2011).

As the larvae are often found feeding on the outer surfaces of the body, non-insect DNA extraction and analysis from the gut is possible. Other molecular applications are discussed in the following section.

Fig. 1.10: Comparison between the size of larvae: (a) *Calliphora* sp. larvae and (b) *M. scalaris* larva (1X). Due to its small size, *M. scalaris* can inhabit places with restricted space.

1.4.5 Molecular applications in forensic entomology

Traditionally, entomologists carry out species identification of immature and mature stages of insects using morphological keys, but many times larval species of forensic interest are very similar to one another making it difficult to identify them accurately (Wells *et al.*, 2001b). Similarly, rearing a larva to a fully-grown adult is time consuming and can sometimes be complicated. Molecular biology finds its application in forensic entomology to overcome such complications (Wells *et al.*, 2001a).

Molecular identification of insects is achieved by amplifying a specific region of the mitochondrial DNA (mtDNA) using PCR by the use of insect universal primers COI+II (Sperling *et al.*, 1994; Ames *et al.*, 2006; Tuccia *et al.*, 2016). As these primer target a fragment of mtDNA, a greater copy number is present than nuclear DNA fragments in each cell. The polymorphic regions are highly stable and are flanked by stable t-RNA genes (Avise, 1994; Byrd & Castner, 2009). In a recent development, the fact that individual insect DNA remains the same throughout all life stages has been used for DNA barcoding, a tool for species identification (Meiklejohn *et al.*, 2013). Apart from the traditional mtDNA use, many studies have shown that a combination of both mtDNA and nuDNA loci can be used for identification (Nelson *et al.*, 2007).

The application of molecular techniques is crucial in cases where perpetrators have moved the body from the crime scene before the arrival of the constabularies. The occurrence of undeveloped stages of carrion-feeding species can give an approximation of when the body was moved from the scene (Vanin, 2016). Empty puraria found at the scene of crime, even after the removal of the body, can be a source of human DNA, which can be extracted and used for identification of the victim (Wells *et al.*, 2001a,b; Marchetti, 2013). Human DNA can be found inside the digestive tract of carrion-feeding larva and successful analysis of the gut contents may provide investigators with information about the victim.

With the advancement of molecular techniques, not only is sex determination of the victim possible, but also the identification of the victim is achievable (Linville *et al.*, 2004; Zenher *et al.*, 2004). In a study conducted by Luise *et al.* (2008), a STR profile was obtained from the DNA extracted from the gut of *Calliphora vicinia*. Similarly, positive STR profiles were also obtained from the larval gut contents of *Protophormia terraenovae* in a study conducted by Njau *et al.* (2016).

1.5 Decoding the encoded evidence: DNA

The human genome has 3 billion base pairs, making it "encoded evidence". Decoding this evidence can assist its use in conviction, exoneration and even in identification of victims of crime and mass disasters (Jobling & Gill, 2004). In 1985, the concept of DNA fingerprinting was introduced by Sir Alec Jeffreys while he was working on repetitive DNA sequences (Jeffreys *et al.*, 1985a,b). He discovered multiple tandem repeated sections on the genome that varied from person to person (Butler, 2005). The first usage of DNA in a forensic context came about in a case of sexual assault of two young girls and their brutal murder in Leicestershire in the mid 1980s. The conviction of the criminal was possible after a positive match of the suspect's DNA with the semen stain found at both crime scenes. More than 3 decades have passed since this first use of DNA to convict criminals, and DNA testing has since seen a tremendous growth in its use in the criminal justice system. Starting with the first use of variable number tandem repeats (VNTRs), today more sensitive and effective tools, such as short tandem repeats (STRs) and single nucleotide polymorphism (SNPs) are used to bring the guilty to justice, and also to exonerate the innocent (Wambaugh, 1989). The use of DNA typing has not only be limited to criminal cases, but has also found application in paternity testing, wildlife crimes, illegal trade and several other non-forensic fields.

1.5.1 DNA: structure

An average human body consists of about 30 trillion cells, all originating from a single cell, the zygote. All the cells in the body contain the same genetic programming, due to the presence of a chemical substance called DNA, which contains information for cell development, function, replication and division. DNA acts as the hereditary unit, containing a genetic blueprint that is passed on from parents to their children. The whole set of DNA of an organism is called its genome (Gunn, 2011).

The structure of DNA was first defined by Watson and Crick in the year 1953. The double helix DNA is a polymer composed of monomeric units made up of 3 components: a nitrogenous base, a sugar and a phosphate forming the backbone of the structure (Watson & Crick, 1953a,b). Four nitrogenous bases, namely: A (adenine), T (thymine), G (guanine) and C (cytosine), are present in the DNA molecule (Fig 1.11). Different combinations of these bases give rise to the diversity among individuals (Butler, 2005).

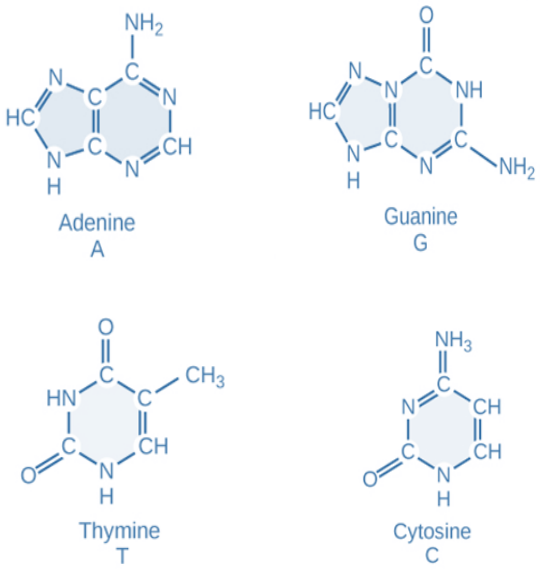


Fig 1.11: The four nitrogenous bases present in the DNA molecule: (A) Adenine and (G) Guanine are purines while (T) Thymine and (C) Cytosine are pyrimidines. The ratio of purines to pyrimidines is always constant (Chargaff, 1951).

In the human cell, apart from mature RBCs, DNA material is present in two regions: the nucleus and the mitochondria (Fig 1.12).

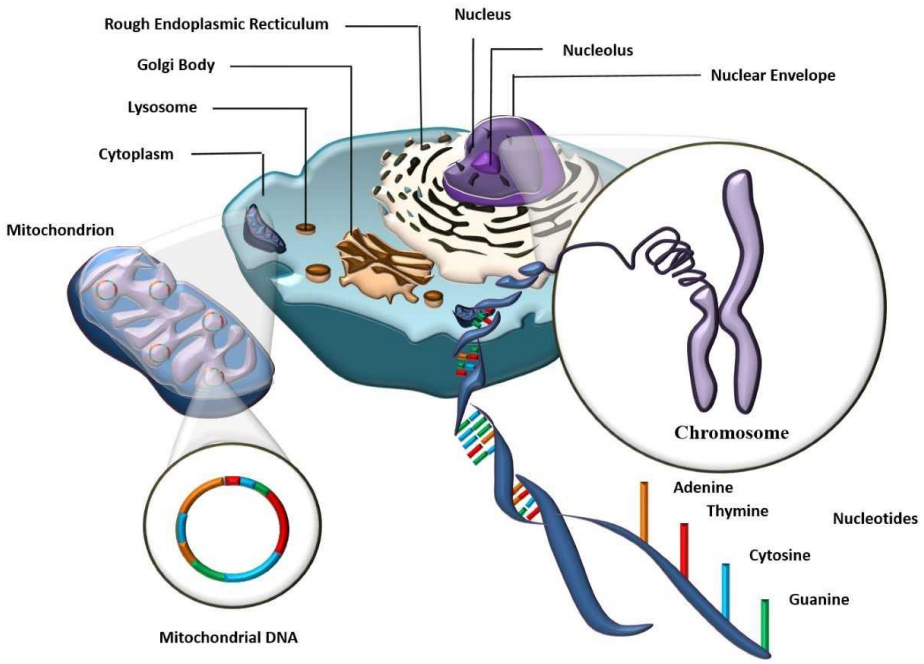


Fig 1.12: Human Cell: (A) Nuclear DNA (nuDNA) is a helical structure with a total length of about 3 billion bp. It is present as 23 pairs of chromosomes including the sex chromosome. (B) Mitochondrial DNA (mtDNA) is a small circular DNA with a length of about 16569bp. Multiple copies of mtDNA are present in each cell (Butler, 2005).

Nuclear DNA

The DNA is made up of two long chains of alternate sugar and phosphate groups along with a nitrogenous base (Watson & Crick, 1953a,b). The strands are linked together by the complementary bases by the process of hybridisation. Adenine forms a double bond with its complementary base, thymine, whereas cytosine forms a triple bond with guanine. Due to this hybridisation (base-pairing) between the complementary bases, the double helix is formed (Fig.1.13). The strands are anti-parallel to each other with one strand polarity reading from 5'-3' whereas the other strand polarity reads as 3'-5' (Farley & Harrington, 1991). The helical structure was strongly suggested by the X-ray picture in an experiment performed by Wilkins *et al.* (1953) and Franklin and Gosling (1953).

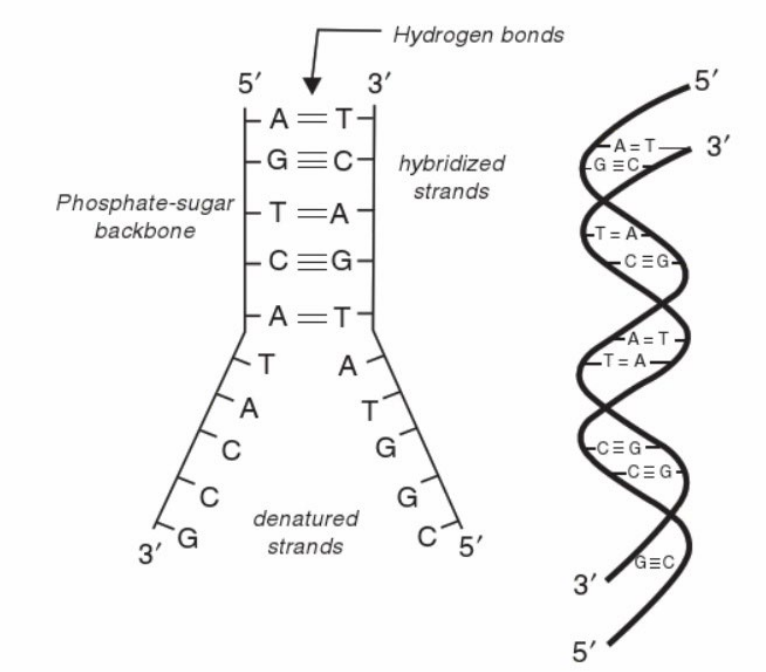
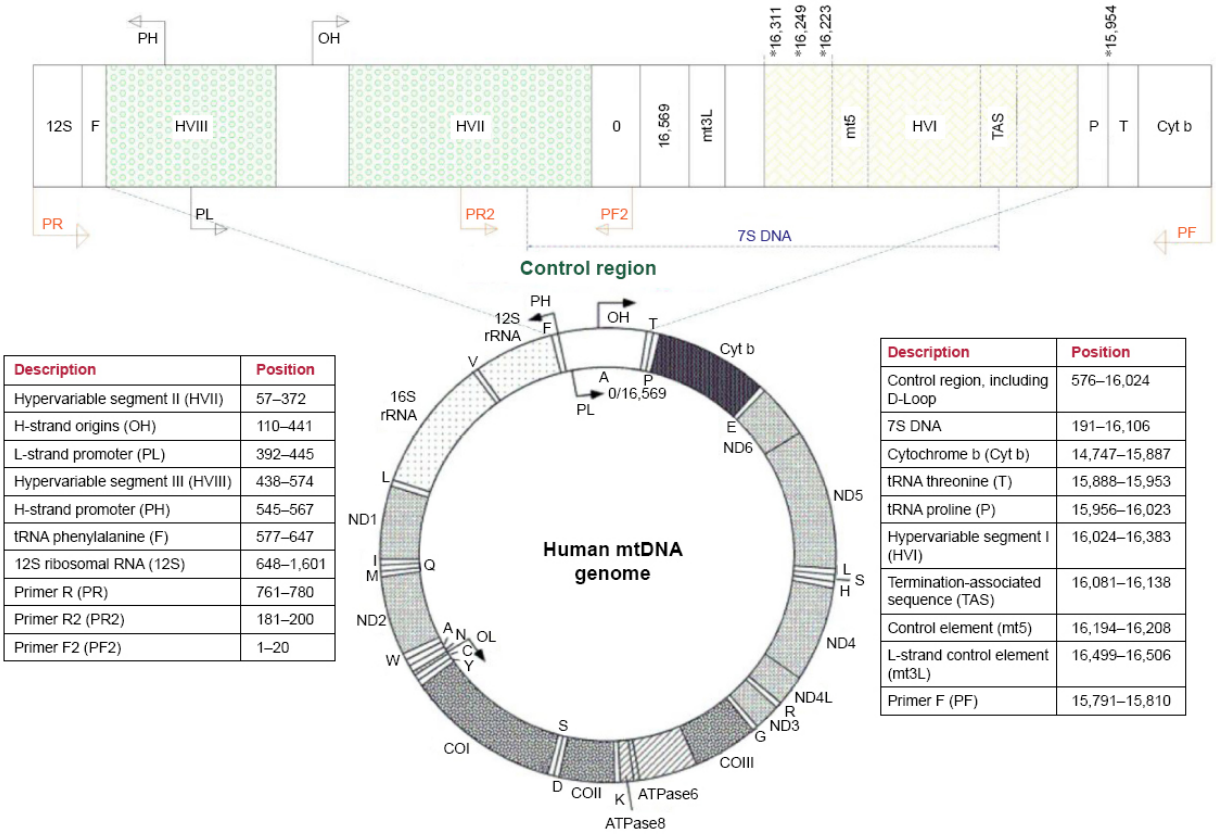


Fig. 1.13: Hybridisation of the DNA strands. Hydrogen bonds formed between the bases hold the strands together. This results in the coiling of the strand around its own axis (Watson & Crick, 1953b; Butler, 2005).

Hybridisation is the fundamental property of a DNA molecule; however, the strands can be separated with the use of elevated temperature, known as denaturation. It is a reversible process, which can be inverted by lowering the temperature, called renaturation. This property of DNA to denature and renature is very useful in the process of PCR, as described in section 1.5.6.

Mitochondrial DNA

Mitochondrial DNA (mtDNA) is circular in shape with a length of 16,569 bp coding for 37 genes (Fig. 1.14). Multiple copies, sometimes even more than 1000, can be found in the cell, making them to easier to detect, even in low amount (Andrews *et al.*, 1999).



Description	Position
Hypervariable segment II (HVII)	57–372
H-strand origins (OH)	110–441
L-strand promoter (PL)	392–445
Hypervariable segment III (HVIII)	438–574
H-strand promoter (PH)	545–567
tRNA phenylalanine (F)	577–647
12S ribosomal RNA (12S)	648–1,601
Primer R (PR)	761–780
Primer R2 (PR2)	181–200
Primer F2 (PF2)	1–20

Description	Position
Control region, including D-Loop	576–16,024
7S DNA	191–16,106
Cytochrome b (Cyt b)	14,747–15,887
tRNA threonine (T)	15,888–15,953
tRNA proline (P)	15,956–16,023
Hypervariable segment I (HVI)	16,024–16,383
Termination-associated sequence (TAS)	16,081–16,138
Control element (mt5)	16,194–16,208
L-strand control element (mt3L)	16,499–16,506
Primer F (PF)	15,791–15,810

Fig 1.14: Genomic Structure of mtDNA (Falah *et al.*, 2017). It has a non-coding region of about 1,100 bp that has a high mutation rate; this region is also known as the hypervariable region and is useful in comparison of DNA samples.

The nuclear DNA and the mtDNA varies in the manner of their heredity. In the case of nuclear DNA, one set of chromosomes is received from the father and the other set from the mother; mtDNA, by contrast, is only transferred from mother to her children as the zygote is only formed by the egg cytoplasm (Houck & Seigel, 2015). As the mtDNA, by contrast, does not go through any recombination, it is a formidable tool in tracking family lines on the maternal side (Fig. 1.15).

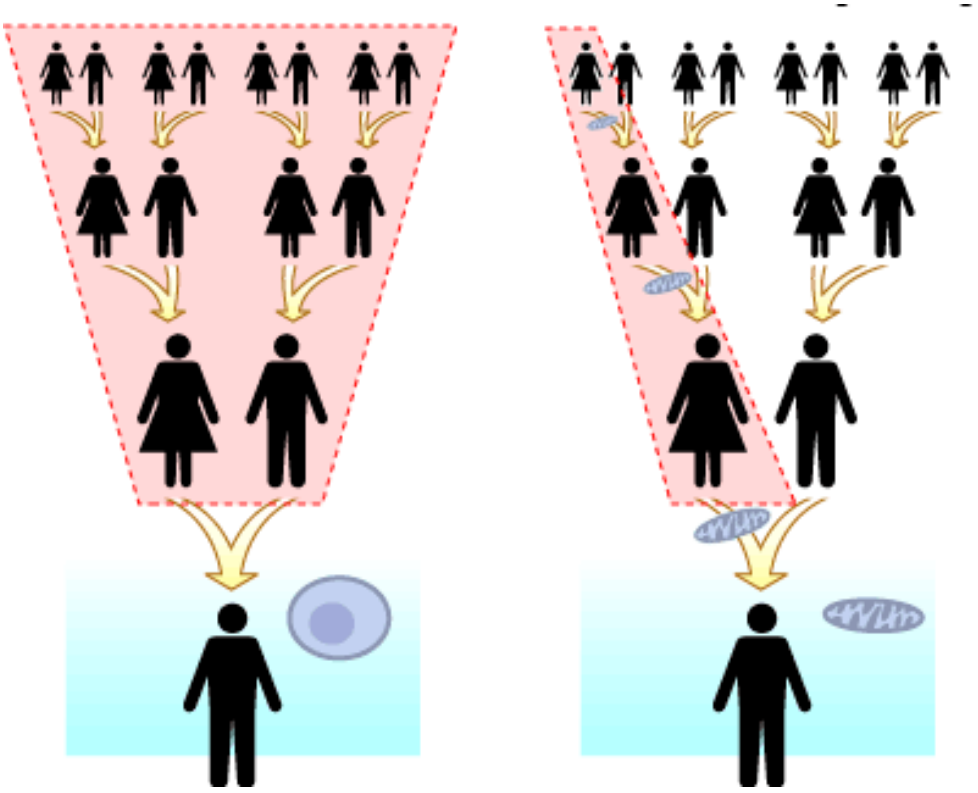


Fig 1.15: Inheritance pattern of nuclear DNA in comparison to mtDNA (University of California Museum of Paleontology's http://www.understandingscience.org); (A) Nuclear DNA is inherited from all ancestors; one pair of chromosomes from the maternal side and one pair from the paternal side. (B) The mtDNA is only inherited from the maternal side and is an example of single lineage inheritance (Andrews *et al.*, 1999a,b).

1.5.2 The use of DNA as evidence

DNA evidence generates unique profiles for each individual (apart from monozygotic twins). The ability to produce such unambiguous profiles is due to the difference that are present at the genetic level (Godwin *et al.*, 2011). Although most of the DNA (99.7%) is common among people, it is the small fraction (0.3%) that makes the difference. This fraction of DNA is highly polymorphic and varies from person to person. It can be traced by looking at tandem repeats (Butler, 2005). These regions are composed by a variable number of repetition of short DNA fragments (3-250bp), which make each individual unique, and are the principle source for the use of DNA as evidence in forensic science (Bond, 2007).

In human DNA, polymorphisms pertaining to forensic genetics are mainly found in two categories: (i) minisatellites (Jeffreys *et al.*, 1985a), also known as VNTRs, and (ii)

microsatellites, also referred to as STRs (Li, 2015). The variations between different individuals is seen due to the difference in the number of repeats, with VNTRs repeat length varying from 6-100bp and STRs varying from 2-8bp. Due to the difference in the length, they are both also known as length polymorphisms (Gerber *et al.*, 2000). SNPs are the simplest form of polymorphism, varying in only a single base, also known as sequence polymorphism (Wambaugh, 1989). Both type of polymorphisims are illustrated in Fig. 1.16. Generally, due to their high discrimination factor and easy process of characterisation, STRs are used widely by forensic investigators in criminal investigations.

In forensic DNA typing, multiple STR loci are generally examined for generating profiles of individuals. A profile frequency is calculated using the product rule, by multiplying each independent individual locus frequency. The higher the frequency, the lower the change of the same profile occurring among two individuals (Butler, 2005).

Fig 1.16: Primary forms of DNA polymorphisms: (A) sequence polymorphisms (SNPs), and (B) length polymorphisms (STRs and VNTRs)

1.5.3 Source of DNA evidence

As DNA is ubiquitously present in humans, there are numerous sources of DNA evidence that should be properly acknowledged while analysing a crime scene. DNA can be left behind at the scene even without the perpetrator realising it, making it even more important for an investigation. For example, the suspect's DNA might be found on the victim's body or deposited on an object around the crime scene. Likewise, the victim's DNA and witness DNA along with suspects DNA can be found around the surroundings, hence investigators should be careful while analysing DNA evidence (Lee *et al.*, 1991; Lee, 1996; Primrose, 1998). The sources of DNA evidence that can be found at scene of crime can be broadly categorised into 'conventional' and 'non-conventional' sources.

1.5.3.1 Conventional sources

Most biological evidence, as described in section 1.3.3.2, is common source of DNA that can be located at a scene of crime. Investigators often submit evidence that has the possibility of containing some body fluids to the forensic laboratory for DNA analysis (Lee *et al.*, 1994). The amount of DNA present generally varies from one sample to another (Table 1.7). Most conventional sources have adequate DNA to establish a positive relationship between the source and the crime. Likewise, kinds of evidence recovered also differ from crime scene to crime scene; for example, in a case of sexual assault, all the clothing present at the scene would be used for DNA analysis, as clothing can contain biological fluids, like semen and blood (Saferstein, 2013). Similarly, in cases of homicide, the weapon by which the crime was commissioned can show presence of blood. The clothing of the victim and the suspect is also important and can be used for analysis. Similarly, in cases of aggravated assault, a weapon, like a baseball bat, can be used to screen for both victim's and the suspect's DNA. The handle of the bat can be analysed for suspect's DNA, while the bottom part for the victim's DNA. Evidence, like masks, facial tissues, stamps, glass bottles, spectacles, bite marks and upholstery can also be used for DNA analysis in cases of forced burglary and kidnapping (Lee *et al.*, 1994). Likewise, a cadaver found at a scene of crime can also act as a conventional source of DNA.

Type of sample	Amount of DNA
Liquid blood	20000-40000 ng/ml
Blood stain	250-500 ng/cm^2
Liquid semen	150000-300000 ng/ml
Postcoital vaginal swab	10-3000 ng/swab
Hair (with root), plucked	1-750 ng/root
Shed hair	1-10 ng/root
Liquid saliva	1000-10000 ng/ml
Oral swab	100-1500 ng/swab
Urine	1-20 ng/ml
Bone	3-10 ng/mg
Tissue	50-500 ng/mg

Table 1.7: DNA content acquired from different samples. Note that the amount of DNA varies upon the environmental conditions, as well as collection and preservation techniques (Lee *et al.*, 1994).

1.5.3.2 Non-conventional sources

Sometimes the above mentioned conventional sources are not found at a crime scene. In such cases, investigators switch to non-conventional evidence. For example, in case of a missing cadaver, although conventional DNA analysis cannot be done to identify the corpse, gut content analysis of the larvae found around the possible crime scene may help in the identification of the possible victim (Campobasso *et al.*, 2005). Similarly, DNA can be also extracted from empty puparia, which are generally found even after the removal of the cadaver (Marcheti *et al.*, 2013). Other entomological that evidence that can act as a non-conventional source includes extraction of DNA from fly speck and fly artefacts (Vanin, 2016). In cases of analysis of fly specks, special care has to be taken while collecting the sample as fly speck are very similar in appearance to small drops of blood (Durdle, 2013). Further, screening of the blood meal of mosquito and lice for DNA evidence is also helpful, not only for identification of the victim, but also for the identification purpose of the perpetrator as well (Lord *et al.*, 1998; Mumcuoglu *et al.*, 2004; Oshaghi *et al.*, 2006; Martínez *et al.*, 2013). Apart from entomological evidence, transfer, trace or touch DNA can also a be on-conventional source of evidence that can be used in casework (Meakin & Jamieson, 2013). Transfer of DNA takes place in two ways: (i) direct transfer, and (ii) indirect transfer (Fig 1.17). Transfer DNA is generally obtained by analysing the shedding of epithelial cells found on substrates like glass, fabric and wood, along with the neighbouring surroundings (Daly *et al.*, 2012). For example, transfer DNA has been successfully found on a knife (study conducted by Meakin *et al.* 2017).

(I) (II)

Fig 1.17: Direct and indirect transfer: (I) Direct, also known as primary transfer, is the contact between two bodies. This can also include the transfer of DNA from an individual without any contact directly, such as talking, coughing, and sneezing. (II) Indirect transfer of DNA is when DNA from an individual is transferred to an item via an intermediary surface. For example, DNA is transmitted from one individual to another and consequently transferred to the item (Meakin & Jamieson, 2013).

DNA evidence is very versatile but it is also very susceptible to degradation and contamination (Capelli *et al.*, 2003). The molecular analysis of a DNA sample begins with the process of extraction, proceeding to quantification and PCR amplification and finally the generation of a DNA profile (Fig 1.18).

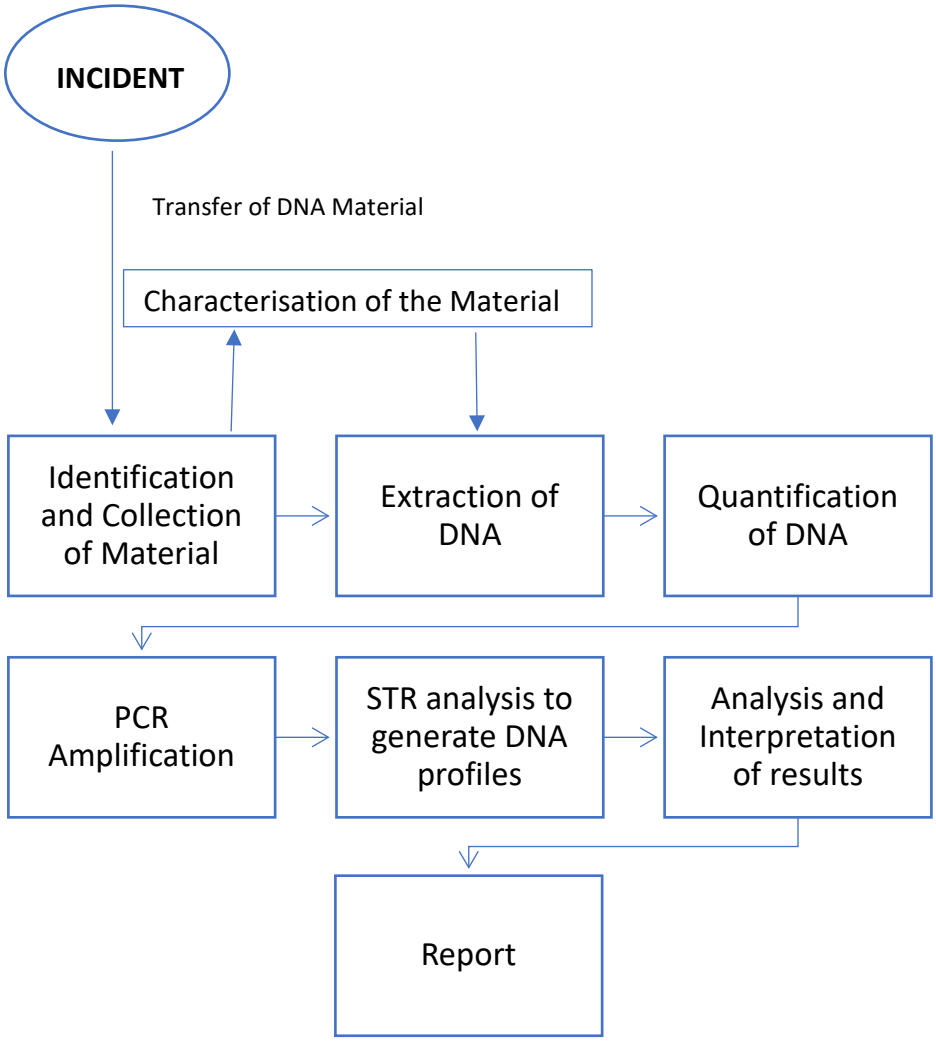


Fig 1.18: Analysis of DNA evidence. Identification and collection of material takes place at the scene of crime, while extraction, quantification, PCR amplification and STR analysis are the molecular techniques performed (Godwin *et al.*, 2011).

1.5.4 Extraction of DNA

Biological samples obtained from the crime scene contain a number of substances along with DNA (Hoff-Olsen *et al.*, 1999). Hence DNA needs to be separated from other cellular materials and contaminates before subsequent analysis (Butler, 2005). The main aim of the extraction process is to: (i) maximize the DNA yield from the sample, sufficient for a full DNA profile, and (ii) extract DNA pure enough for the downstream process, like PCR amplification and STR amplification (Godwin *et al.*, 2011). Hence it is very important to find a suitable process for DNA isolation for successful completion of the above-mentioned aims. Poor quality DNA can result in failure to obtain a full DNA profile, even hampering subsequent analysis (Li, 2015). Independent of the method of extraction used, DNA extraction can be broadly divided into three main stages, including: (i) breaking of cell membranes resulting in cell lysis, (ii) protein denaturation, and (iii) separation of DNA from cellular components following with its elution (Holland *et al.*, 2003).

Methods of extraction

(i) Organic extraction (Phenol-Chloroform)

Organic extraction has been a widely-used method for many years by forensic laboratories for the extraction of the DNA. In this process, a series of reagents are added, including Sodium Dodecyl Sulphate (SDS) and proteinase K for cell lysis and protein degradation, and Phenol-Chloroform for separation of DNA from the protein molecules (Fig. 1.19). DNA is eluted in distilled water (Vandenberg *et al.*, 1997). An improved version of organic extraction, differential extraction, is used for the separation the female epithelial cell from sperm cells in cases of sexual assault, first described in 1985 by Gill *et al.* (1985).

This method can be used successfully to extract large amounts of double stranded DNA, but it is time consuming and involves the use of dangerous chemicals, like phenol, which can inhibit PCR amplification (Schrader *et al.*, 2012).

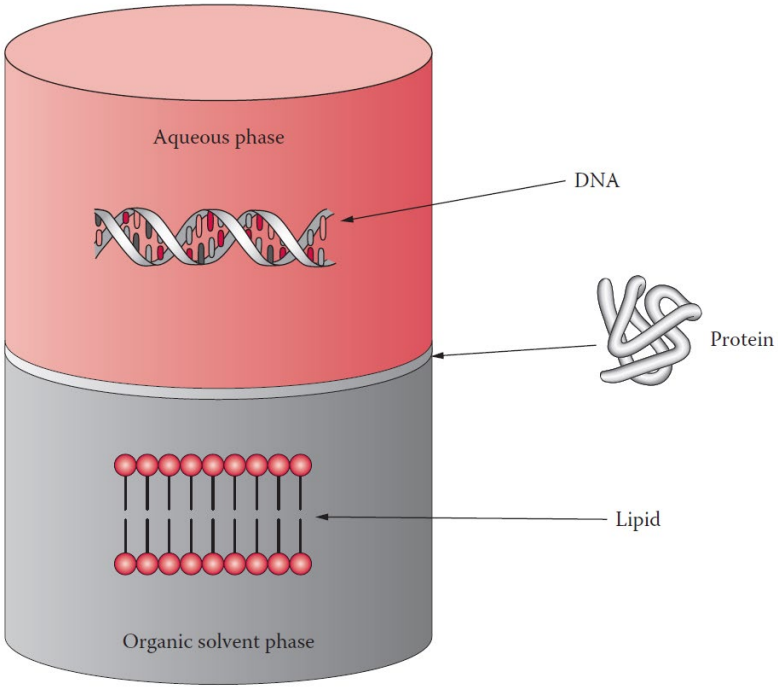


Fig 1.19: Organic extraction of DNA (Li, 2015). The DNA is present in the aqueous phase, while the lipids and other cellular debris are present in the organic solvent. A thin layer of protein separates the two layers.

(ii) Chelex extraction

In 1991, an alternate and cheap method of DNA extraction, known as Chelex® extraction (Bio-Rad laboratories, Hercules, CA), was introduced (Li, 2015). In this method, the samples are added to the Chelex® suspension containing styrene divinylbenzene copolymers, preventing DNA degradation from endogenous DNase (Fig. 1.20). Subsequent boiling and centrifuge steps separates the Chelex® resin and other debris from the DNA, which is suspended in the supernatant, which is subsequently used for PCR amplification (Walsh *et al.*, 1991). Though this method is cheap and rapid, boiling of the sample affects the chromosomal DNA making it single stranded, and making it unsuitable for STR analysis.

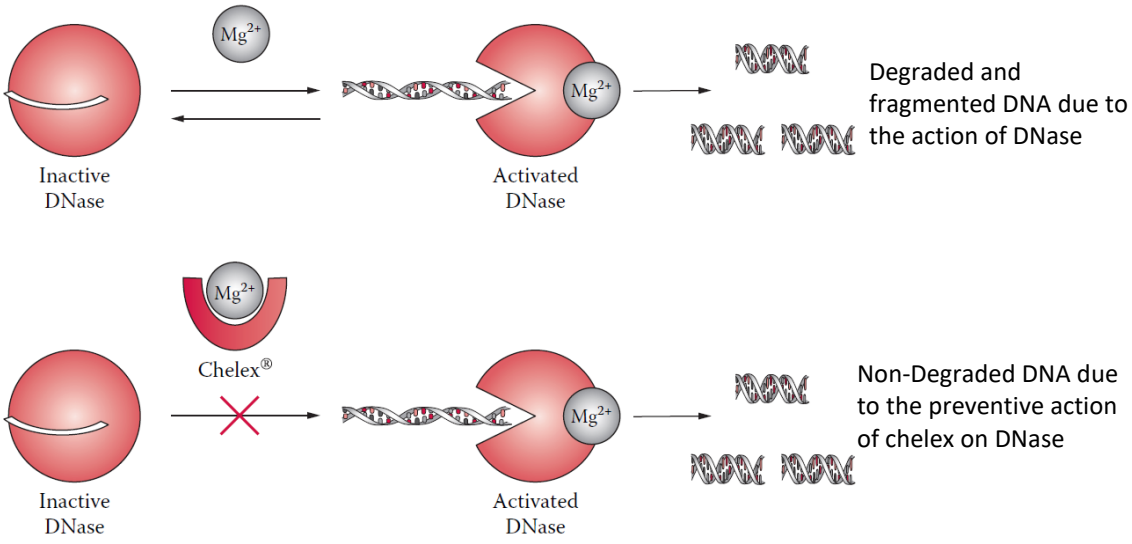


Fig. 1.20: Effect of Chelex® on DNA (Li, 2015). It inactivates the DNase with the help of divalent molecules such as Mg^{2+}.

(iii) Solid phase DNA extraction

In recent years, with the advancement of automation, solid phase DNA extraction methods have been developed. These are high-throughput methods resulting in high purity DNA samples which are suitable for downstream processes like PCR amplification and STR analysis. One active method is the silica-based extraction generally used in Qiagen® columns found in the kits provided for DNA extraction (Greenspoon *et al.*, 1998). In this approach, the nucleic acid is bound on the silica beads in the presence of high concentrations of guanidine hydrochloride, guanidine isothiocyanate, sodium iodide, and sodium perchlorate (Chaotropic salts), resulting in the denaturation of the protein and stabilising the DNA molecule (Fig.1.21). Several washes are performed to eliminate proteins and other cellular debris (Duncan *et al.*, 2003). Later, the DNA is suspended in an elution buffer and stored until further use. Apart from silica-based methods, magnetic particle DNA extraction is also used by forensic laboratories. The Prepfiler® Magnetic Kit was introduced in 2008 by Applied Biosystems for high-quality DNA extraction of forensic grade (Butler, 2005). These above methods can be automated with the use of suitable platforms; for example, the QIA cube for Qiagen® DNA extraction, and the Tecan Freedom EVO® platform for for Prepfiler® extraction (Montpetit *et al.*, 2005).

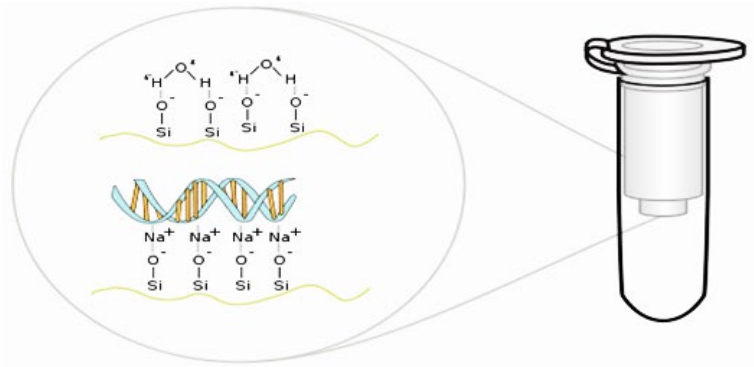


Fig. 1.21: Silica-based DNA extraction columns used in the extraction kits by Qiagen (Soni, 2016); the silica binds the DNA and, after washing until buffer, the DNA is eluted in elution buffer.

Extracted DNA is typically stored at -20°C, making it suitable to use for subsequent analysis, like PCR, and for long-term storage (Butler, 2005).

1.5.5 Quantification of DNA

The next step, after extraction of DNA, is determination of the amount of DNA extracted. This is very essential for its use in PCR-based assay for DNA testing (Nicklas & Buel, 2003). A number of methods are used for the estimations of the DNA quantity, including visualisation on agarose gels, slot blot, UV absorbance and spectrometry, fluorescent dye assay and q-PCR or RT-PCR.

1. Agarose gel electrophoresis (AGE) is a quick and simple method for estimating both quality and quantity. The DNA is separated with the use of electricity in an electrophoretic tank. Dyes intercalating the backbone of the DNA molecule are used, like Midori green or Ethidium Bromide, both of which are visualised under UV light (Godwin *et al.*, 2011). A DNA ladder of known length is run alongside for estimation of fragment size. The brightness of the amplification bands on the gels can be used for quantity estimation (Issaq *et al.*, 1997).

2. Similarly, the slot blot technique is used to detect human genomic DNA, in which a small amount of sample is spotted, using a slot blot device, transferred onto a nitrocellulose membrane. The DNA sample is hybridised with a labelled complimentary probe with a primer-specific DNA sequence. This is visualised with the use of streptavidin and horseradish peroxidase conjugate giving a colorimetric reaction (Budowle *et al.*, 1995). A more sensitive

test of fluorescence assay, using PicoGreen® dye, can also detect ds-DNA to 25pg/ml (Hopwood *et al.*, 1997).

3. UV absorbance and spectrometry analysis of DNA can also help in estimating the quantity of DNA, along with its purity, as DNA is absorbed maximally at 260nm. The spectrometric analysis is undertaken by putting the sample in a cuvette, which is kept in the analysis chamber. Though it is widely used, this method fails to give accurate results with small amounts of DNA, and so it is unsuitable for forensic analysis.

All the above mentioned methods can be used for estimating the DNA quantity, but a more sensitive and accurate way of measuring quantity is q-PCR or RT-PCR. The process and components of this technique are described in section 3.8.

1.5.6 Polymerase Chain Reaction (PCR)

PCR is an enzymatic procedure in which a particular region of the DNA (template) is amplified in an exponential manner to produce multiple copies of a particular sequence of DNA. This process of molecular 'Xeroxing' was introduced by Kary Mullis in 1985 while working on amplifying the β-globin gene for diagnosis of sickle cell anaemia (Mullis *et al.*, 1986; Mullis & Faloona, 1989).

Initially, after the introduction of minisatellites (VNTRs) by Sir Alec Jeffreys, most DNA forensic casework revolved around it (Jefferys *et al.,* 1985a,b). But the analysis of VNTRs by restriction length fragment polymorphism (RLFP) is time consuming, and it requires a large amount of DNA for analysis, making it unsuitable for some forensic cases. In an attempt to overcome these limitations, PCR a more sensitive and high-throughput method, was used on VNTR loci. Alleles of size between 5kb and 10kb were positively amplified from fresh material (Jeffreys *et al.*, 1988), thus making PCR as the base of several forensic DNA assay, including DNA quantitation, STR profling, and mtDNA sequencing (Li, 2015).

Soon, PCR technology was incorporated into forensic casework analysis. In 1988, it was first used in the examination of the skeletal remains of a 3-year-old child by amplifying the polymorphic HLA-DQα locus (Stoneking *et al.*, 1991; Blake *et al.*, 1992).

1.5.6.1 Components of PCR

A PCR reaction is made by mixing numerous discrete constituents and then adding nuclease-free deionised water to reach the required volume and concentrations (Butler, 2005). The components of the PCR reaction are: (i) *Template DNA*: the extracted DNA acts as the template DNA. In most cases of forensic profiling, successful amplification is achieved even with a low amount of template (Gill *et al.*, 2000). (ii) *Taq DNA polymerase*: a thermostable polymerase isolated from the bacterium *Thermus aquaticus* is used, increasing the specificity, sensitivity and total yield of the reaction (Chien *et al.*, 1976). Taq works optimally at 72°C-80°C, and a hot start (≅95°C) can be used to minimise non-specific binding, resulting in better yields (Daquila *et al.*, 1991). (iii) *Primers*: one pair of oligonucleotide sequences that flank the specific region of the DNA template that is to be copied. The melting temperature of each primer in pair usually varies by less than 5°C (Li, 2015). Generally, during design of primers for forensic purposes, it is important to keep in mind the conserved regions of DNA and, therefore, primers are designed in a manner that can be used to amplify human DNA from all populations (Budowle *et al.*, 2001). (iv) *Reaction buffer (including $MgCl_2$):* a buffer is used to generally to maintain the pH of the reaction around 8.3- 8.8 at room temperature, while divalent cations, like $MgCl_2$, are used to stabilise the primer-template complex formed. (v) *Deoxynucleoside Triphosphates (DNTP's):* the building blocks of PCR are dNTPs, which are merged into the nascent DNA strand during amplification. Typically, equimolar amounts (200 µM) of dATP, dCTP, dGTP and dTTP are present in a PCR assay reaction (Godwin *et al.*, 2011).

1.5.6.2 Process of PCR

The PCR amplification is a process involving heating and cooling of samples in a specific thermal cyclic pattern. Each cycle of the PCR is divided into three main phases: Denaturation, Annealing and Extension, which are repeated over around 30-35 cycles (Fig. 1.22). (i) *Denaturation*: the two complementary strands of the template DNA are separated at high temperature (95°C), causing melting as the weak hydrogen bonds between the nitrogenous bases are broken. (ii) *Annealing*: the temperature is reduced to the annealing temperature specific to the primers used. This temperature is usually around 50°C- 60°C and it allows the primers to anneal to the complimentary sequences on the single stranded template DNA, making it primed for the amplification process. (iii) *Extension*: the temperature is increased

to 72°C (optimum temperature) where *Taq* polymerase adds nucleotides to the template DNA at a rate of about 40-60 nucleotides every second, proceeding with the amplification process (Takagi *et al.*, 1997).

PCR amplification is carried out using an instrument called thermocycler, composing of a metal block of heating and cooling elements. The temperature of the thermocycler is controlled by a microprocessor, making cyclic temperatures changes during the amplification process. Generally, the lid of the metal block housing the plastic PCR tubes is heated to 105°C, preventing evaporation and condensation of the PCR reaction mix, and thus keeping the reaction stable (Godwin *et al.*, 2011).

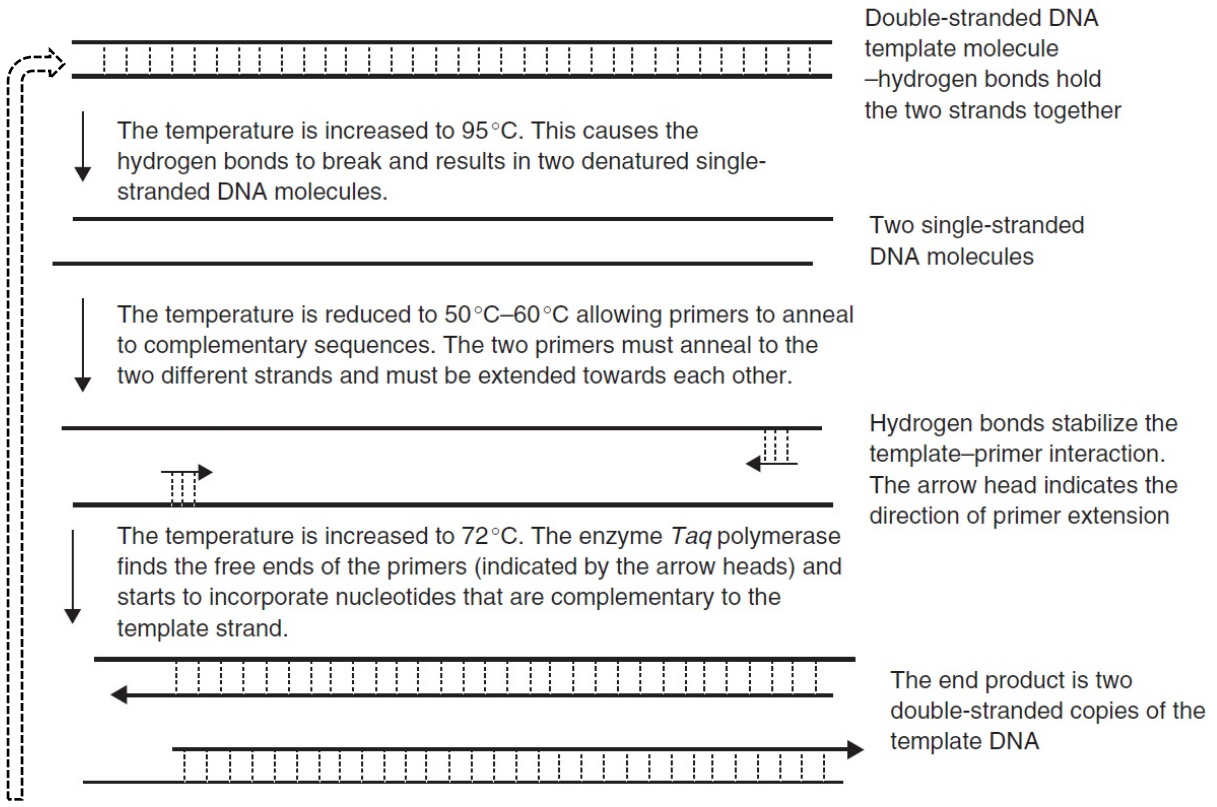


Fig. 1.22: The PCR reaction (adapted from Godwin *et al.*, 2011). It consists of 3 phases: denaturation at 95°C, annealing at 50°C-60°C, and extension at 72°C. These continuous cycles of heating and cooling are repeated over at least 30 cycles.

Apart from traditional PCR amplification, the approach of Nested PCR (N-PCR) is often used to increase the specificity and yield of the desired amplicon. In this approach, two subsequent PCR reactions are carried out with the use of two pairs of primers. The first pair is the outer

primer set containing the specific amplicon, while the second pair of primers (nested) corresponds to the specific region of the desired amplicon (Fig. 1.23).

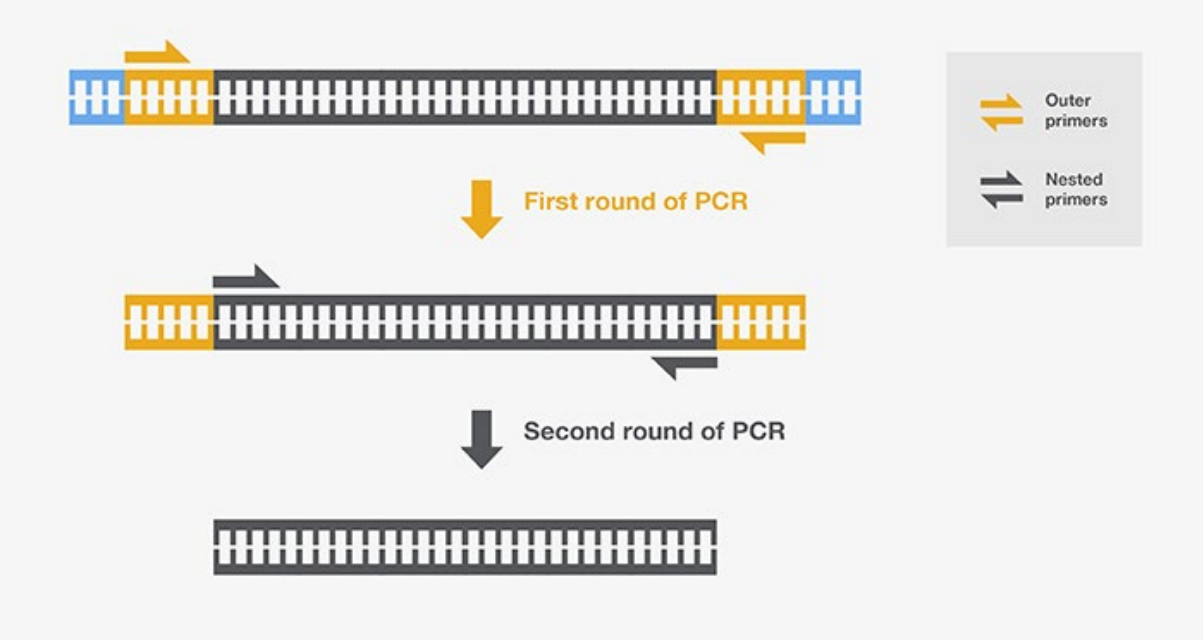


Fig. 1.23: Nested PCR (ThermoFisher Scientific). in cases of where nonspecific sections are amplified due mispriming by the outer primers, it is not very likely for the same nonspecific section to be amplified by the nested primer set, so specificity is still endorsed by the nested set of primers when recognising the intended amplicon.

In theory, one PCR amplification reaction containing about 32 cycles should produce about one billion copies of the targeted region (Butler, 2005). But several factors, like template degradation along with low copy number (LCN) of the template, can affect the rate of reaction. PCR inhibitors, like phenol along with other contaminants, have an adverse effect on the efficiency of the reaction. The relationship between the number of PCR cycles needed for desired copies of DNA template can be expressed as the following equation:

$$\mathbf{Nx = N_0\,(1 + E)^x}$$

where X is the number of PCR cycles; Nx is the copy number of the amplicon after x cycles of PCR; N_0 is the initial copy number of the template; and E is the PCR efficiency of the *Taq* polymerase. In cases where the efficiency is 100%, the PCR product is doubled in each cycle. The PCR product formed at the end of the amplification process is generally visualised using agarose gel electrophoresis (AGE). In AGE, DNA fragments are separated on the basis of their fragment length in comparison to a DNA ladder that is ran alongside with it (Johansson, 1972).

1.5.7 Real Time PCR (RT-PCR)

The development of RT-PCR was done in the 1990s to analyse per cycle change in the fluorescence signal caused due the amplification of the desired sequence of DNA (Li, 2015). This was introduced by Higuchi, along with his co-workers at the Cetus Corporation, who referred to it as 'kinetic analysis'; it is an extension on the work of Kary Mullis who first described PCR in 1985 (Higuchi *et al.*, 1992).

1.5.7.1 Components of RT-PCR

The components of RT-PCR are similar to that of PCR (already described in section 1.5.4.1). As well as the common components, a fluorescent reporter dye is also added to monitor the fluorescence level during the PCR amplification process. Generally, the fluorescence of the reporter molecule rises as products accumulate after every cycle of amplification.

The detection of the fluorescent signals is undertaken by the use of two methods, namely (i) TaqMan probe assay and (ii) SYBR green assay (Fig 1.24). The TaqMan probes are labelled with two different fluorescent dyes, the quencher and the reporter, which have different wavelengths. The probe sequence is intended to hybridise specifically to the target region of DNA between the two PCR primers (Wang & Brown, 1999). Typically, in a deliberate attempt for proper annealing, the probe is designed to have a slightly higher annealing temperature compared to the PCR primers. During the SYBR Green™ detection method, the fluorescent probe intercalates to double stranded DNA amplicons and, upon excitation, the fluorescence emission intensity can be detected by the instrument (Zipper *et al.*, 2004). Although the TaqMan probe assay is specific and more sensitive, it is costly and designing the specific assay can sometimes be complicated. On the other hand, SYBR green is cheap and commercially available. However, SYBR green dye intercalates to all the doubly bonded DNA amplicons, making it less specific, while TaqMan is highly specific, only binding to its complimentary sequence on the target DNA (Tajadini *et al.*, 2014). Therefore, the use of the SYBR green assay is beneficial in cases where no interfering amplicon exists at the same level of florescence (Arikawa *et al.*, 2008).

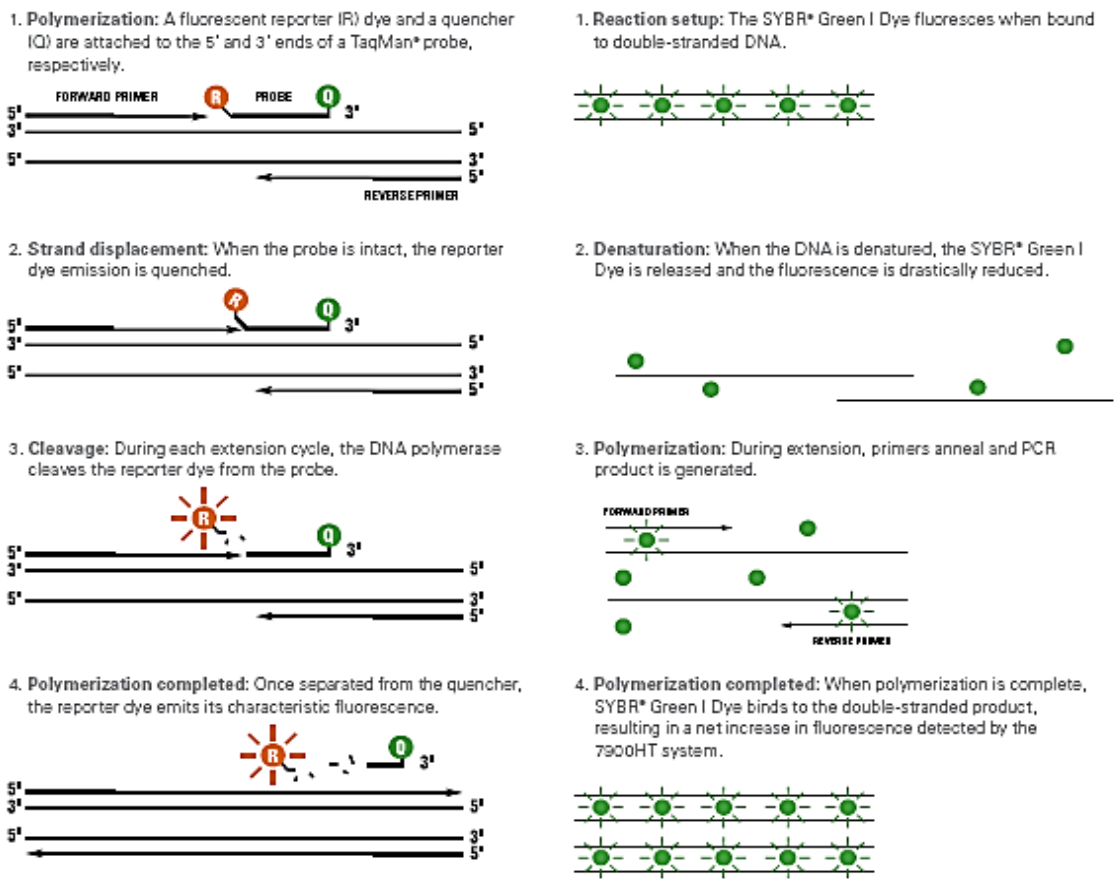


Fig 1.24: Comparison of the chemistry of the (A) TaqMan probe and the (B) SYBR green assay. In the TaqMan probe, a quencher and a reporter is present and, as the amplification process takes place, the reporter cleaves the quencher leading to fluorescence. SYBR green binds to the double bonded DNA strands, giving fluorescence as the amplification proceeds (retrieved from Biosynthesis, USA; Arikawa *et al.*, 2008).

1.5.7.2 Process of RT-PCR

The process of RT-PCR is divided into three distinct phases: (i) geometric/exponential amplification, (ii) linear amplification, and (iii) the plateau region (Fig. 1.25). These regions can be seen as a sigmoid-shaped amplification plot between fluorescence versus PCR cycle number, using a computer software (Swango *et al.*, 2006). Throughout the first phase, exponential amplification, a high extent of precision, with an efficiency close to 100% of formation of new PCR products, is observed. A plot is generated by the software in which cycle number (C_T) is plotted against log scale of the DNA concentration, resulting in a linear relationship during this phase. This phase is followed by the linear phase of amplification

process (Andréasson *et al.*, 2002; Butler, 2005). As the reaction proceeds, some components, like dNTPs and primers, are depleted leading to decrease in their concentration, hence turning them into a limiting factor for the reaction. In the final phase of RT-PCR, the plateau region, most of the components are either used up or degraded enough to not play an effective part in the assay leading to accumulation of PCR products. This slowly halts the assay, leading to phasing out of the fluorescence (Higuchi *et al.*, 1993; Li, 2015).

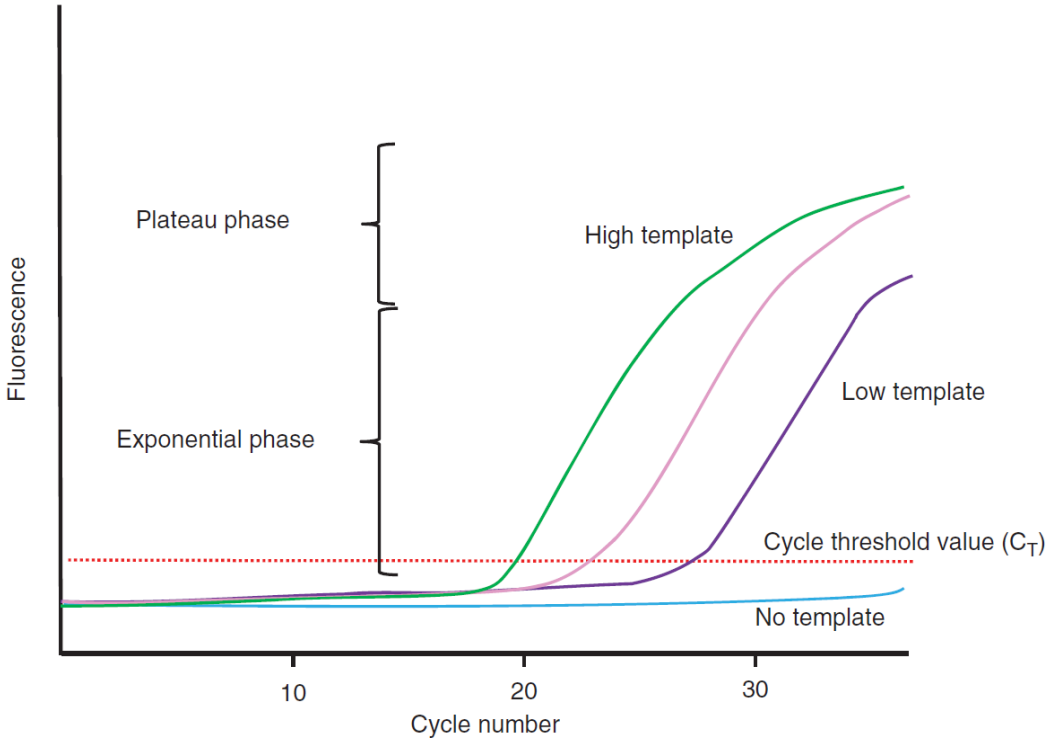


Fig 1.25: Amplification Plot of DNA (Godwin *et al.*, 2011). Four template showing different amounts of fluorescence. At the C_T value, the sample enters the exponential phase. The later the sample crosses the C_T, the lesser the amount of template in the sample (C_T -Cycle threshold).

Real-time PCR software uses the cycle threshold (C_T) value to plot a standard curve for the quantification of samples (Niederstätter *et al.*, 2007). An increase in fluorescence signal is observed during the cleavage of the TaqMan probes and intercalation of the SYBR green dye. This increase in fluorescence is correlated to the initial template (DNA) amounts when compared with with templates of known DNA concentration (Rutledge, 2004). A sample with an unknown DNA quantity can be compared to this standard curve to calculate its initial DNA template quantity (Fig 1.26). The melt curve analysis plot is formed during the analysis of SYBR green assay, due to its non-specific nature. In this, the temperature is slowly elevated after the amplification process is completed and the fluorescence is evaluated as the function of

temperature. When the sample reaches the melting temperature (Tm), the fluorescent dye separates leading to drop in fluorescence instantaneously. A characteristic melting peak at a particular Tm distinguishes the amplicon from its primer-dimers that melt at lower temperatures forming broader peaks.

Several real-time PCR assays like Plexor HY from Promega Corporation are available in the market for determining the amount of human DNA (Krenke *et al.*, 2008).

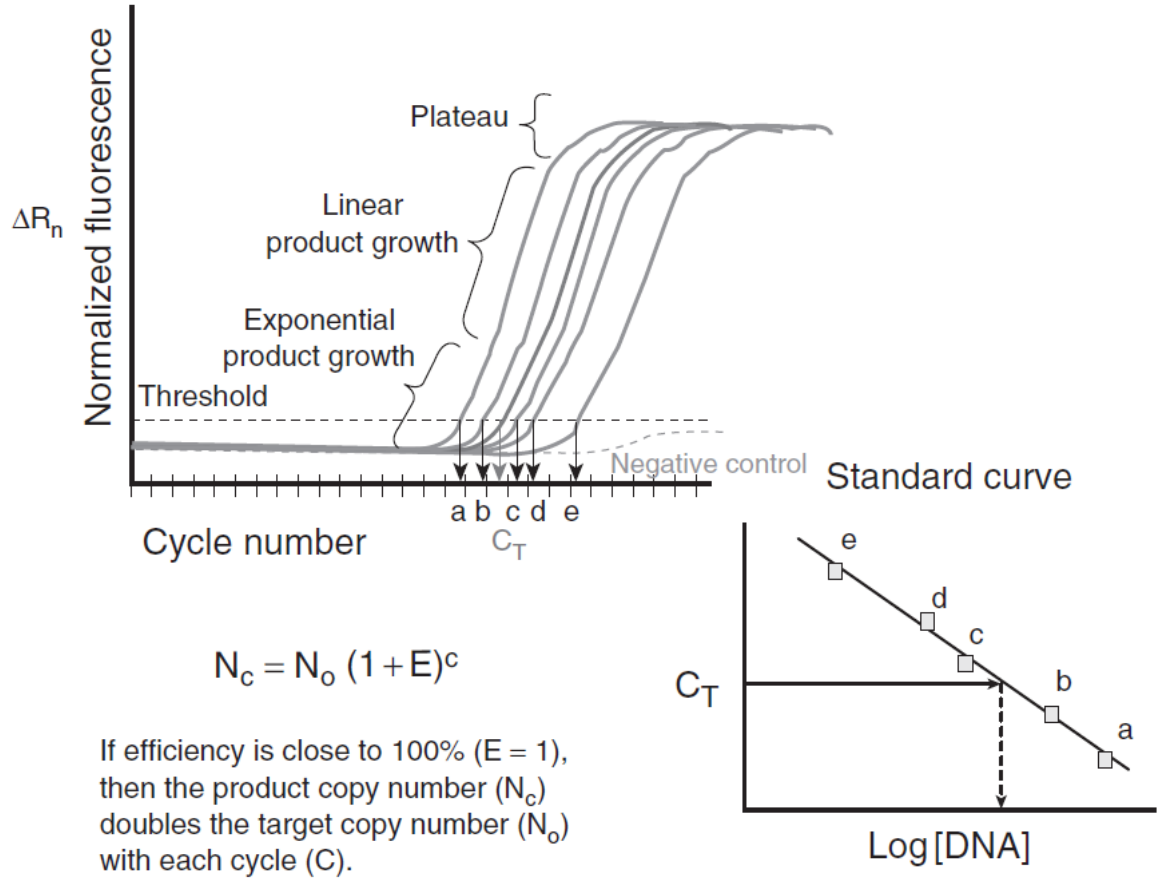


Fig 1.26: Real time PCR plot and the use of data to plot the standard curve (Butler, 2005); a, b, c, d, e are the DNA standard samples with a known quantity of DNA. A standard curve is prepared using these data and the unknown sample is plotted against them, thus giving the quantification of the unknown DNA.

1.5.8 DNA profiling - A Tool for Forensic Casework

The majority of the human genome is identical in every individual, but the presence of tandem repeats, like minisatellites and microsatellites, makes regions of the DNA polymorphic, and so a multidimensional tool in forensic casework.

Minisatellites were the first class of the tandem repeats to be described in the 1980s (Jeffreys *et al.*, 1985a), and they are commonly known as VNTRs. The analysis of the VNTRs is done by RFLP. The analysis uses specific restriction endonucleases which cleave unambiguous sites flanking the VNTR loci on the human DNA, producing restriction fragments of variable length (Gill *et al.*, 1985; Jeffreys *et al.*, 1985a,b). The fragments are separated by electrophoresis, and then transferred to a nitrocellulose membrane hybridising with a radioactive probe. Complementary bands binding to the probe are visualised with autoradiography. The length variation among different restriction fragments can be detected, making it useful for forensic casework. But, as already described in section 1.5.6, it is a time-consuming process. Moreover, the samples recovered from the crime scene are generally degraded, making it difficult for RFLP analysis as it requires the genomic DNA to be intact. It even requires a large amount of DNA for the analysis, making it less practical for use in forensic DNA analysis (Pejic *et al.*, 1998).

To overcome these problems, PCR has been used on the VNTR fragments, and is known as amplified fragment length polymorphism (AFLP). In this process, the VNTR loci, with sizes less than 1kb, are amplified using PCR, and fragments are visualised using silver stain after polyacrylamide agarose gel electrophoresis (PAGE) separation (Baechtel *et al.*, 1995). This process is fast and requires less material for analysis than RFLP; moreover, this method is also suited for degraded DNA. However, the discriminating power of the AFLP is reduced due to the presence of alleles which are common among populations, hence paving the way for multiplex system STR analysis in the late 1990s (Li, 2015).

Microsatellites are regions of DNA with a tandem repeat sequence of about 3-8 bp long. They are commonly known as short tandem repeats (STRs) or simple sequence repeats (SSRs) (Butler, 2005). STRs have become crucial in forensic casework as the analysis is simple and STRs are easily amplifiable using general PCR techniques. Moreover, STRs can be used with highly degraded samples, making it better than VNTRs analysis (Li, 2015). STR sequences can be found scattered all over the genome, and the number of repeats can be highly variable

among individuals, making it a very effective tool in human identification (Ellegren, 2004). In the genome, tandem repeats are present as dimeric, trimeric, tetrameric, pentameric and hexameric forms, but most of the STRs used for forensic purposes have a tetrameric nature (Urquhart *et al.*, 1994).

1.5.8.1 Components of STR analysis

The analysis of STRs begins with a multiplex PCR amplification. A commercially available kit contains all the loci required for the forensic case work. The loci are selected keeping in mind the discriminating power of the STR, which is calculated by the population match probability (Pm) the lower the Pm, the less chance of it to occur within a population. To keep the Pm low, high variable unlinked STR loci are selected. To make them useful in the analysis of degraded samples, short amplicon size loci are preferred for multiplex STR analysis kits.

The first STR based typing system was developed in the UK by the Forensic Science Services (FSS) in 1994, and contained 4 STR loci (Lygo *et al.*, 1994). To increase the discriminating power, the 4-locus system developed into 16 locus systems generally used by investigators around the globe (Table 1.8). Apart from the autosomal STR loci, the use of the amylogen locus to determine the sex of the individual has also been included in all commercially available kits (Godwin *et al.*, 2011).

QUAD	SGM	SGM® Plus	Identifiler®	PowerPlex®16
vWA	*Amelogenin*	*Amelogenin*	*Amelogenin*	*Amelogenin*
TH01	*vWA*	*D3S1358*	*D3S1358*	*D3S1358*
F13A1	*D8S1179*	*vWA*	*vWA*	*vWA*
FES	*D18S51*	*D16S359*	*D16S359*	*D16S359*
	TH01	*D8S1179*	*D8S1179*	*D8S1179*
	FGA	*D8S1179*	*D8S1179*	*D8S1179*
		D18S51	*D18S51*	*D18S51*
		TH01	*TH01*	*TH01*
		FGA	*FGA*	*FGA*
			D13S317	*D13S317*
			CSF1PO	*CSF1PO*
			D7S820	*D7S820*
			TPOX	*TPOX*
			D5S818	*D5S818*
			D2S1338	*PENTA D*
			D19S433	*PENTA E*

Table 1.8: The growth of STR systems. The quadruplex (QUAD) and SGM system were developed by the FSS, UK, with 4 and 7 STR loci respectively. The SGM Plus was developed in 1998 with 9 loci, and has been adopted for routine forensic casework by a large number of laboratories. The Identifiler and PowerPlex16 are the modern STR analysis kits, including the 13 CODIS loci. It also has its application in paternity disputes.

1.5.8.2 Process of STR analysis

The STR loci primers, labelled with fluorescent dyes, are amplified using PCR (already described in section 1.5.4.2). The amplified product is then separated by capillary electrophoresis with the use of a genetic analyser instrument. The instrument detects the different fluorescent dyes and the corresponding peaks are resolved with the use of computer software (data collection software). An electropherogram is generated, showing peaks with the corresponding STR locus, representing its size and data points (Fig 1.27).

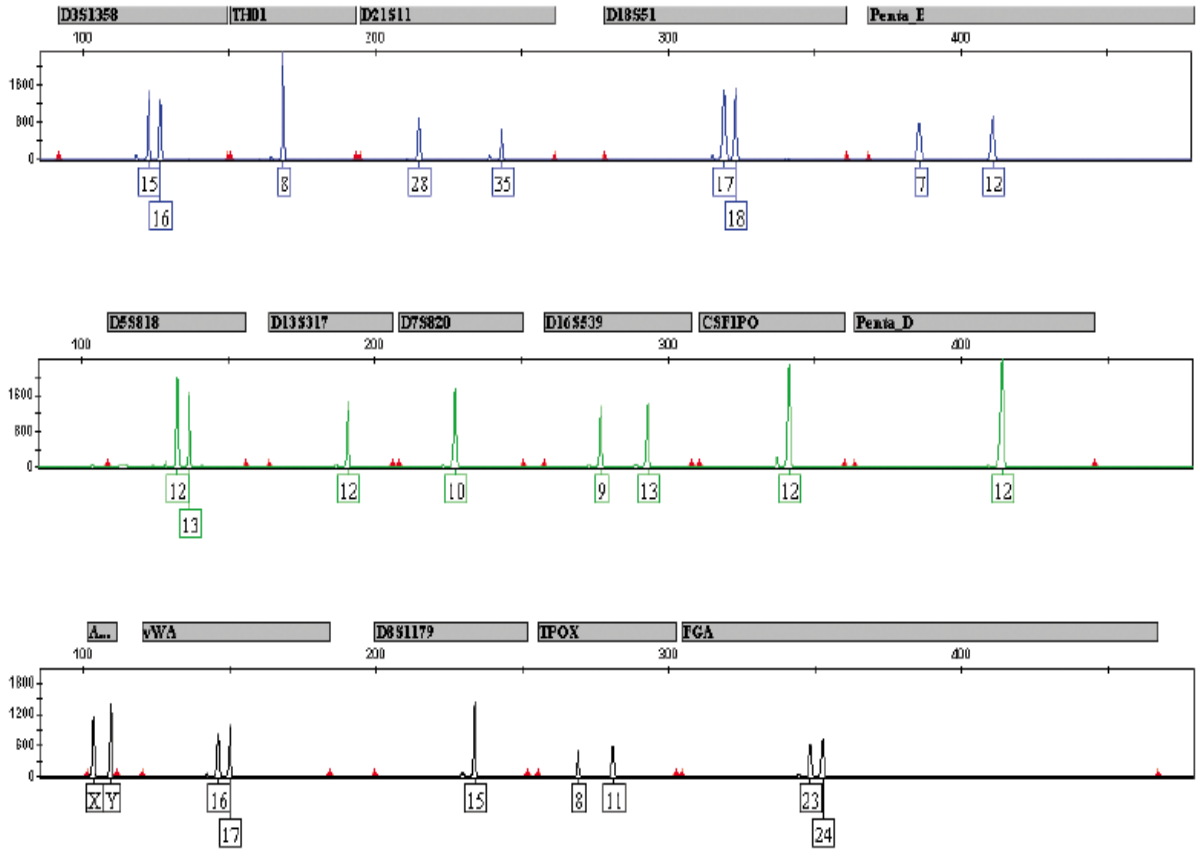


Fig 1.27: A typical electropherogram generated with the use of Powerplex®16 STR kit; The grey bands denote the STR loci along with their corresponding peaks (retrieved from Onwon Biotechnology Ltd.)

1.5.8.3 Interpretation of STR profiles

The data interpretation of electropherograms is very important in forensic DNA analysis. After data collection, the raw data is processed in Genemapper or Genescan software. These remove overlapping peaks and calculates the final size, as shown in Fig 1.27. The sizing is done with the help of an internal size standard (Liz™). The size standard is processed along with

the sample so any difference affecting electrophoresis can be easily identified (Godwin *et al.*, 2011). The height of the peaks is calculated in relative fluorescence units (RFU), which is generally set by the examining laboratory (Edwards *et al.*, 1991). After the analysis of the raw data, the profile is generated.

In criminal cases, one profile is made from the questioned sample and one from the reference sample. A match between the profiles establish a relation between the two profiles. Similarly, in cases of paternity disputes, the questioned sample of the progeny is matched with both the potential parents.

2. Aims

Since its inception into the criminal justice system, forensic DNA analysis has acted as a multifaceted tool in crime scene investigation over the last couple of decades due to its extraordinary power of discrimination. DNA materials as evidence are routinely collected from conventional sources (body fluids) from a wide range of crime scenes. In the absence of such conventional sources, DNA evidence can also be obtained from non-conventional sources, such as touch DNA (Meakin & Jamieson, 2013), empty pupariam (Marchetti *et al.*, 2013) and also from the gut contents of larvae (Wells *et al.*, 2001a). Most previous studies have focussed on the use of conventional methods of DNA extraction as a tool of investigation rather than the use non-conventional sources.

Megaselia scalaris is an important Dipteran of forensic interest with a cosmopolitan distribution (Disney, 2008). In cases of indoor crime scenes and buried corpses, the access of large necrophagus species of Calliphoridae and Sarcophagidae to the corpse is obstructed (Vanin & Huchet, 2017). However, smaller insects like *M. scalaris,* with its ability to crawl through tight spaces, is generally found colonising these bodies (Amendt *et al.*, 2004; Campobasso *et al.*, 2004; Pastula & merritt, 2013; Bugelli *et al.*, 2015), making it an important species for forensic investigators.

While most of the study of insects and their larval stages obtained from the crime scene has been undertaken for PMI estimation, like the study conducted by Bugelli *et al.* (2015), the use of gut contents from *M. scalaris* (Diptera, Phoridae) larvae for human identification has not been looked at.

My study aims are:

(i) develop a comprehensive framework to extract non-insect DNA from the gut contents of larvae of *M. scalaris*, fed on *Sus scorfa* meat, and using it for STR analysis, thus making it a tool for human identification.

(ii) identify a suitable fixing method for the collected larvae, not only for ease of dissection but also to maximise the yield of extracted DNA from the sample, further aiding crime scene officers in proper collection and preservation of samples.

3. Materials and Methods

3.1 Breeding of flies

Megaselia scalaris adults were taken from lines of flies bred from 2011 in the Forensic Laboratory for Entomology and Archaeology (FLEA), School of Applied Sciences, University of Huddersfield. Insects were maintained in a temperature-controlled cooled incubator (Panasonic MIR-I54) at 25°C in dark conditions. The flies were reared in glass jars (Byrd & Castner, 2009) and fed on pork meat (*Sus scrofa;* Linneaus 1758), obtained from a local butcher in Huddersfield, UK.

3.2 Preparation of larvae

Third instar larvae fed on pork meat were fixed using following 5 different protocols:

1. The larvae were kept in hot water (>80°C) for 40 seconds. Standard method described by Amendt *et al.* (2007).
2. The fresh larvae were directly stored at -20°C overnight. The larvae were then used for DNA extraction (Li *et al.,* 2011).
3. Fresh larvae were stored in EtOH (98%) in a glass bottle. The bottle was stored at -20°C overnight before DNA extraction (Linville *et al.,* 2004).
4. The larvae were stored in a glass bottle at -20°C for 4 hours and then EtOH (98%) was added to the bottle. The bottle was stored at -20°C (Linville *et al.,* 2004) overnight.
5. The larvae were kept in hot water (>80°C) for 40 seconds (Amendt *et al.,* 2007) and then EtOH (98%) was added. This was stored at -20°C in the freezer before DNA extraction (Di Luise *et al.,* 2008).

A schematic representation of the above methods is reported in Figure 3.1.

The fixed larvae were dissected using the protocol described by Tuccia *et al.* (2016) and the gut along with its contents were taken for examination. The dissection was carried out using sterilised pins and needles under a stereomicroscope (Leica MRZ). The larvae gut contents were weighed using an electronic balance (Thermofisher). The dissected gut contents were transferred to autoclaved 1.5 ml tubes containing extraction buffer, as described in section 3.3. The tubes were kept at room temperature before proceeding to DNA extraction.

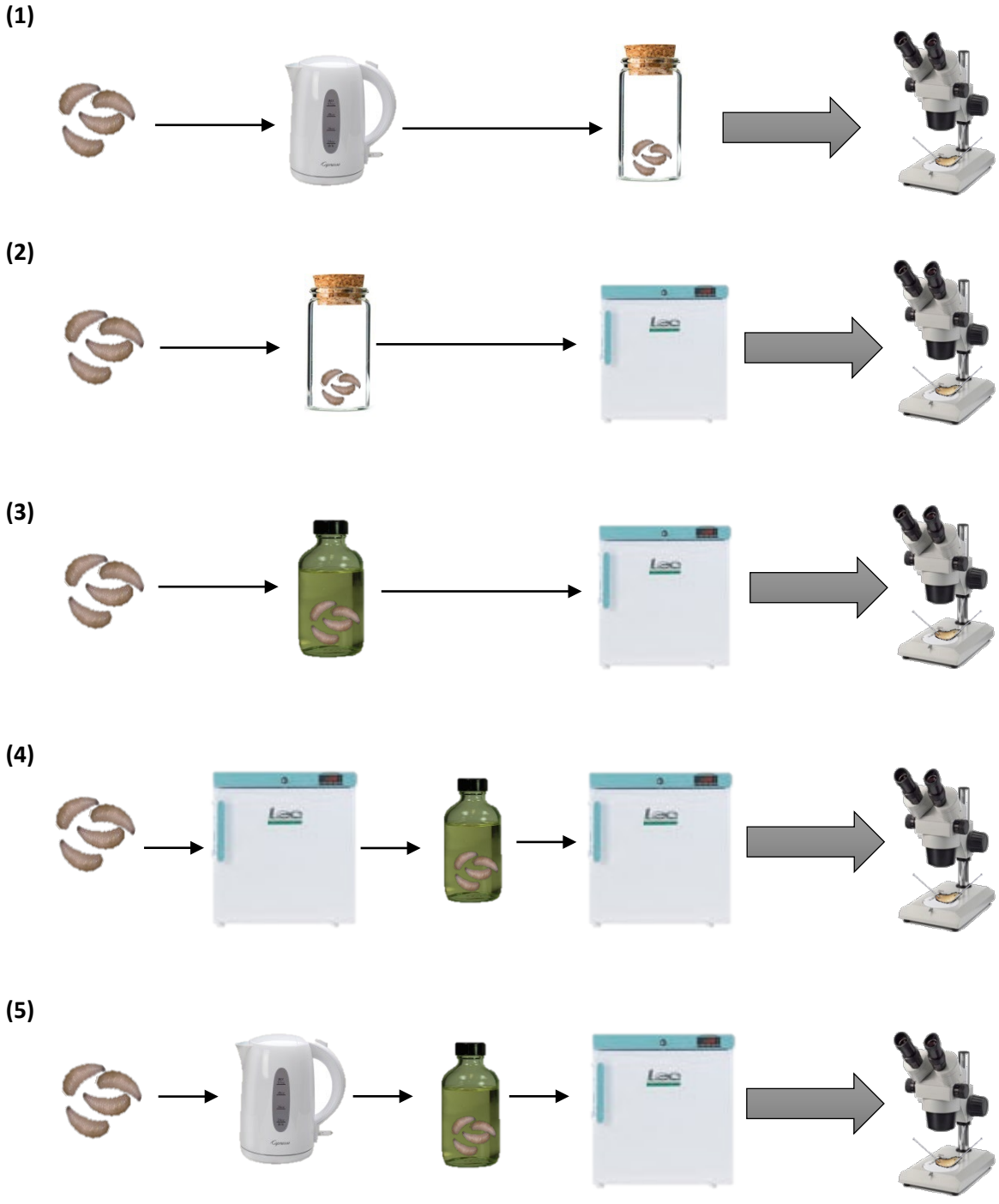


Fig.3.1: Fixing techniques for preparation of the larvae used. **(1)** The larvae were kept in hot water for 40 seconds (Amendt *et al.,* 2007) and were used for dissection. **(2)** Larvae were directly kept in the freezer at -20°C (Li *et al.,* 2011) overnight. **(3)** The larvae were kept in a glass bottle containing EtOH (98%) (Linville *et al.,* 2004) which was stored at -20°C overnight before proceeding to the dissection. **(4)** Larvae were kept at -20°C for four hours and then EtOH (98%) was added. The glass bottle was then kept at -20°C overnight (Linville *et al.,* 2004). **(5)** Larvae were kept in boiling water (Amendt *et al.,* 2007) and then transferred to a glass containing EtOH (98%) and stored at -20°C (Di luise *et al.,* 2008) overnight before dissection

3.3 Extraction of DNA

Before starting the process of extraction, the workspace was cleaned with DNA Away (Applied Biosystem) and EtOH (70%). Sterile aerosol-free micropipette and filter tips were used for the whole process of the extraction. Powder-free sterilised rubber gloves were used.

Three different types of DNA extraction kit were used to extract DNA from the gut contents of the larvae.

The first kit used was QIAamp® DNA Mini Extraction Kit and the second kit was Qiagen QIAamp® DNA Investigator Kit (Qiagen, Netherlands). The third kit was PrepFiler® Forensic DNA Extraction Kit (Applied Biosystems, USA). All three kits are solid-phase methods for DNA extraction, with Qiagen® kits having silica-based columns (Greenspoon *et al.*, 1998) while PrepFiler™ has magnetic particles (Barbaro *et al.*, 2009).

The extractions were carried out according to the user manual procedures provided by the kit suppliers, with some volumetric modifications adapted by the laboratory to improve the yield of DNA as described in the following sections.

3.3.1 DNA Extraction using QIAamp® DNA Mini Extraction Kit

The larvae gut contents were placed into a 1.5ml tube containing 180µl of Buffer ATL. The suspended tissue was crushed into smaller pieces using a sterile plastic pestle. 20µl proteinase K (100µg/ml) was added and was mixed by vortexing for 15 seconds. The samples were incubated at 56°C overnight for better yield of extracted DNA. Thermoshakelite™ with temperature at 56°C and 300RPM was used for the incubation process. After an overnight incubation, the samples were briefly centrifuged. To obtain RNA-free genomic DNA, 4µl RNase A (4mg/ml) (Promega) was added and mixed by pulse-vortexing for 15 seconds. The sample was then incubated for 2 minutes at room temperature (20–25°C). The tube was briefly centrifuged and 200µl Buffer AL was added to the sample. The samples were mixed again by pulse-vortexing for 15 seconds, and incubated at 70°C for 10 minutes. After a brief centrifugation step, 200µl EtOH (98%) was added to the sample, and mixed by pulse-vortexing for 15 seconds. All the lysate (along with the precipitate) was transferred to a QIAamp Minispin column (in a 2ml collection tube) and centrifuged at 8000RPM for 1 minutes. The QIAamp Minispin column was placed in a clean 2ml collection tube, discarding the filtrate. Each spin column was closed to avoid aerosol formation during centrifugation. 500µl Buffer

AW1 was added to the QIAamp Minispin column. The tube was centrifuged at 8000RPM for 1 minute. The QIAamp Minispin column was placed in a clean 2ml collection tube, and the collection tube containing the filtrate was discarded. 500µl Buffer AW2 was added to 1.5ml tube, and it was centrifuged at full speed (14000RPM) for 3 minutes. The QIAamp Minispin column was placed in a clean 1.5ml tube and the collection tube containing the filtrate was discarded. The DNA sample was eluted in 200µl Buffer AE, incubated at room temperature for 1-3 minutes, and centrifuged at 8000RPM for 1 minute. The eluted samples were stored at -20°C untill being used for further analysis (Qiagen, 2016).

3.3.2 DNA extraction using QIAamp® DNA Investigator Kit

The dissected sample was transferred to a 1.5ml micro-centrifuge tube, which already contained 180µl Buffer ATL. 20µl proteinase K (Qiagen®) was mixed by pulse-vortexing the micro-centrifuge tube for 15 seconds. The 1.5ml tube was placed in a thermomixer incubator at 56°C overnight. 200µl Buffer AL was added alongside 4µl carrier RNA to the sample. 200µl EtOH (98%) was added to the sample and it was mixed thoroughly by pulse vortexing the tube for 15 seconds. The sample was incubated for 5 minutes at room temperature. The samples were briefly centrifuged to remove drops from the inside the lid. Carefully the entire lysate was transferred to the QIAamp MinElute column (in a 2ml collection tube) without wetting the rim and was centrifuged at 8000RPM for 1 minute. The QIAamp MinElute column was placed in a clean 2ml collection tube and the collection tube containing the flow-through was discarded. Carefully the QIAamp MinElute column was opened and 500µl Buffer AW1 was added without wetting the rim. The lid was closed and the column was centrifuged at 8000RPM for 1 minute. The QIAamp MinElute column was again placed into a clean 2ml collection tube and the collection tube containing the flow-through was discarded. 700µl Buffer AW2 was added to the QIAamp MinElute column. The sample was centrifuged at 8000RPM for 1 minute. The QIAamp MinElute column was placed in a clean 2ml collection tube, and the collection tube containing the flow-through was discarded. After carefully opening the QIAamp MinElute column, 700µl of EtOH (98%) was added. It was centrifuged at 8000RPM for 1 minute. The MinElute column was placed in a collection tube and, centrifuged at 14000RPM to ensure all the ethanol had been spun through. The QIAamp MinElute column was then placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the

flow-through was discarded. The sample was incubated at room temperature for 10 minutes. 100 µl Buffer ATE was added to the sample in the centre of the membrane in order to elute DNA. The lid was closed and the sample was incubated at room temperature for 1 minute. The sample was centrifuged at full speed (14000RPM) for 1 minute. The eluted DNA was stored at -20°C for downstream processing (Qiagen, 2012).

3.3.3 DNA extraction using PrepFiler® Forensic DNA Extraction Kit (Applied Biosystems™)

The dissected sample was placed in a 1.5ml tube, and 250µl PrepFiler™ Lysis Buffer and 3µl of 1M DTT was added. The tube was mixed by pulse vortexing for 5 seconds. The tube was then incubated at 70°C on a Thermalshakelite™ at 900RPM for 30 minutes. Temperature equilibrium of the sample with the environment was obtained by leaving it for 5 minutes at room temperature. 15µl of magnetic particles were added to the sample lysate and it was centrifuged briefly before adding 180µl of isopropanol (100%). The sample was mixed at room temperature at 1000RPM for 10 minutes, and was vortexed to re-suspend the magnetic particles. The sample tube was placed in a magnetic stand for 2 minutes. The liquid phase was discarded with the help of a pipette. 300µl of PrepFiler™ Wash Buffer was added to the tube and it was vortexed for 5 seconds. The tube was once again placed in the magnetic stand for 2 minutes, and the liquid phase was discarded. The above two steps were repeated three times. The magnetic-bound DNA was dried at room temperature for 10 minutes. For elution of DNA, the sample tube was placed in a thermal shaker kept at 70°C with ramp speed 900RPM for 5 minutes, while 50µl elution buffer was added to the sample. After the incubation, the tube was vortexed briefly and was placed in the magnetic stand for 2 minutes. The liquid phase was transferred to a fresh 1.5ml tube. This liquid phase contained the extracted genomic DNA (Applied Biosystems, 2008), which was stored at -20°C for downstream processes.

Control samples were also extracted for each type of sample following the previously mentioned methods. In addition, DNA was extracted directly from the meat that was feed to the insects, and from the larval tissue.

3.4 Quantification using Invitrogen™Qubit® 3.0 (Life technologies, USA)

The assay was performed according to the user guide recommended by the manufacturer (Qubit® fluorometer - dsDNA Assay User Guide, 2015). A solution was prepared using 199µl of Qubit® dsDNA High Sensitivity Buffer and 1µl of Qubit® Fluorophore per sample. For the analysis, 199µl of the solution was taken and 1µl of the extracted DNA sample was added to Qubit® assay tubes. The assay tubes were inserted into the fluorometer for analysis. The readings, expressed in ng/µl, were taken in triplicate. The average and SD of the readings were calculated and recorded.

3.5 Polymerase chain reaction

The PCR workstation was cleaned and sterilised using DNA Away (Applied Biosystem) and EtOH (70%). Sterile aerosol-free tips and micropipettes were used for the process. Powder-free rubber gloves were used during the whole process of the PCR.

All the extracted DNA samples were subjected to PCR amplification. Multiple PCR assays were setup and a specific primer set was used for each type of sample (gut contents and larval tissue). PCRs were carried out on a Bio-Rad C1000 thermocycler (Bio-Rad Laboratories, USA).

3.5.1 Designing of specific primers

Species-specific primers were designed for each type of sample after reviewing the literature (Tuccia *et al.* 2016). Samples of DNA extracted from the larval tissue were amplified using the universal invertebrate specific COI primers (Folmer, 1994) targeting a conserved region (658 bp) within the mitochondrial gene coding for Cytochrome Oxidase subunit 1. Similarly, a nucleotide sequence of 1140 bp internal to the mitochondrial cyt *b* gene was amplified using primers designed by Naidu *et al.* (2011). This primer set is specific to mammals and was used to target DNA in the gut contents. Another set of cyt *b* primers (Verma *et al.*. 2002) (mammalian specific) was used to amplify a DNA sequence of 472 bp within the conserved region of 1140 bp described, above performing a nested PCR in order to increase the specificity of the reaction. Based on the different size of the targets produced by the two set of cyt *b* primers, the words “long” and “short” will be used further in this dissertation referring respectively to the 1140 bp and 472 bp amplicons. Pig specific cyt *b* primers (Soares *et al.* 2013) were used to amplify a portion of 149 bp of the mitochondrial gene. Another pig specific

primer set (Lee *et al.*. 2016) was used to amplify a sequence of the 16S ribosomal RNA gene (138bp). Specific primers used in multiple PCR assay with their probe name, probe sequence and fragment size are summarised in Table 3.1.

Primer	Primer Name	Primer Sequence (5'-3')	Fragment size(bp)
COI (Folmer, 1994)	LCO	GGTCAACAAATCATAAAGATATTGG	658bp
	HCO	TAAACTTCAGGGTGACCAAAAAATCA	
Cyt *b* (long) Mammals (Naidu *et al.*., 2011)	MTCB-F	CCHCCATAAATAGGNGAAGG	1140bp
	MTCB-R	WAGAAYTTCAGCTTTGGG	
Cyt *b* (short) Mammals (Verma *et al.*, 2002)	mcb398	TACCATGAGGACAAATATCATTCTG	472bp
	mcb869	CCTCCTAGTTTGTTAGGGATTGATCG	
Cyt *b* Pig (Soares *et al.*, 2013)	Cyt *b* pork F	ATGAAACATTGGAGTAGTCCTACTATTTACC	149bp
	Cyt *b* pork R	CTACGAGGTCTGTTCCGATATAAGG	
16s rRNA Pig (Lee *et al*., 2016)	16S SFI11 Pig F	CAACCTTGACTAGAGAGTAAAACC	138bp
	16S SFI11 Pig R	GGTATTGGGCTAGGAGTTTGTT	

Table 3.1 Different primers used for specific PCR assays in this study. COI (Folmer, 1994) were used for DNA of larval tissue samples. Cyt *b* (long) Mammals (Naidu *et al*., 2011), cyt *b* (short) Mammals (Verma *et al*., 2002), cyt *b* pig (Soares *et al.,* 2013) and 16s rRNA pig (Lee *et al*., 2016) were used for DNA extracted from gut contents of the larvae.

3.5.2 DNA amplification using PCR

(i) PCR

PCR amplifications were carried out in a final volume of 20µl in PCR assay tubes (Star Lab, UK) containing 4µl of DNA template. The PCR assay mix contained 4µl of 5X GoTaq Flexi® Buffer (Promega, USA), 4µl of 25mM $MgCl_2$ (Promega, USA), 0.5µl each of forward and reverse primer (10 pmol/µl), 0.5µl of dNTPs (10mM), 0.25µl GoTaqG2 (5u/µl) polymerase and 6.25µl of Ultrapure™ PCR grade water (Invitrogen, USA). After an initial denaturation, for all the steps (Denaturation, Annealing and Elongation) the thermal cycler was set at different temperatures and for different durations for each primer set (Table 3.2). This was followed by a final elongation step of 72°C for 10 minutes. Each amplification was repeated over 30 cycles.

Primer Set	Sample type used for amplification	Denaturation temperature and duration	Annealing temperature and duration	Extension temperature and duration	Number of cycles
COI	Larval tissue	95°C 10 minutes	49.8°C 1 minute	72°C 1 minute	30
Cyt *b* long	Gut contents	95°C 10 minutes	55°C 1 minute	72°C 1 minute	30
Cyt *b* short	Gut contents	95°C 10 minutes	51°C 1 minute	72°C 1 minute	30
Cyt *b* pig	Gut contents	95°C 10 minutes	60°C 1 minute	72°C 1 minute	30
16s rRNA pig	Gut contents	95°C 10 minutes	58°C 1 minute	72°C 1 minute	30

Table 3.2: Different temperatures and durations of each step of PCR for specific primer set.

(ii) Nested PCR (N-PCR)

As previously described in the introduction (Section 1.5.6), this is a three-step PCR, with amplification using the combination cyt *b* (long) and *cyt b* (short) primer sets. In the first step, the sample was amplified using the primer set MTCB-F and MTCB-R (*cyt b* long) in a final volume of 20µl containing 4µl of DNA template. The PCR assay mix was made with the same specifications as described above. PCR amplifications proceeded after an initial denaturation step of 10 minutes at 95°C, for 30 cycles. Each cycle composed of three steps of 95°C for 30s, 55°C for 30s and 72°C for 45s. This was followed by final elongation step at 72°C for 10 minutes. The PCR product was stored at 4°C for further use. In the second step, the PCR product was purified using a PCR purification kit (Qiagen®, Netherlands). This procedure was followed according to the user manual provided by the kit supplier (QIAQuick®, 2015). The purified product was stored at -20°C until further use. In the final third step, a PCR assay was set up in a final PCR volume of 20µl with 4µl of the DNA template obtained from step 2. The PCR assay mix was made with the same specification as described, but with the primer set of mcb398 and mcb869. Amplifications were preceded after an initial denaturation step of 10 minutes at 95°C, for 30 cycles. Each cycle composed of three steps of 95°C for 30s, 51°C for 30s and 72°C for 45s. This was followed by final elongation step at 72°C for 10 minutes. The product was stored at 4°C for further usage.

3.6 Gel electrophoresis

The visualisation of the amplified PCR products was undertaken via electrophoresis using a 1.5% W/V agarose gel. The gel was made using 1.5g of agarose (Sigma-Aldrich) in 100µl of 1X TBE running buffer. 3µl of Midori green (Nippon Genetics) visualisation dye was added. The gel was placed in an electrophoresis unit (Bio-Rad) containing 1X TBE running buffer. 5µl of PCR product and 2µl Blue/orange dye 6X (Promega, USA) was mixed together and loaded into the wells of the gel. The running voltage was set at 100 V for 45 minutes.

The gel was visualised on a UV gel doc system (Syngene Bio Imaging System) using Gene Snap (SynGene) software. The size of the fragments was estimated using a 100bp DNA ladder (Promega, USA).

3.7 DNA purification

The PCR products were purified using a PCR purification kit (Qiagen®, Netherlands). The procedure was followed according to the user manual provided by the kit supplier (QIAQuick®, 2015). The sample was eluted in 30µl AE elution buffer. It was stored at room temperature. It was sent for sequencing to Eurofins Genomics (Ebersberg, Germany). The sequences obtained were analysed using blast (Altschul *et al.,* 1990).

3.8 Real Time PCR / Quantitative PCR

Apart from the end-point PCR, Real Time PCR (RT-PCR) of the DNA extracted from the gut contents was also performed. The RT-PCR was performed on a 7500 Fast Real Time PCR instrument (Applied Biosystems, USA).

3.8.1 Designing of specific primers

The primers used were cyt *b* pig (Soares *et al.* 2013) and 16s rRNA pig (Lee *et al*. 2016), specific to *Sus scrofa* (Table 3.2).

3.8.2 PCR amplification

The amplification was undertaken in the total volume of 20µl on a MicroAmp® Fast optical reaction plate (Life Technologies, USA) containing 5µl of DNA template. The PCR assay mix was made using 10µl of SYBR® green (Applied Biosystems, USA), 0.5µl each of forward and reverse primers and 4µl of Ultrapure™ PCR grade water (Invitrogen, USA). The plate was sealed using MicroAmp™ Optical Adhesive Film (Applied Biosystems, USA), centrifuged (1200RPM) for 40 seconds, and loaded into the instrument. Amplification was proceeded, after the initial holding stage of 95°C for 10 minutes, for 40 cycles. Each cycle composed of two steps of 95°C for 15 seconds and 60°C for 60 seconds. The amplification was followed by melt curve analysis, with an initial holding phase of 95°C for 15 seconds, followed by 60°C for 1minute, 95°C for 30 seconds and with 60°C for 15 seconds (Fig. 3.2).

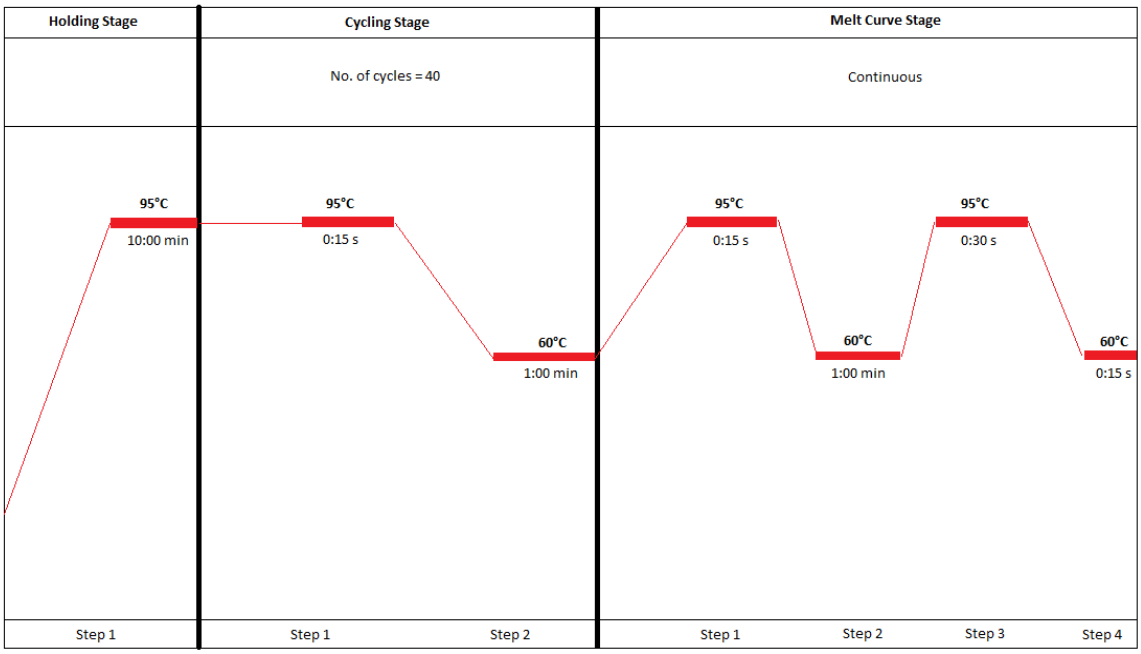


Fig. 3.2: Graphical view of the run method using 7500 software v2.3 on 7500 Fast RT-PCR instrument (Applied Biosystems, USA).

3.8.3 RT-PCR analysis software

The reaction was setup using the 7500 software v2.3. A standard quantification run method with standard (~2h) ramp speed was selected. The amplification curve, standard curve and quantification of the samples were obtained using the above method.

3.9 Genetic Analysis

The samples of DNA extracted from the gut contents of larvae were genotyped for a set of 12 microsatellites (*387A12F, S0655, SBH1, SBH2, SBH4, SBH10, SBH13, SBH18, SBH19, SBH20, SBH22, SBH23 X/Y*) recommended by the International Society of Animal Genetics (ISAG) for a STR profile (Table 3.3). The genetic profiling was performed using an automatic sequencer, 3130 Genetic Analyser (Applied Biosystems, USA).

3.9.1 PCR amplification

Biotype®Animaltype Pig PCR Amplification Kit (Biotype® GmbH, Germany), containing the above locus-specific primers, was used for the analysis. The procedures were carried out according to the user handbook provided by the kit supplier with some volumetric modification (Biotype®, 2016). The amplification was performed in the final volume of 25µl containing 3µl of DNA template. The PCR assay mix was made using 14.1µl of nuclease-free water, 5µl of reaction mix D, 2.5µl of primer mix and 0.4µl of Multi Taq2 DNA polymerase. For the positive control and the negative control, control DNA DL 157 and nuclease free water provided by the supplier was used respectively. The total amount of DNA used in the above reaction was 5ng. The amount of the DNA template was adjusted to appropriate volume of 3µl by diluting it with nuclease-free water.

The amplification was proceeded on a thermocycler (Bio-Rad C1000) after an initial activation step of 94°C for 4 minutes for 30 cycles. Each cycle composed of three steps of 94°C for 20s, 60°C for 40 seconds and 72°C for 30 seconds, followed by a final elongation step 70°C for 60 minutes. The PCR products were stored at 4°C until further analysis.

Locus	Repeat Motif of the Reference Allele	Reference Allele	Allele range
387A12F	$[TTCT]_2$ CT $[TTCT]_{19}$	21	9-21
S0655	$[GGAA]_{12}$	12	5-22
SBH1	$[CTTT]_{13}$	13	7-18
SBH2	$[AGAA]_{24}$AA [AGAA}	25	6-34
SBH4	$[GAAA]_2$GGAA $[GAAA]_2$A $[GAAG]_7$[GAAA] [GAAG] [AAAG] $[AGAG]_5[AAAG]_6$ AA$[AAAG]_4$A $[AAAG]_3$AA $[AAAG]_4$A $[AAAG]_{21}$AG $[AAAG]_3$AGAG $[AAAG]_2$	64	47.3-66.1
SBH10	$[TAGA]_{15}$ $[CAGA]_{12}$ $[TAGA]_7$TACA [TAGA]TACA [TAGA]TACA $[TAGA]_2$TACA $[TAGA]_2$TACA $[TAGA]_2$CAAA	48	31-50
SBH13	$[TATC]_{15}$	15	8-18
SBH18	$[AGGA]_{15}$	15	9-23
SBH19	$[GTCT]_4[ATCT]_{10}$	14	10-16
SBH20	$[CTTT]_{14}$CTTC $[CTTT]_2$CTTC $[CTTT]_2$CTTC $[CTTT]_3$	24	19-49
SBH22	$[ATAG]_6$ ATG $[ATAG]_{11}$ ATG $[ATAG]_3$	20	18-28
SBH23 Y			-
SBH23 X			-

Table 3.3: Locus-specific information of Animaltype Pig Locus. The repeat motifs are concordant with the International Society for Forensic Genetics (ISFG) guidelines for the use of microsatellite markers (Bär *et al*., 1997).

3.9.2 Capillary Electrophoresis

Analysis was undertaken on an automated analyser 3130 (Applied Biosystems, USA) with POP-4 polymer (Applied Biosystems, USA). 1µl of the above PCR product was mixed with 12µl of Hi-Fi™ formamide (Applied Biosystems, USA), and 0.2µl of ROX labelled DNA size standard (Biotype® GmbH, Germany) was loaded into MicroAmp® Fast optical reaction plate (Life Technologies, USA). The plate was sealed using a 96 well plate septa (Life Technologies, USA) and was briefly centrifuged at 1000RPM. The samples were denatured at 95°C for 3 minutes and cooled at 4°C for three minutes using a thermocycler (Bio-Rad C1000). The MicroAmp® Fast optical reaction plate combined with Fast Plate retainer and base (Life Technologies, USA) was loaded into the plate bay of the analyser. Analysis of the samples was done according to the protocols prescribed by the kit supplier, illustrated in Table 3.4 (Biotype®, 2016).

Run Module Editor	Values
Oven Temperature	60 °C
Current Stability	6 µA
Pre run voltage	15 kV
Pre run time	180 s
Injection voltage	3 kV
Injection time	5 s
Run voltage	15 kV
Run time	1440 s

Table 3.4: Run module editor. It defines the conditions of the 3130 Genetic Analyser (ABI) used during the capillary electrophoresis (Biotype®, 2016)

3.9.3 Data collection

The plate setup and data collection of the samples was done using data collection software v2.3 according to the user manual supplied by the kit supplier (Biotype®, 2016). DNA fragments of the sample resulted in sequencer electropherograms, which were analysed using Genemapper v 3.2 software (Applied Biosystems, USA). For every sample, 50 RFU was setup as the threshold for peak detection and allele calling, a rule established by the laboratory where these analyses took place.

3.10 Statistical analysis

The weight of the dissected larval guts and the quantification result of the extracted DNA samples, statistical analysis was carried out in order to evaluate the results, using the IBM® SPSS Software. Comparison between the samples was performed with an ANOVA test (one-way and two-way), which was then followed by a Post Hoc analysis (Tukey HSD). The significance value was set up at 0.05. P values obtained higher than this number were considered as non-significant (acceptance of the null hypothesis, Ho, of no difference between the samples). Values obtained below the threshold of 0.05 denoted significance (rejection of the Ho). All of the descriptive values are reported as mean ± standard deviation (SD).

4. Results

4.1 Weight of gut of larvae fixed by different techniques

The larvae were dissected as already discussed (section 3.2) and the gut content obtained was weighed using an electronic balance (Thermo Fisher, USA). The dry weight of the samples was also recorded after briefly (40 seconds) keeping them in the oven at 60°C. All readings were recorded in triplicate.

4.1.1 Wet weight

According to the evaluation performed, after the dissection, wet weight of the gut content of the larvae fixed with hot water (>80°C) (method 1) was found out to be the maximum, while the larva fixed first by boiling and then storing in EtOH at -20°C (method 4) gave the least amount. A one-way ANOVA test was conducted. It demonstrated the presence of a significant statistical variation among the different methods used for fixing the larvae (F=369.000, df=4, p-value=0.000). A post-hoc statistical analysis (Tukey HSD) was also performed to identify the variation amongst the methods used. The analysis showed that the wet weight of gut content obtained after the dissection of the larva with method 1 (larva fixed with boiling water) and with method 3 (larva fixed with only EtOH at -20°C) varied significantly to other methods (p-value= 0.000) (Fig. 4.1). The detailed post-hoc analysis results can be found in Appendix 1, Table 1.

A two-way ANOVA was performed, which revealed that, individually, temperature (frozen or hot water) and EtOH (presence or absence) had a significant effect (p-value= 0.000) on the amount of gut content obtained. Moreover, the interaction between these individual variables also displayed a significant statistical variation (p-value=0.000) (Table 4.1).

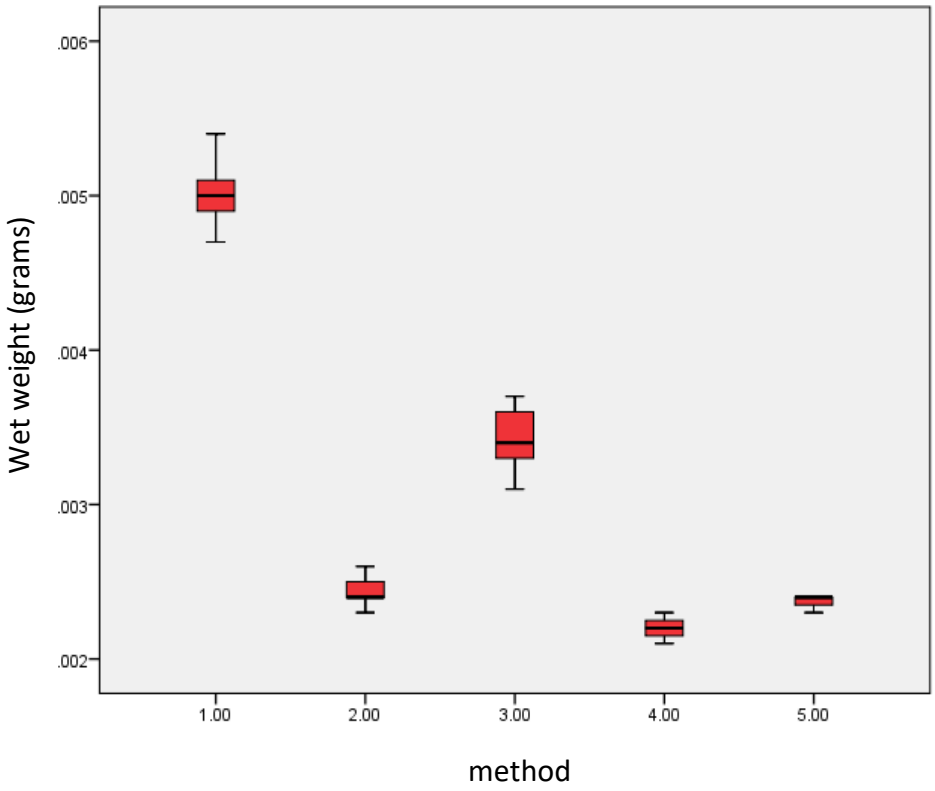


Fig. 4.1: Wet weight (gram) comparison of 10 dissected larval crops fixed with different methods: (1) larvae fixed by hot water (>80°C); (2) larvae fixed by freezing at -20°C; (3) larvae fixed in EtOH at -20°C; (4) larvae first fixed by hot water and then storing at EtOH at -20°C; and (5) larvae frozen at -20°C and stored in EtOH at -20°C.

Source	df*	F	Significance(p-value)
Temperature	1	35.165	0.000
EtOH	1	61.220	0.000
Temperature * EtOH	1	171.492	0.000

*degree of freedom

Table 4.1: Two-way ANOVA of temperature and EtOH (wet weight). Effect of temperature and EtOH, and the interaction between them, has a significant statistical variation (p-value< 0.05) in the wet weight of the gut contents of the larvae.

4.1.2 Dry weight

Upon analysis, the dry weight of the gut content of the larvae fixed with freezing at -20°C followed by storage in EtOH at -20°C (method 5) displayed the maximum amount, while the larva fixed by first boiling and then storing in EtOH at -20°C (method 4) gave the least amount. Similar to wet weight analysis, a one-way ANOVA test was conducted, which confirmed the presence of a significant statistical variation ($F=10.157$, $df=4$, p-value=0.000) among the different methods used for fixing the larvae. A post-hoc statistical analysis (Tukey HSD) was also performed to identify the variation amongst the methods used. Though the analysis exhibited variation amongst the methods, no method varied statistically from the other methods. The dissection of the larva with method 5 (larvae frozen at -20°C and stored in EtOH at -20°C) and with method 1 (larva fixed with boiling water) varied statistically with the three other methods of fixation, with a p-value < 0.05 (Fig. 4.2). The detailed Post-Hoc analysis results report can be found in Appendix 1, Table 2.

A two-way ANOVA was performed, which revealed that, individually, temperature (frozen or hot water) and EtOH (presence or absence) had no significant effect (P-value> 0.05; temp. p-value$= 0.384$; EtOH p-value$= 0.291$) on the amount of gut content obtained. In contrast, the interaction between these individual variables displayed a significant statistical variation (p-value<0.05) on the amount of gut content obtained (Table 4.2).

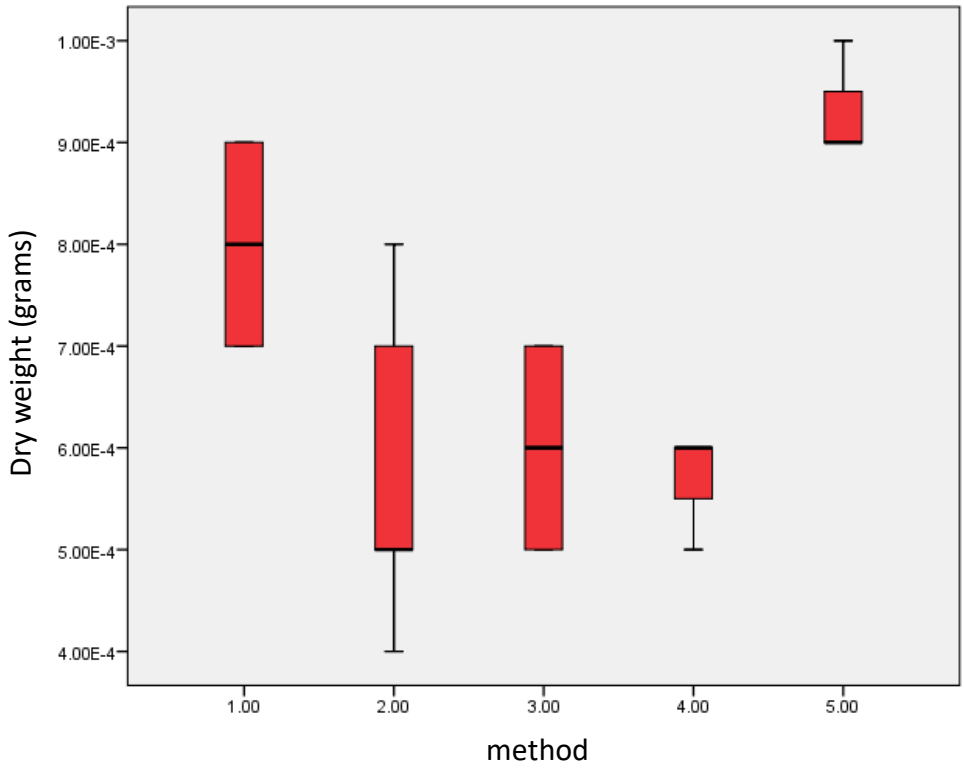


Fig. 4.2: Dry weight (gram) comparison of 10 dissected larval crops fixed with different methods: (1) larvae fixed by hot water (>80°C); (2) larvae fixed by freezing at -20°C; (3) larvae fixed in EtOH at -20°C; (4) larvae first fixed by hot water and then stored at EtOH at -20°C; and (5) larvae frozen at -20°C and stored in EtOH at -20°C.

Source	df*	F	Significance (p-value)
Temperature	1	0.781	0.384
EtOH	1	1.156	0.291
Temperature * EtOH	1	7.549	0.010

*Degree of freedom

Table 4.2: Two-way ANOVA of temperature and EtOH (dry weight). Effect of temperature and EtOH does not have a significant statistical variation, while their interaction had a significant statistical variation (p-value< 0.05) on the dry weight of the gut contents of the larvae.

4.2 DNA quantification

In the first part of the quantification, DNA extracted from the gut content of larvae fixed with hot water (>80°C), using three different DNA extraction kits as described (section 3.2), was quantified using Invitrogen™ Qubit® 3.0 in triplicate. The extraction was performed in set of 1 crop, 3 crops and 5 crops. A one-way ANOVA and post-hoc (Tukey HSD) analysis of DNA extracted per crop using three different kits revealed a significant statistical difference (p-value= 0.000) amongst them (Table1-3, Appendix 2). DNA extracted using QIAamp® DNA Mini Extraction kit gave the minimum quantity, whereas PrepFiler® Forensic DNA Extraction Kit (Applied Biosystems™) gave the maximum amount (Fig. 4.3).

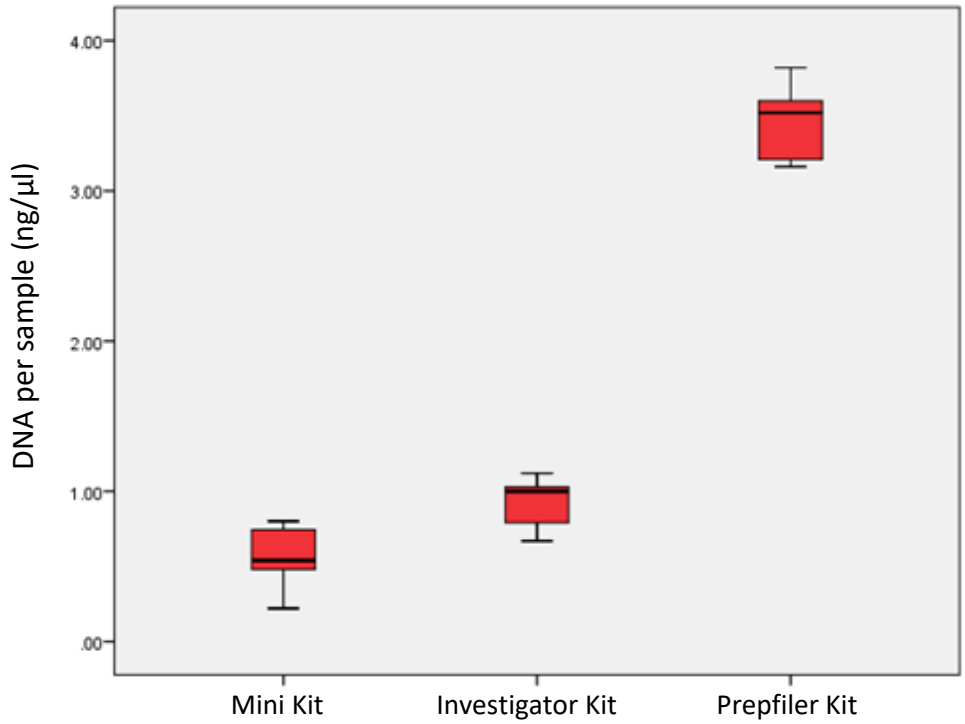


Fig. 4.3: DNA obtained per sample (ng/µl) using 3 different DNA extraction kits: (1) QIAamp® DNA Mini Extraction Kit; (2) QIAamp® DNA Investigator Kit; and (3) PrepFiler® Forensic DNA Extraction Kit.
*NOTE: Sample number indicates crop of larva examined

In the second part of the quantification, DNA extracted from the gut content of the larvae was fixed with 5 different methods with QIAamp® DNA Investigator Kit (Qiagen®), as described (section 3.2), and was quantified using Invitrogen™ Qubit® 3.0 in triplicate (Fig 4.4). The extraction was performed in sets of 1 crop, 3 crops and 5 crops. A one-way ANOVA and post-hoc (Tukey) analysis of DNA extracted per crop using different methods revealed a significant statistical difference (p-value= 0.000) amongst the methods used (Appendix 2). The DNA from the gut content of larvae fixed with method 2 (freezing at -20°C) gave the maximum DNA per sample, whereas the method 1 (hot water >80°C) gave the least DNA per sample.

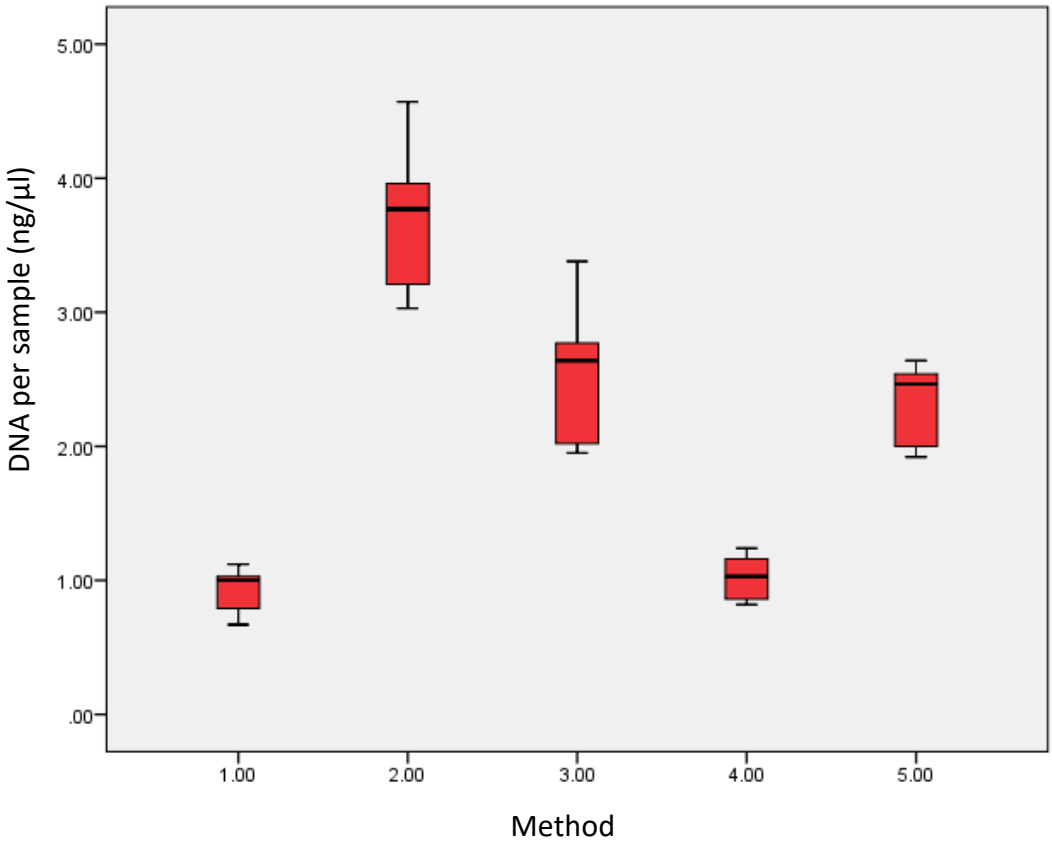


Fig 4.4: DNA per sample (ng/µl) using 5 different methods using QIAamp® DNA Investigator Kit: (1) larvae fixed by hot water (>80°C); (2) larvae fixed by freezing at -20°C; (3) larvae fixed in EtOH at -20°C; (4) larvae first fixed by hot water and then stored at EtOH at -20°C; and (5) larvae frozen at -20°C and stored in EtOH at -20°C. *NOTE: Sample number indicates crop of larva examined

A two-way ANOVA was performed, which revealed that, individually, temperature (frozen or hot water) and EtOH (presence or absence) had a significant effect (p-value= 0.00) on the amount of DNA obtained per sample (Table 4.3).

Source	df*	F	Significance (p-value)
Temperature	1	790.974	0.000
EtOH	1	60.198	0.000
Temperature * EtOH	1	80.306	0.000

Table 4.3: Two-way ANOVA (DNA quantification). Effect of temperature and EtOH, and the interaction between them, have a significant statistical variation (p-value< 0.05) in the DNA per sample (ng/µl) from the gut contents of the larvae fixed using 5 different methods.

Moreover, the interaction between these individual variables also displayed a significant statistical variation (p-value< 0.05) on the DNA obtained with different methods. Frozen samples displayed a high DNA amount per sample (ng/µl), both in the presence and absence of EtOH, whereas larvae fixed with hot water (>80°C) gave lower DNA amount (ng/µl) both in the presence and absence of EtOH. In the case of the frozen sample, sample without EtOH gave a higher DNA amount per sample, whereas in the case of larvae fixed with hot water (>80°C), samples with EtOH gave slightly a higher DNA amount per sample (Fig 4.5).

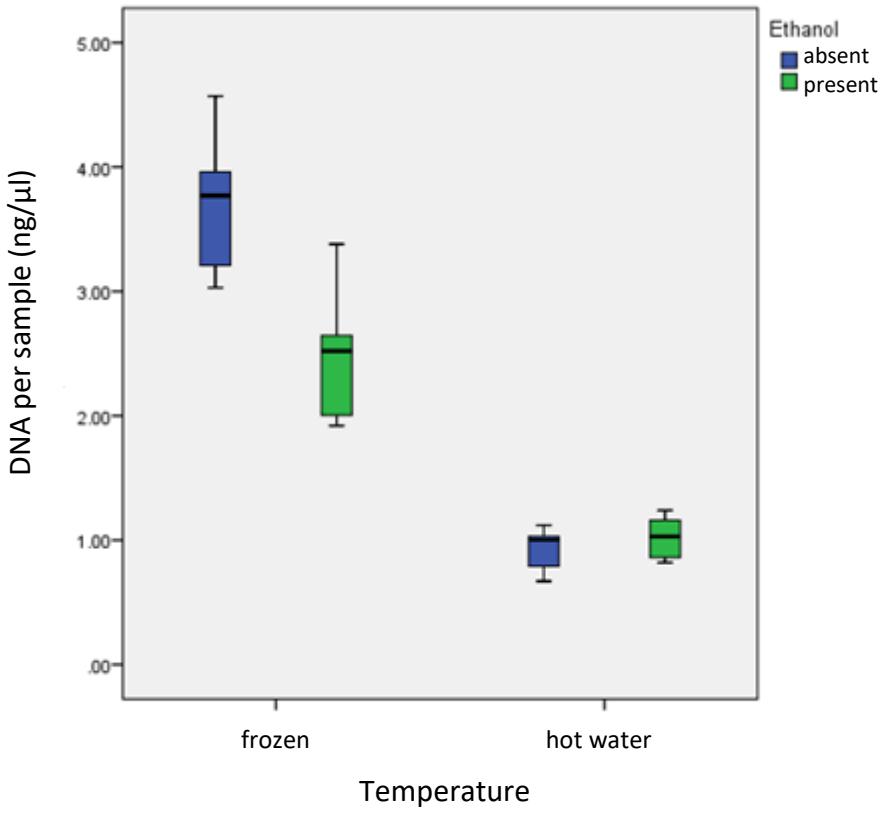


Fig. 4.5: Interaction between temperature (FRZ: Frozen, HW: Hot water) and EtOH (Blue= Absence, Green= Presence).

Invitrogen™Qubit® 3.0 (Life technologies, USA) does not give species specific quantifications, therefore, paramount care was taken to avoid contaminating the gut content with any host (*M. scalaris*) DNA while dissecting. Nonetheless, it is may be likely that DNA from some larval tissue, which surrounds the crop, may exist along with the DNA from the gut content.

4.3 PCR results

First electrophoresis was carried out to distinguish larval DNA from mammalian DNA from the larva fixed with hot water (>80°C). The primer set COI (LCO, HCO) was successful in amplifying *M. scalaris* DNA, which was extracted using QIAamp® DNA Mini Extraction Kit (Fig. 4.6). A control of the DNA obtained from the gut content gave a negative result.

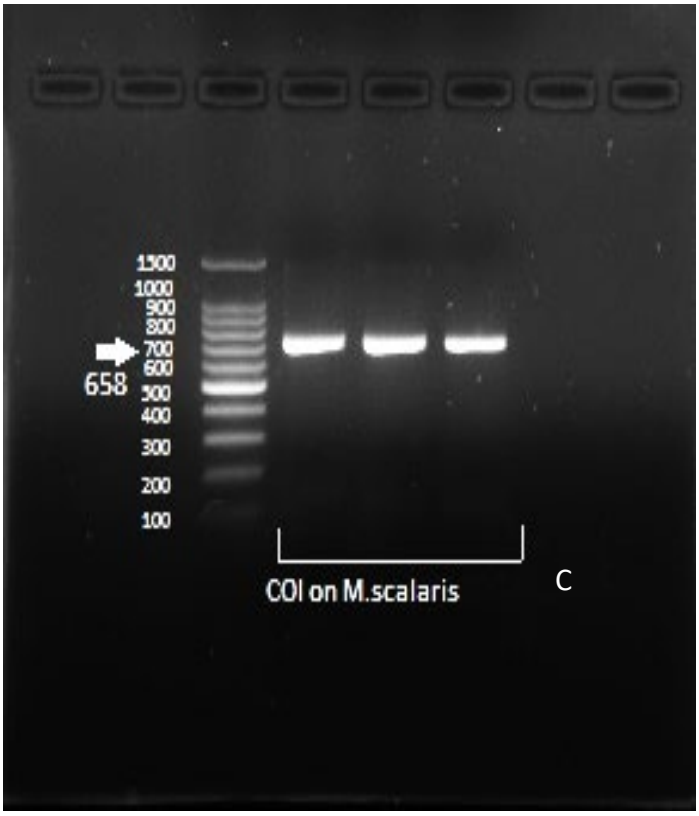
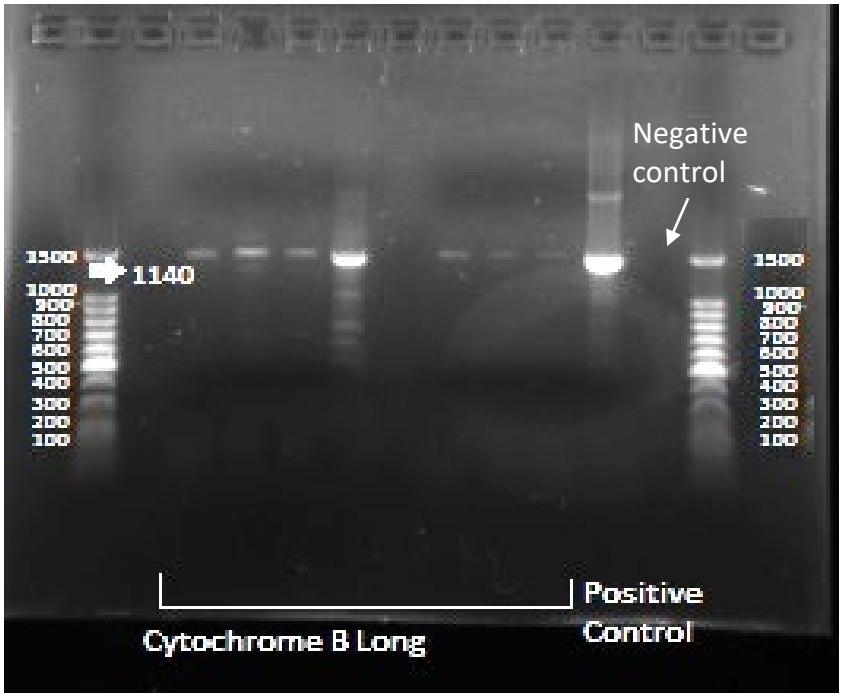
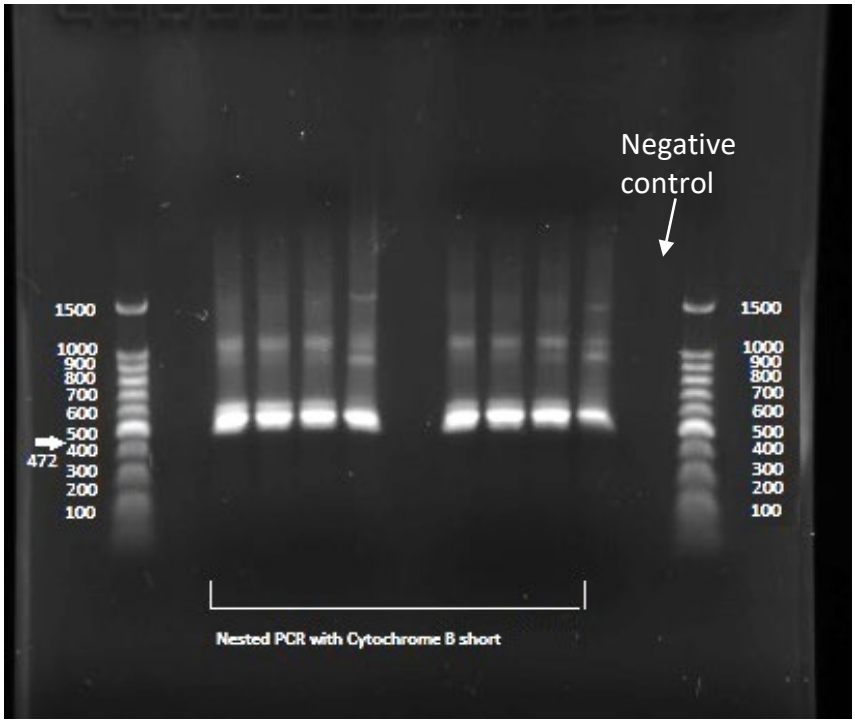


Fig 4.6: Visualisation of agarose gel electrophoresis of *M. scalaris* DNA with primer set COI (658 bp). 5µl DNA was stained using Midori Green Advanced stain. C = DNA from gut content.

Following this, DNA extracted from *M. scalaris* gut content was amplified using primer set *cytochrome b* Long. However, gel visualisation showed that the primer was unable to amplify the intended targeted region. Therefore, in order to maximise the specificity of mammalian DNA and reduce the template size, a nested PCR was carried out (section 3.5.2). The PCR product obtained from amplification by cytochrome *b* Long was used as a template and the primer set cytochrome *b* short was used for the second amplification. Gel electrophoresis showed that it provided positive results and was able to effectively amplify the mammalian DNA (Fig. 4.7 A & B).



(A)



(B)

Fig. 4.7: Agarose gel visualisation of mammalian DNA before and after nested PCR; (A) No positive result was obtained with Primer cyt *b* long (1140 bp), while (B) positive result was obtained by primer cyt *b* short (472 bp)

After confirming the presence of mammalian DNA in the larval gut content, the DNA present in gut content was again extracted using three different kits, as described above, and was amplified using pig specific primers, cyt *b* pig (149 bp) and 16s rRNA pig (138 bp). Visualising results after AGE indicated that QIAamp® DNA Investigator Kit gave the best results upon comparison (Fig.4.8 a and b) and hence, for reasons already discussed above, it was used for further DNA extractions from gut content of larvae fixed with different methods.

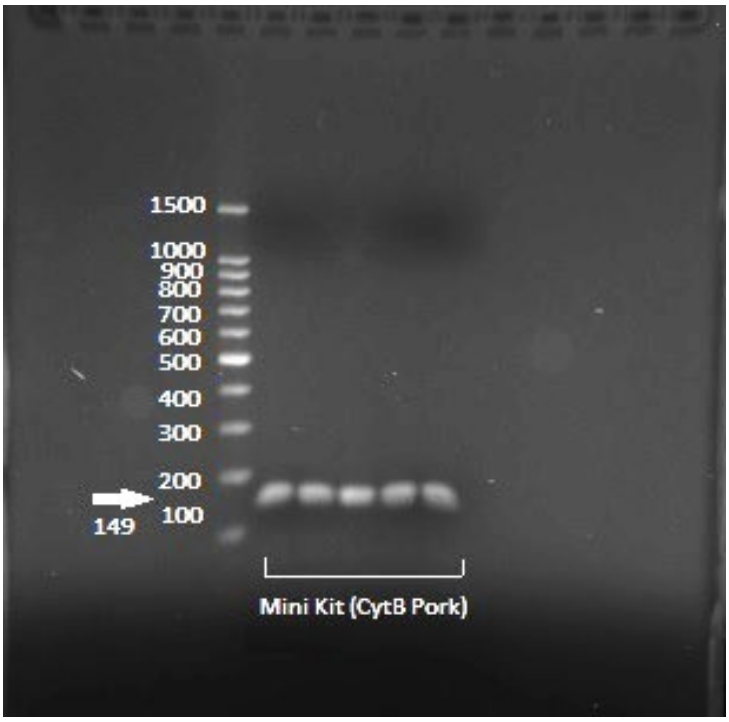


Fig. 4.8 (a): Visualisation of agarose gel electrophoresis of DNA samples extracted from larvae using QIAamp® DNA Mini Extraction Kit fixed by hot water (>80°C) with cyt *b* pig (149 bp).

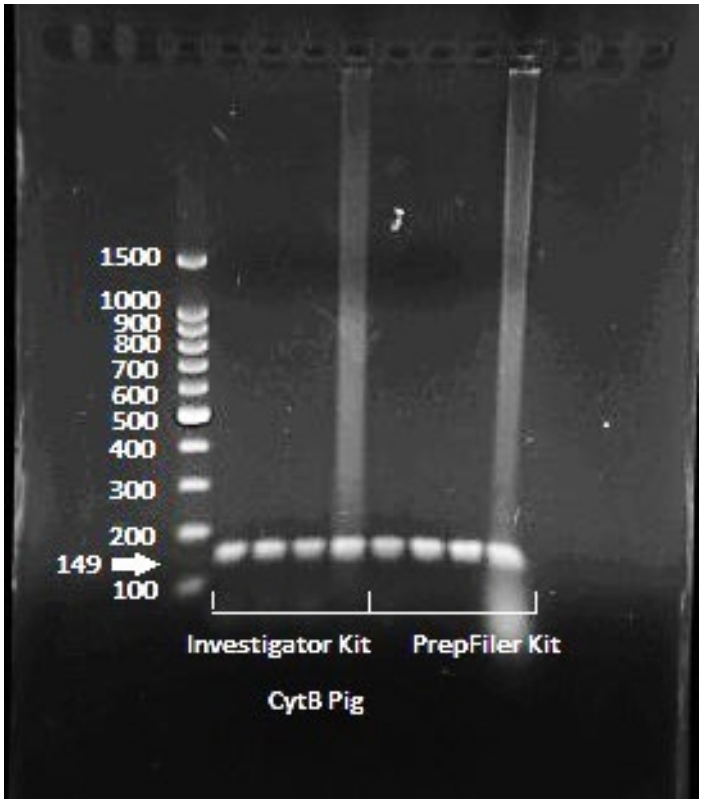


Fig. 4.8 (b): Visualisation of agarose gel electrophoresis of DNA samples extracted from larva using QIAamp® DNA Investigator Kit and PrepFiler® Forensic DNA Extraction Kit (Applied Biosystems™) fixed by hot water (>80°C) with cyt *b* pig (149 bp).

Several fixation methods were used to fix the larvae, and subsequent dissection was performed to obtain the crop contents (Fig. 3.1). The DNA in the gut content was extracted using the QIAamp® DNA Investigator Kit and was amplified using primers cyt *b* pig (149 bp) and 16S rRNA pig (138 bp). Both primers showed positive amplification with most of the samples [Fig. 4.9 (a)(b) and 4.10 (a)(b)].

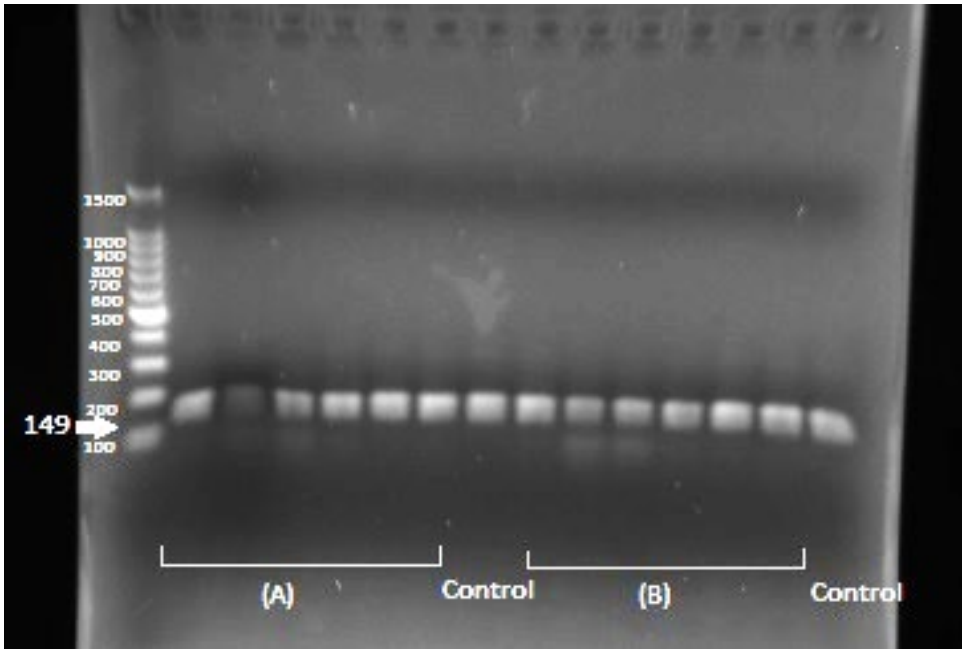


Fig. 4.9 (a): Visualisation of agarose gel of DNA extracted from gut of larvae. (A) Fixed by directly freezing at -20°C, and (B) fixed by immediately placing larvae in EtOH then at -20°C; amplified with primer cyt *b* pig (149 bp). DNA extracted from *Sus scrofa* meat was used as positive control.

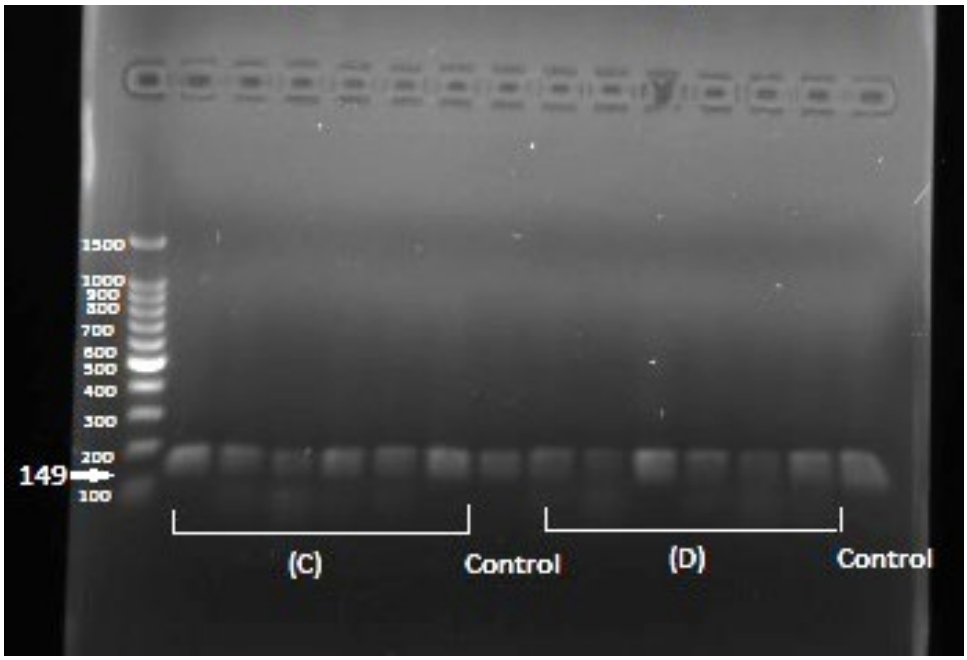


Fig. 4.9 (b): Visualisation of agarose gel of DNA extracted from gut of larvae: (C) fixed by pouring hot water for 30s then placing in EtOH, and (D) fixed by freezing larvae at -20°C then placing them in EtOH, amplified with primer cyt *b* pig (149 bp). DNA extracted from *Sus scrofa* meat was used as positive control.

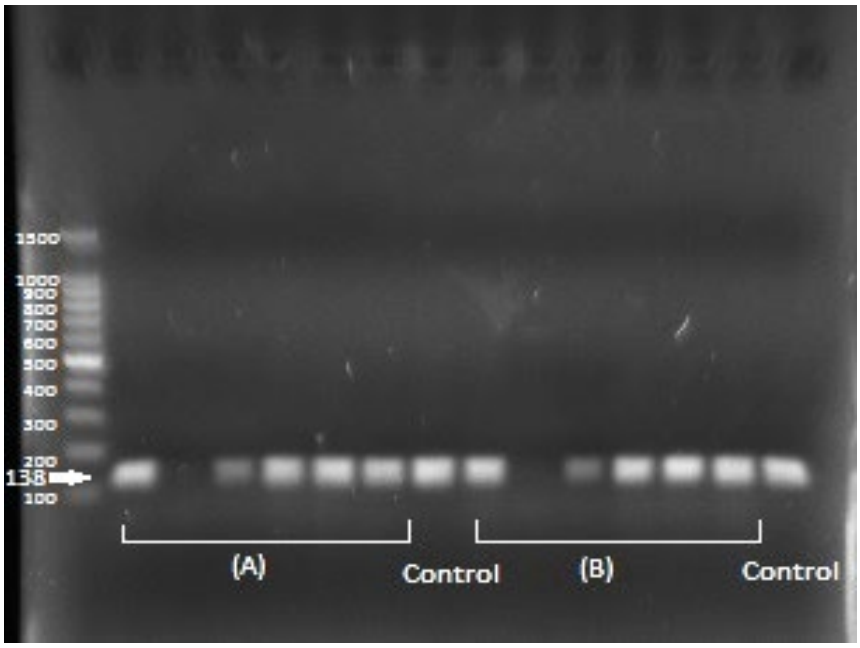


Fig 4.10 (a): Visualisation of agarose gel of DNA extracted from gut of larvae: (A) fixed by directly freezing at -20°C, and (B) fixed by immediately placing larvae in EtOH then at -20°C, amplified with primer 16S rRNA pig (138 bp). DNA extracted from *Sus scrofa* meat was used as positive control.

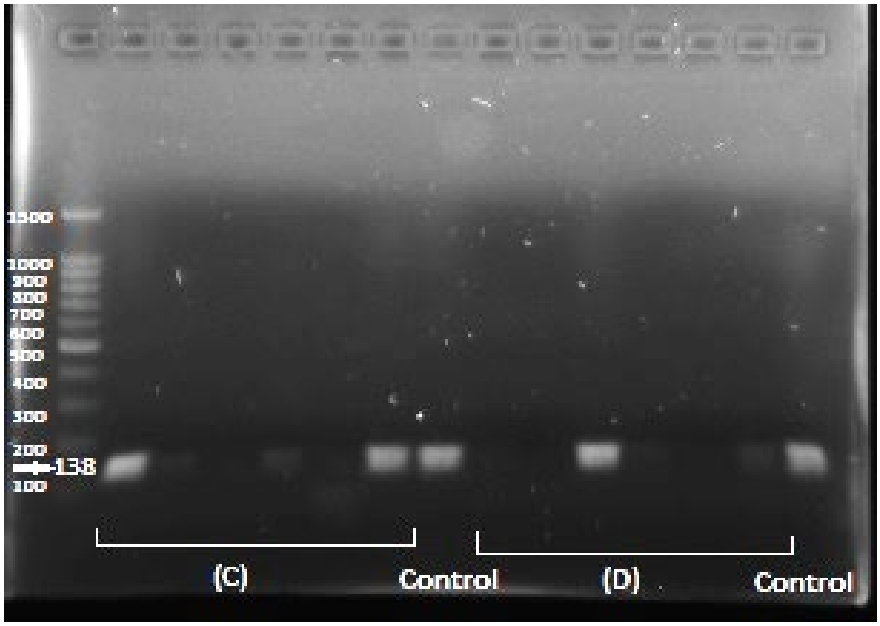


Fig.4.10 (b): Visualisation of agarose gel of DNA extracted from gut of larvae: (C) fixed by pouring hot water for 30s then placing in EtOH, and (D) fixed by freezing larvae at -20°C then placing them in EtOH, amplified with primer 16S rRNA pig (138 bp). DNA extracted from *Sus scrofa* meat was used as positive control.

4.4 Genome Sequencing

All samples that showed positive results in gel electrophoresis were purified as described in materials and methods (section 3.7) and sent for Sanger sequencing to Eurofins Genomics (Ebersberg, Germany). BlastN of all the sequences (Appendix 3) showed most of the samples amplified with cyt *b* pig and 16s rRNA pig were from *Sus scrofa* (Table 4.4 and 4.5).

Cyt *b* Pig

Sample name	Sequencing result cyt *b* pig (149bp)	max score	total score	Query Cover	E-value	indent	Acces. no.
1	*Sus scrofa*, 113bp	191	191	93%	4.00E-45	99%	MF143597.1
2	*Sus scrofa*, 115bp	113	113	96%	8.00E-22	98%	EF545592.1
3	*Sus scrofa*, 113bp	191	191	96%	4.00E-45	98%	AM492573.1
4	*Sus scrofa*, 114bp	189	189	92%	1.00E-44	99%	MF143597.1
5	*Sus scrofa*, 108bp	187	187	96%	5.00E-44	99%	MF143597.1
6	*Sus scrofa*, 114bp	189	189	92%	1.00E-44	99%	MF143597.1
7	*Sus scrofa,* 110bp	182	182	91%	2.00E-42	99%	AM492573.1
8	*Sus scrofa*, 106bp	183	183	98%	6.00E-43	98%	MF143597.1
9	*Sus scrofa*, 106bp	187	187	98%	5.00E-44	99%	MF143597.1
10	*Sus scrofa,* 68bp	121	121	95%	5.00E-24	100%	MF143597.1

Table 4.4: BlastN results of samples amplified with primer cyt *b* pig (149 bp). All samples belonged to *Sus scrofa.* Note that, due to use of Sanger sequencing the results obtained are less than 149 bp due to loss of sequence during sequencing.

16S rRNA

Sample name	Sequencing result 16s rRNA Pig (138bp)	max score	total score	Query Cover	E-value	indent	Acces. no.
1	*Sus scrofa*, 95bp	161	161	91%	3.00E-36	100%	MF143597.1
2	*Sus scrofa*, 88bp	145	145	100%	3.00E-31	97%	KJ746666.1
3	*Sus scrofa*, 97bp	145	145	100%	3.00E-31	97%	KJ746666.1
4	*Sus scrofa*, 89bp	134	134	96%	6.00E-28	95%	MF143597.1
5	*Sus scrofa*, 96bp	163	163	94%	8.00E-37	99%	KJ746666.1
6	*Sus scrofa*, 95bp	154	154	91%	5.00E-34	99%	MF143597.1
7	*Sus scrofa* : 97bp	163	163	90%	8.00E-37	100%	MF143597.1
8	*Sus scrofa* : 96bp	161	161	90%	3.00E-36	100%	MF143597.1
9	*Sus scrofa* : 94bp	161	161	100%	3.00E-36	98%	MF143597.1
10	*Sus scrofa* : 97bp	165	165	91%	2.00E-37	100%	MF143597.1

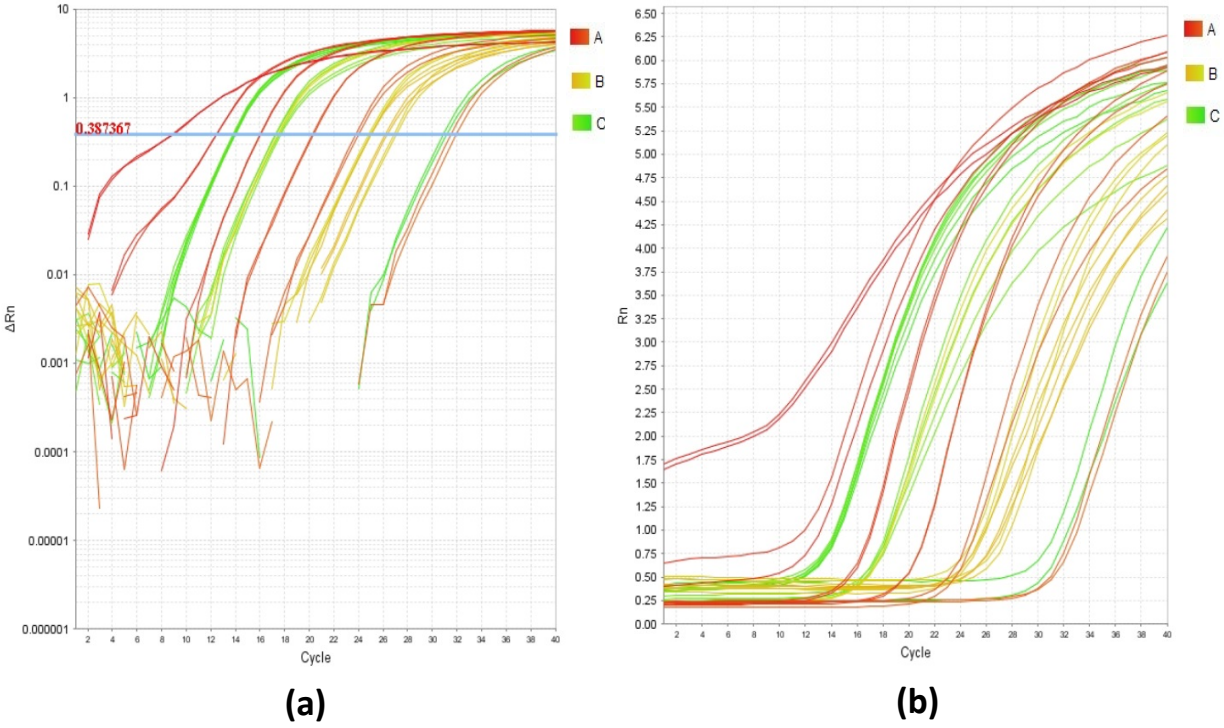
Table 4.5: BlastN results of samples amplified with primer 16S rRNA Pig (138bp). All samples belonged to *Sus scrofa.* Note that, due to use of Sanger sequencing, the result obtained are less than 138 bp due to loss of sequence during sequencing.

4.5 Quantitative / Real time PCR

4.5.1 Amplification Plots

As discussed in the materials and methods (section 3.8), a standard RT-PCR run was performed on a 7500 Fast Real Time PCR Instrument (Applied Biosystems, USA) using SYBR Green, which resulted in successful amplification of target regions cyt *b* pig and 16S rRNA pig in the samples (Fig. 4.11 and Fig. 4.12).

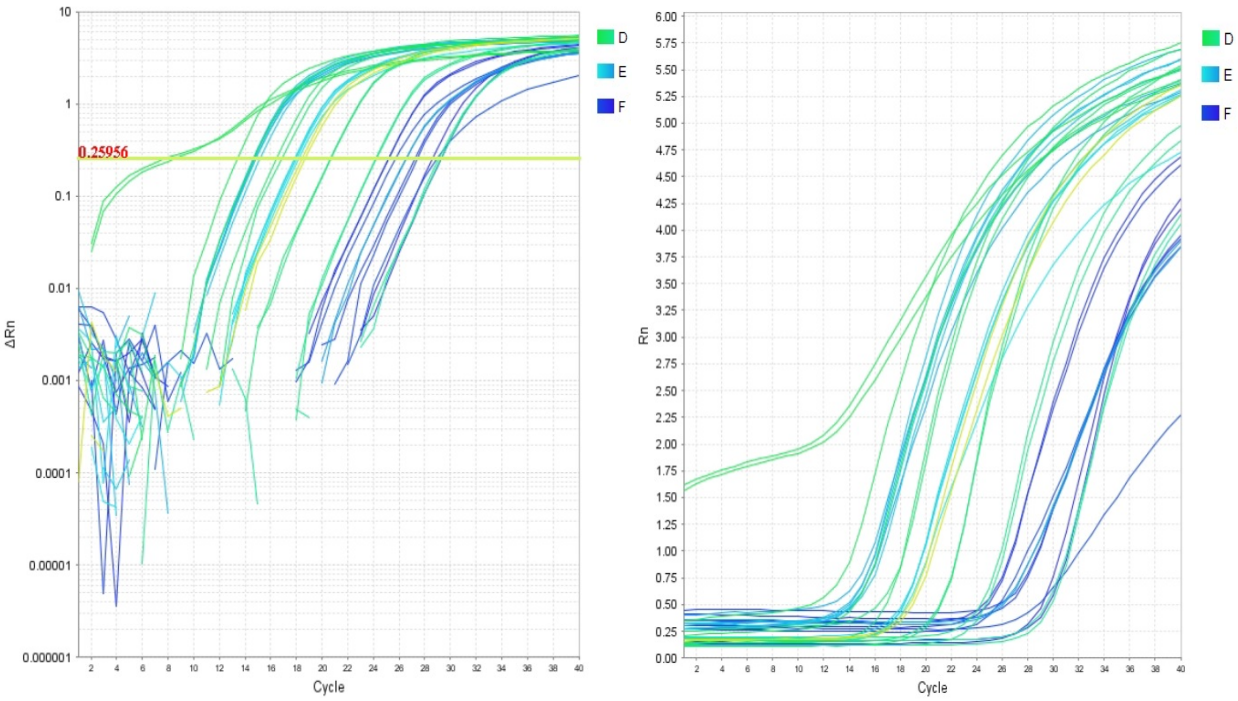
Cyt b *pig*



(a) **(b)**

Fig.4.11: Amplification plot of samples with primer set cyt *b* pig (149 bp); (a) ΔRn vs Cycle amplification plot of samples amplified with cyt *b* pig showing the cycle threshold value of 0.39 for the reaction. (b) A normalised report of Rn Vs Cycle amplification plot of samples amplified with cyt *b* pig. A (red) - Standard sample; B (yellow) - unknown sample; C (green) - unknown sample and negative control (NTC).

16S rRNA pig



(a) **(b)**

Fig.4.12: Amplification plot of samples with primer set 16S rRNA (138 bp): (a) ΔRn vs Cycle amplification plot of samples amplified with 16S rRNA Pig showing the cycle threshold value of 0.26 for the reaction; (b) A normalised report of Rn Vs Cycle amplification plot of samples amplified with 16S rRNA Pig. D (fl. green) - standard sample; E (light blue) - unknown sample; F (dark blue)- unknown sample and negative control (NTC).

4.5.2 Standard curve plots

A standard curve was plotted (Fig. 4.13 a & b) based on standards manually prepared from *Sus scrofa* meat. Using this, the quantity of unknown samples was determined (Tables 4.6 and 4.7) and, from those, samples appropriate for STR analysis were selected (quantity ≥ 0.005 ng/µl).

Cyt b *pig*

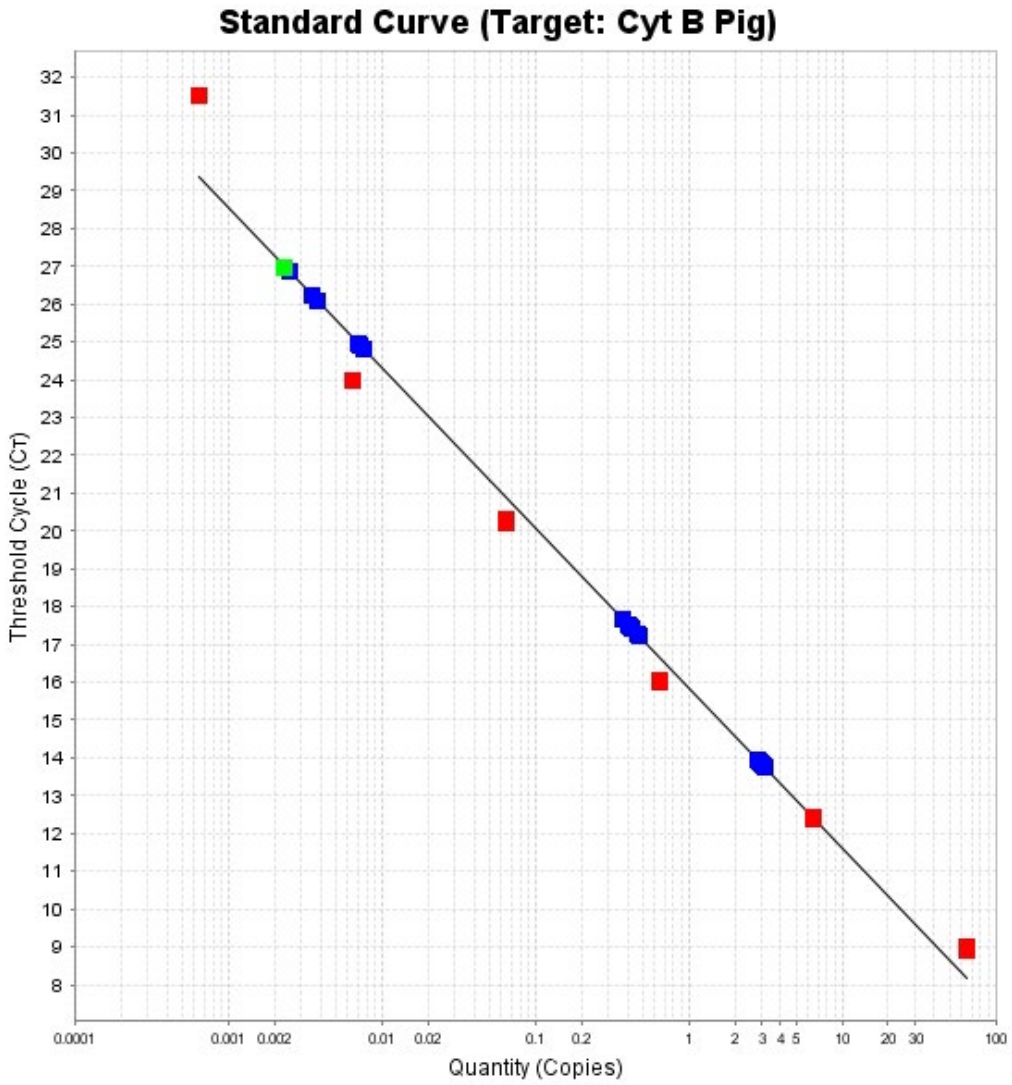


Fig. 4.13(a): Standard curve plotted from threshold cycle (C_T) versus quantity (cyt *b* pig). Standard curve plotted to determine quantity of unknown samples with respect to standards of known quantity for the primer set cyt *b* pig (149 bp). Here: ■ = Standard; ■ = Unknown; ■ = Unknown (flagged). Note that Unknown flagged are samples with multiple Tm and also no template sample like the Negative Control (NTC). Slope:-4.231; Y-Intercept: 15.865; R^2: 0.981.

16S rRNA pig

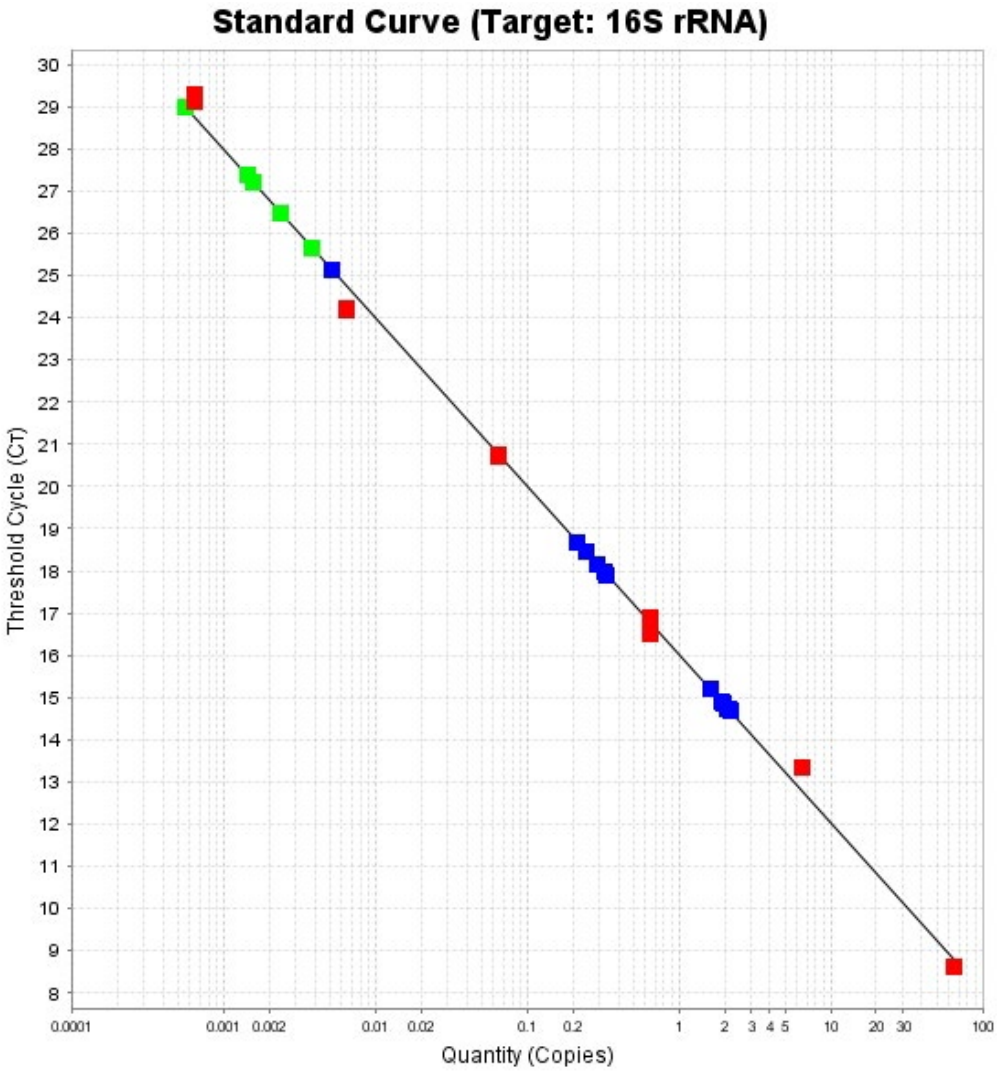


Fig. 4.13(b): Standard curve plotted from threshold cycle (C_T) versus quantity(16S rRNA), Standard curve plotted to determine quantity of unknown samples with respect to standards of known quantity for the primer 16S rRNA Pig. Here: ■ = Standard; ■ = Unknown; ■ = Unknown (flagged). Note that Unknown flagged are samples with multiple Tm and also no template sample like the Negative Control (NTC). Slope: -3.996; Y-Intercept: 16.022; R^2: 0.996.

4.5.3 Melt curve analysis plot

The melt curve analysis displayed one peak in most of the samples, therefore verifying the amplification of only the target region (Fig.4.14).

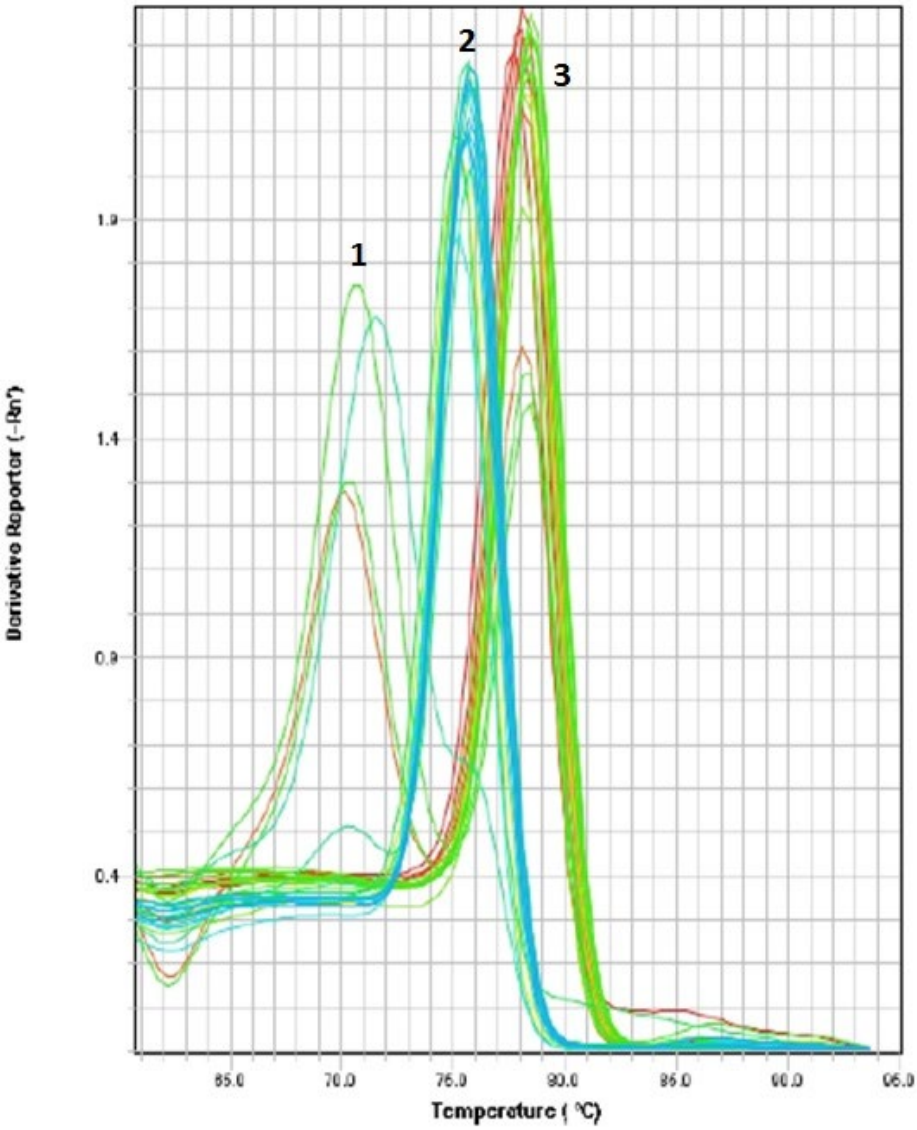


Fig. 4.14: Melt curve analysis: (1) Detection of primer-dimer formation in the negative control (water); (2) The melt curve for all samples amplified with 16S rRNA pig. As only one peak is observed, it confirms the amplification of only the target region (3) The melt curve for all samples amplified with cyt *b* pig. As only one peak is observed it confirms the amplification of only the target region.

4.5.4 Quantification table of unknown samples

With the help of the standard curve obtained above (Fig. 4.13 and Fig. 4.14), the quantity of the unknown samples was determined. The standards used in the examination were prepared manually from known quantities, starting from 65 ng/µl serially diluting with a factor of 1:10. The analysis was undertaken in sets of three samples comprising of 1 crop, 3 crops and 5 crops respectively for each type of method used for fixing the larvae as described in the materials and methods (section 3.2). Upon evaluation, samples 19-21, fixed with only EtOH at -20°C, gave the maximum per crop quantification, followed by the samples 16-18, fixed with freezing the larvae at -20°C (Tables 4.6 and 4.7). This was observed in the amplification process with both the cyt *b* pig primer set as well as 16s rRNA pig primer set. Further, the Tm values were clustered for each type of primer (or cyt *b* pig about 78.1°C, and for 16s rRNA about 75.8°C), indicating only one region of amplification.

Cyt *b pig*

Sample Name	Target	Sample type	Quantity (ng/µl)	Quantity (ng/µl) per sample*	CT	Tm (°C)
1	cyt *b* pig	STANDARD	65	-	8.912	77.732
2	cyt *b* pig	STANDARD	6.5	-	12.434	77.905
3	cyt *b* pig	STANDARD	0.65	-	16.039	78.078
4	cyt *b* pig	STANDARD	0.065	-	20.327	78.250
5	cyt *b* pig	STANDARD	0.0065	-	23.749	78.078
6	cyt *b* pig	STANDARD	0.0007	-	31.520	78.078
7	cyt *b* pig	UNKNOWN	0.0028	0.0028	26.988	78.250
8	cyt *b* pig	UNKNOWN	0.0046	0.0015	26.069	78.078
9	cyt *b* pig	UNKNOWN	0.3851	0.0770	17.687	78.078
10	cyt *b* pig	UNKNOWN	0.0031	0.0031	26.844	78.423

Continued.

Sample Name	Target	Sample type	Quantity	Sample Name	Target	Sample type
11	cyt *b* pig	UNKNOWN	0.0083	0.0028	24.999	78.195
12	cyt *b* pig	UNKNOWN	0.4156	0.0831	17.543	77.905
13	cyt *b* pig	UNKNOWN	0.0083	0.0083	24.960	78.250
14	cyt *b* pig	UNKNOWN	0.4396	0.1465	17.437	78.250
15	cyt *b* pig	UNKNOWN	2.9111	0.5822	13.858	78.423
16	cyt *b* pig	UNKNOWN	0.0084	0.0084	24.934	78.250
17	cyt *b* pig	UNKNOWN	0.4828	0.1609	17.259	78.078
18	cyt *b* pig	UNKNOWN	3.0027	0.6005	13.800	78.423
19	cyt *b* pig	UNKNOWN	0.0090	0.0090	24.804	78.423
20	cyt *b* pig	UNKNOWN	0.4919	0.1640	17.224	78.423
21	cyt *b* pig	UNKNOWN	3.0682	0.6136	13.759	78.423
22	cyt *b* pig	NTC	-	-	30.895	70.648

*crop of the larva

Table 4.6: Quantification of the unknown samples of DNA obtained from the standard curve (cyt *b* pig). Samples 1-6 are standards of known quantity serially diluted in the ratio of 1:10. In accordance to these results, samples 9 (5 larval crops fixed first by freezing then placed in EtOH), 11 (3 crops of larvae fixed first with hot water then placed in EtOH), 12 (5 crops of larvae fixed first with hot water then placed in EtOH), 13 (1 crop of larva fixed by pouring hot water), 14 (3 crops of larvae fixed by pouring hot water), 15 (3 crops of larvae fixed by pouring hot water), 16 (1 crop of larva fixed by freezing only), 17 (3 crops of larvae fixed by freezing only), 18 (5 crops of larvae fixed by freezing only), 19 (1 crop of larva fixed by placing directly in EtOH), 20 (3 crops of larvae fixed by placing directly in EtOH) and 21 (5 crops of larvae fixed by placing directly in EtOH) were selected for an STR analysis as all of them have quantities >0.005ng/µl.

16S rRNA pig

Sample Name	Target	Sample type	Quantity (ng/µl)	Quantity (ng/µl) per sample*	C_T	Tm (°C)
1	16S rRNA	STANDARD	65	-	7.9160	75.140
2	16S rRNA	STANDARD	6.5	-	13.360	75.659
3	16S rRNA	STANDARD	0.65	-	16.517	75.831
4	16S rRNA	STANDARD	0.065	-	20.697	75.831
5	16S rRNA	STANDARD	0.0065	-	24.164	75.831
6	16S rRNA	STANDARD	0.0007	-	29.295	71.512
7	16S rRNA	UNKNOWN	0.0040	0.0040	25.665	75.659
8	16S rRNA	UNKNOWN	0.0025	0.0008	26.493	75.831
9	16S rRNA	UNKNOWN	0.0084	0.0017	27.485	75.659
10	16S rRNA	UNKNOWN	0.0006	0.0006	28.978	70.821
11	16S rRNA	UNKNOWN	0.0025	0.0008	26.485	75.659
12	16S rRNA	UNKNOWN	0.0025	0.0005	26.485	75.659
13	16S rRNA	UNKNOWN	0.2594	0.2594	18.456	75.486
14	16S rRNA	UNKNOWN	0.3391	0.1130	17.994	75.659
15	16S rRNA	UNKNOWN	2.2513	0.4503	14.726	75.831
16	16S rRNA	UNKNOWN	0.3556	0.3556	17.911	75.486
17	16S rRNA	UNKNOWN	1.7235	0.5745	15.187	75.659
18	16S rRNA	UNKNOWN	2.2725	0.4545	14.710	75.659

Continued.

Sample Name	Target	Sample type	Quantity (ng/µl)	Quantity (ng/µl) per sample*	C_T	Tm (°C)
19	16S rRNA	UNKNOWN	0.3087	0.3087	18.156	75.140
20	16S rRNA	UNKNOWN	0.3575	0.1192	17.902	75.659
21	16S rRNA	UNKNOWN	2.3251	0.4650	14.670	75.831
22	16S rRNA	NTC	-	-	29.319	71.512

*crop of the larva

Table 4.7: Quantification of unknown samples of DNA obtained from the standard curve (16s rRNA). Samples 1-6 are standards of known quantity serially diluted in the ratio of 1:10. In accordance to these results, samples 9 (5 larval crops fixed first by freezing then placed in EtOH), 13 (1 crop of larva fixed by pouring hot water), 14 (3 crops of larvae fixed by pouring hot water), 15 (3 crops of larvae fixed by pouring hot water), 16 (1 crop of larva fixed by freezing only), 17 (3 crops of larvae fixed by freezing only), 18 (5 crops of larvae fixed by freezing only), 19 (1 crop of larva fixed by placing directly in EtOH), 20 (3 crops of larvae fixed by placing directly in EtOH) and 21 (5 crops of larvae fixed by placing directly in EtOH) can be considered appropriate for STR analysis, as all of them have quantities >0.005ng/µl. It is also seen that the results corresponding to both the primer sets cyt *b* pig and 16S rRNA match.

4.6 Genetic profiling

An STR analysis of samples concluded with samples 17, 18, 19 and 21 giving full DNA profiles for all 12 loci, and samples 16 and 20 giving partial DNA profiles. Table 4.8 describes the genotyping of all positive samples that were extracted from larvae fixed by freezing at -20°C and from larvae fixed by placing them directly in ethanol. The corresponding DNA profiles have been attached in Appendix 4 for reference. The positive result with 1 crop of larvae preserved in EtOH gave a positive match with the Standard (the food source) (Fig. 4.15).

Locus	Samples							
	Standard 1 Allele Calling at 50 RFU	17 (frozen)	18 (frozen)	21 (EtOH)	Standard 2 Allele Calling at 50 RFU	16 (frozen)	19 (EtOH)	20 (EtOH)
387A12F	***14.1, 15.1***	14.1, 15.1	14.1, 15.1	14.1, 15.1	**9, 21**	9, 21	9, 21	9, 21
S0655	***13, 13***	13, 13	13, 13	13, 13	**11, 13**	ND	11, 13	ND
SBH1	***13, 14***	13, 14	13, 14	13, 14	**14, 14**	14, 14	14, 14	14, 14
SBH2	***6, 26***	6, 26	6, 26	6, 26	**26, 27**	26, 27	26, 27	26, 27
SBH4	***56, 57***	56, 57	56, 57	56, 57	**56, 66.1**	56, 66.1	56, 66.1	56, 66.1
SBH10	***49, 49***	49, 49	49, 49	49, 49	**46, 49**	46, 49	46, 49	46, 49
SBH13	***11, 14***	11, 14	11, 14	11, 14	**11, 15**	11, 15	11, 15	11, 15
SBH18	***12, 12***	12,12	12,12	12,12	**9,ND**	ND	9, ND	ND
SBH19	***14, 15***	14, 15	14, 15	14, 15	**12, 14**	14	12, 14	14
SBH20	***23, 37***	23, 37	23, 37	23, 37	**36, 37**	36, 37	36, 37	36, 37
SBH22	***23, 23***	23, 23	23, 23	23, 23	**23, 23.3**	23, 23.3	23, 23.3	23, 23.3
SBH23 Y	***Y***	Y	Y	Y	**Y**	Y	Y	Y
SBH23 X	***X***	X	X	X	**X**	X	X	X

ND- not detected

Table 4.8: Genotyping results at 50 Relative fluorescence unit (RFU) of the positive samples in comparison to the genotyping of DNA extracted from *Sus scrofa* meat that was fed to the larvae while breeding (standards 1 and 2) (frozen and stored in EtOH). Samples 16, 17, 18 were obtained from gut content of larvae, which were fixed by only freezing at -20°C and . Samples 17 and 18 show exact matches and sample 16 shows m in 09 out of 12 loci, proving the DNA extracted from the gut content was similar to the one the larvae fed on. While, samples 19, 20, 21 were obtained from gut content of larvae, which were fixed by directly placing the larvae at EtOH and then storing at -20°C. Samples 19 and 21 show exact matches, and sample 20 shows a shows match in 9 out of 12 loci. This proves the DNA extracted from the gut content was similar to the one fed to the larvae.

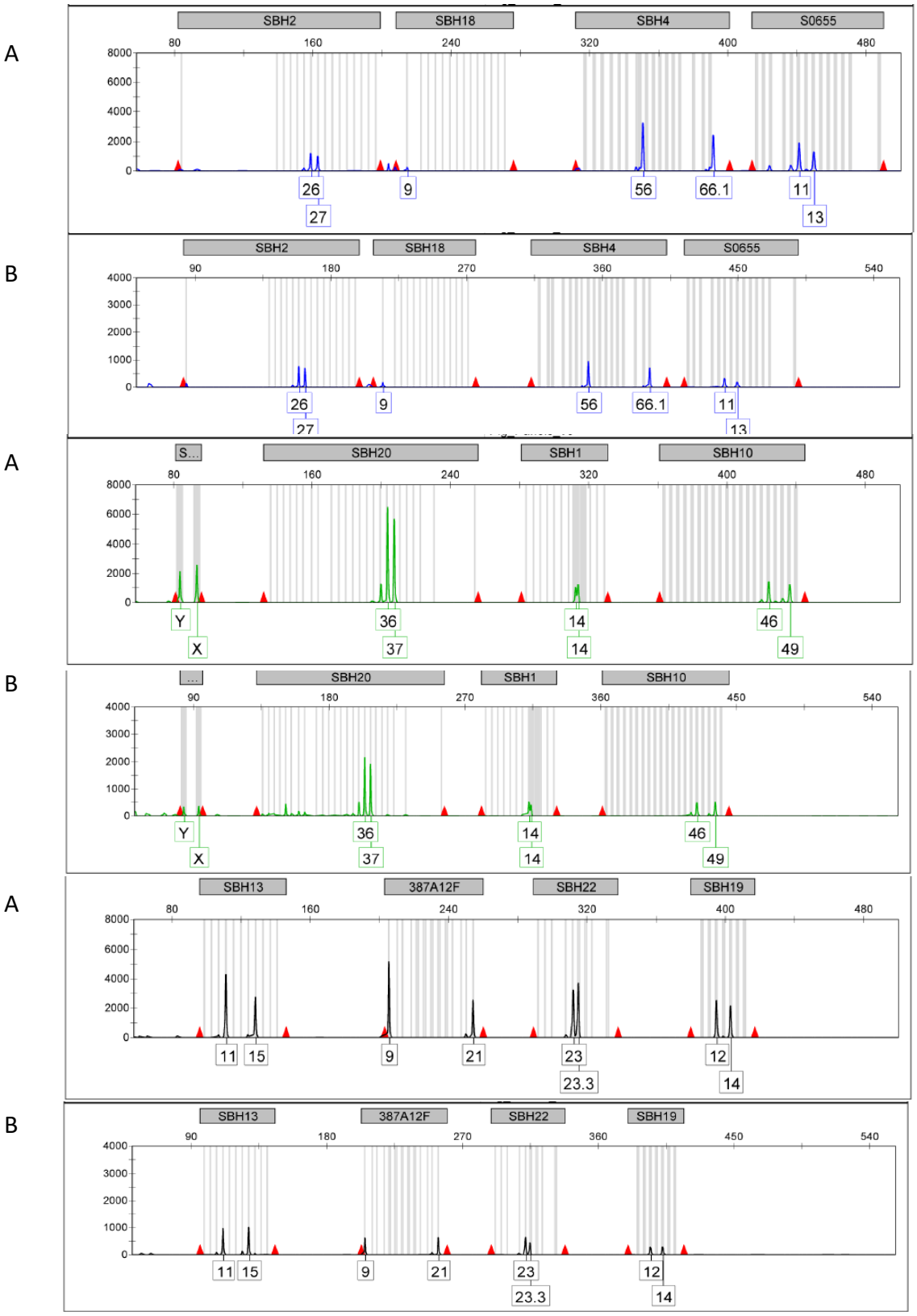


Fig 4.15: Electropherogram comparison of Standard 2 with 1 crop of larvae fixed in EtOH; (A) Standard (B) Sample.

5. Discussion

In this study, an attempt was made to extract DNA from the gut content of *M. scalaris* (Diptera, Phoridae) larvae, a non-conventional source of DNA evidence, and assess its reliability as a tool for human DNA identification forensic context.

Forensic investigators around the world typically depend on conventional sources, like body fluids, in an attempt to recover DNA evidence from the scene of crime. They routinely use DNA to establish a relationship between the questioned sample and the scene of incidence (Butler, 2005; Li, 2015). However, in some cases, like extreme cadaver decomposition or the absence of the cadaver, the use of traditional methods of DNA evidence collection and its analysis is not advantageous for the establishment of the identity of the victim (Wells *et al.*, 2001a,b). To overcome this problem, investigators need to use a different approach for the collection of DNA evidence. The analysis of the gut contents of necrophagous larvae (a non-conventional source of DNA evidence) can be of importance in such investigations. In instances where investigators discover maggots but no corpse from a possible scene of crime, gut content analysis of the larvae (maggots) can be useful to establish the identification of the missing corpse (Wells *et al.*, 2001a,b). Successful non-insect DNA extraction from the gut contents of larvae of Calliphoridae (blow flies) and Sarcophagidae (flesh flies), which are generally the first colonisers of a body, have been repeatedly demonstrated in the literature. In a study conducted by Li *et al.* (2015), human DNA was successfully extracted from the gut contents of *Aldrichina graham* (Calliphoridae) found on a corpse in central-southern China. Likewise, in the study conducted by Wells *et al.* (2001a,b), successful extraction and amplification of human DNA with mitochondrial gene markers possible from the gut content of *Cynomyopsis cadaverina* (Calliphoridae). Although larvae of Calliphoridae (blow flies) and Sarcophagidae (flesh flies) are first colonisers on decomposing corpses, in cases of a buried corpse or indoor crime scene, *M. scalaris* is found to be the primary coloniser due its small size and its ability to crawl into tight spaces (Disney, 2008), thus making it an important fly of forensic interest (Pastuala & Merritt, 2013; Bugelli *et al.*, 2015). As previously mentioned, most studies have focused on the extraction of DNA from Calliphoridae and Sarcophagidae, with no work yet undertaken on extraction of DNA from the larval gut content of fly species of small size like scuttle flies (Phoridae). Hence, this study expands the source of DNA

extraction, aiding criminal investigations by successfully extracting and amplifying non-insect DNA from the gut content of *M. scalaris* (Phoridae) larva fed on *Sus scrofa* meat, and hence, associating the larvae with the corpse.

In the initial phase of the study, post-feeding 3rd instar larvae of *M. scalaris* were taken and fixed using hot water (>80°C) for 40s, a standard method of fixation of larvae of forensic interest, as described by Amendt *et al.* (2007). The larvae were dissected by the method described by Tuccia *et al.* (2016) in groups of 1 crop, 3 crops and 5 crops. Qiagen® DNA Mini Extraction Kit was used to successfully extract DNA from the gut content of the larva. The DNA thus obtained was first amplified with the invertebrate specific COI primer set, followed with the mammalian specific cyt *b* primers (Long and Short). The COI primer set gave positive amplifications from DNA extracted from the *M. scalaris* larval tissue; however, negative results with no amplification of the intended targeted DNA region were obtained with the control sample from the DNA extracted from the gut content of larvae. Further, PCR amplification with mammalian specific cyt *b* primer set was performed using the DNA extracted from the gut, positive results were obtained, confirming the presence of mammalian DNA. This result was in accordance with the suggestion already published in a review by Campobasso *et al.* (2005) on forensic genetic analysis of gut contents. The result also proves that non-insect DNA extraction was possible from gut content of smaller larvae (0.1-0.25 cm), like that of *M. scalaris*. Subsequently, successful amplification bands were obtained not only from sets of 3 crops and 5 crops, but also from the set of 1 crop. The DNA found in the gut content provided important information about the 'last meal' of the larvae (Wells *et al.*, 2001b; Campobasso *et al.*, 2005; Zuha and Omar, 2014). Hence, DNA analysis can provide information on possible relocation of the corpse (if maggots are present where no food source is apparently present indicates possible relocation) and also information about the actual food source in presence of multiple food sources.

After intensive analysis of the literature, three different types of DNA extraction kits (QIAamp® DNA Mini Extraction Kit, QIAamp® DNA Investigator Kit and PrepFiler® Forensic DNA Extraction Kit) were used to extract DNA from the 3rd instar larvae fixed with hot water (>80°C) for 40s (Amendt *et al.*, 2007). Although positive results were obtained from all the kits, there was a statistical significant difference (p-value < 0.05) in the yield of the DNA obtained per sample from each kit. While the Minikit gave the least amount of DNA

(0.057±0.02 ng/μl), the Prepfiler Kit gave the maximum amount of DNA (3.48±0.02 ng/μl) per sample. The statistical variations were also confirmed by AGE, after amplifying the DNA samples extracted from each kit with pig specific mitochondrial gene primer cyt *b* pig (149 bp). The brightness of the amplified bands varied from kit to kit with Minikit showing the least bright bands and Prepfiler Kit and Investigator Kit both giving the brightest bands, as DNA extraction with Minikit gave very low DNA amount per sample, it was not used for further analysis. Diffrences in the amount of non-insect DNA extraction from immature stages of Diptera is also reported by Marchetti *et al.* (2013). Marchetti and the collaborators (2013) found out that the highest amount of the victim DNA was obtained using Prepfiler Kit whereas Chelex-100 (Bio-Rad) method of DNA extraction did not provide any useful results. This result was also validated in this study. All the positive samples were sent for sequencing, and the sequences compared through BlastN, giving a positive match with *Sus scorfa* mitochondrial region (cyt *b* pig, 149 bp. Thus, these results successfully link the DNA extracted from the larval gut contents to its food source i.e. *Sus scrofa*. The DNA obtained can be further used for STR analysis and also for Y-chromosome analysis for identification and sex determination respectively (Clery, 2001; Zehner *et al.* 2004).

During RT-PCR, multiple Tm peaks were obtained during the melt curve analysis of the sample strongly suggesting presence of fragmented DNA sample with multiple sites being amplified simultaneously. The negative result also suggested the presence of inhibitors during the process of RT-PCR (Opel *et al.*, 2010). This result was in contrast with the validation studies of the Prepfiler kit conducted by Brevnov *et al.* (2009), which suggested that PCR inhibitors are generally removed with the use of this kit. However, DNA samples extracted with the Investigator Kit gave a positive result during RT-PCR, and only one Tm peak was obtained during the melt curve analysis, implying amplification of one specific targeted region of the mitochondrial gene, cyt *b* pig. The successful use of the QIAamp® DNA Investigator Kit was, therefore, validated for forensic casework on samples with low amount of DNA (Sturk-Andreaggi *et al.*, 2011). Its effectiveness has also been seen in case of mass disaster victim identification (Watherston *et al.*, 2018). Given its effectiveness in the extraction of samples with low amount DNA, and failure of the PrepFiler kit to give positive result with RT-PCR, the Investigator Kit was used for further studies and subsequently for STR profiling.

After deciding on the extraction kit and confirming the possibility to extract DNA from *M. scalaris* larval gut content, further DNA extractions were carried out using the Investigator Kit to test which method of fixation of larvae gave the maximum yield of DNA. Generally, investigators use hot water (>80°C) to fix larvae as a standard procedure for preservation of the larval samples collected from the scene of crime, as described Amendt *et al.* (2007). This process is preferred when morphological analysis is to be performed, but due to liquefaction of the gut it causes difficulties during dissection. Furthermore, post dissection, a lot of host tissue is attached with the larval gut due to liquefaction. This affects the further molecular analysis of the gut content. Hence, to derive a better method for dissection, further in the study the larva was fixed using 5 different protocols.

The dry and wet weight of crops attained after dissection of larvae fixed by freezing at -20°C and by placing in EtOH at -20°C, was lesser when compared to the hot water (>80°C) and frezzing at -20°C and stored in EtOH methods. In addition, these two methods of fixation resulted in an easier dissection. The gut remained intact when dissected and was surrounded by lesser host tissue. As reported by Linville *et al.* (2004), EtOH results in dehydration by replacing all the water molecules, and dissection after freezing the larvae may result in leaking of gut content if pricked. Hence, care was taken while dissecting the larvae with these methods. Storing larvae in EtOH is also not recommended if visual examination is to be carried out, as it causes changes in larval morphological features by shrinking the tissues, or extracting cellular components, like lipids, causing colour disruptions (Carter 2003).

Quantification results, showed that the fixing methods hugely affect the DNA extraction process. Some fixing process like use of ethanol is very helpful for DNA extraction but it is not suitable for morphological analysis, making it very important for the investigators to use the correct method to facilitate further analysis.

As stated by Linville *et al.* (2004), preserving larvae at lower temperatures helps to lessen or eliminate bacterial growth, and reduces or eliminates bacterial development and enzymatic activity, which helps in preservation of DNA. EtOH works in a similar way as well. The results obtained after STR analysis were also in accordance to the results obtained after weight and quantifications. Methods of only freezing the larvae and only placing in EtOH not only provided a good amount of weight of crop, they also provided the maximum amount of DNA

as observed after quantification and showed the brightest PCR amplification bands for both primers cyt *b* pig and 16S rRNA pig.

A number of studies, such as by Haskell *et al.* (2001), suggest the use of formaldehyde containing Kahle's solution for preservation of larvae. This method is preferred when morphological analysis of the larva is required, but it is not recommended when DNA analysis is to be performed, as the formaldehyde results in tissue degradation as shown by Tokuda *et al.* (1990). This study describes the use of only EtOH or only freezing the larvae as preferred methods of fixation for molecular analysis, which is in contradiction to the entomological guidelines by Amendt *et al.* (2007), who proposed pouring hot water on larvae and immersing them for 40s as an ideal method of fixation. The methods described in this study (only EtOH or only freezing) are also more convenient and practicable for crime scene officers and can help in speedier collection of larvae in cases where further molecular analysis is required.

Animaltype Pig (Biotype) STR multiplex kit was successful in STR analysis of the samples and the results displayed complete STR profiles for samples 17 (3 crops), 18 (5 crops), 19 (1 crop) and 21 (5 crops) for all 12 loci and samples 16 (1 crop) and 20 (3 crops) giving partial DNA profiles. The alleles were compared to the ones obtained after STR analysis of the sample of DNA derived from *Sus scrofa* meat that was fed to the flies while breeding them. Table 4.8a and 4.8b shows the genotyping results for all the samples which displayed complete and partial DNA profiles in comparison to the DNA extracted from the standard (meat fed to the respective flies while breeding). This establishes the fact that *Sus scrofa* DNA extracted from the gut of *M. scalaris* larva was exactly the one that was fed to them. This is concurrent to studies previously conducted on larvae of species of bigger size by Campobasso *et al.* (2005), Di Luise *et al.* (2008), Wells *et al.* (2001a,b), Zehner *et al.* (2004) and several others.

6. Conclusion

Three decades ago, the development of DNA fingerprinting changed the perception of criminal investigation. Since then, the development of modern methods in molecular genetics, statistics and development of massive databases have helped investigators in accurate criminal convictions and exonerations. From time to time, lack of conventional sources of DNA evidence in crime scenes has necessitated investigators and forensic scientists in development and identification of newer sources of DNA evidence. This horizon has been expanded in this field with successful DNA extraction and STR profiling of the gut content of *M. scalaris* larvae. Although, traditionally, larvae were only used for PMI estimation, with the advent of molecular techniques, larval gut content analysis for human identification has been developed. This study concludes that, regardless of the size of the larvae, DNA can be extracted from a minimum of one small sized larva using proper preservation and extraction methods. Furthermore, the study also concludes that fixing the larvae by placing it directly in ethanol, or by directly freezing it, not only helps in easier dissection but is also useful in DNA analysis, both in terms of quality and quantity. This result suggests methods for fixing the larvae, which can not only help investigators in collecting them from the crime scene, but also provide good quality DNA for analysis from its gut content. Although *Sus scrofa* was used as the study model, the results obtained can be expanded to human identification from larvae obtained from crime scenes. A positive DNA profile could be used in multiple cases, establishing a relationship between the suspected movements of the body from the crime scene, or establishing the source of the last meal of larvae, in the case of presence of multiple food sources. Finally, this study concludes that, although the larvae were very small, non-insect DNA could be extracted and used for STR analysis, making these small insects a tool in criminal justice system for human identification.

Further research

This study opens a non-exhaustive list of further research. Firstly, determination and analysis of the time period following the feeding of the 3rd instar larvae of *M. scalaris*, untill which DNA can be successfully extracted and genotyped is a very important parameter that could further be studied. Research could also be employed on larvae that have fed on human tissue, so as to extract and characterise human DNA, as better quality genotyping kits with higher

sensitivity are available for human DNA. Also factors of the STR analysis, like injection time, injection volume along with injection voltage, could be tested to determine if samples are capable of providing complete STR profiles. The search for other methods of fixation that could affect DNA analysis could also be further investigated.

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Appendix 1

Table 1. Results of Post-Hoc Analysis (Tukey HSD) of wet weight of larvae fixed with 5 methods performed to identify the variation amongst the methods used.

(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Significance (p-value)
1	2	0.0026*	0.00008	0.000
	3	0.0016*	0.00008	0.000
	4	0.0028*	0.00011	0.000
	5	0.0027*	0.00011	0.000
2	1	-0.0026*	0.00008	0.000
	3	-0.0010*	0.00008	0.000
	4	0.0003	0.00011	0.159
	5	0.0001	0.00011	0.923
3	1	-0.0016*	0.00008	0.000
	2	0.0010*	0.00008	0.000
	4	0.0012*	0.00011	0.000
	5	0.0011*	0.00011	0.000
4	1	-0.0028*	0.00011	0.000
	2	-0.0003	0.00011	0.159
	3	-0.0012*	0.00011	0.000
	5	-0.0002	0.00013	0.722
5	1	-0.0027*	0.00011	0.000
	2	-0.0001	0.00011	0.923
	3	-0.0011*	0.00011	0.000
	4	0.0002	0.00013	0.722

* The mean difference is significant at the 0.05 level.

Table 2. Results of Post-Hoc Analysis (Tukey HSD) of dry weight of larvae fixed with 5 methods showing statistical variation amongst the methods. No method varied completely statistically from all the other methods.

(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Significance (p-value)
1	2	-0.0001	0.00005	0.414
	3	-0.0002*	0.00005	0.020
	4	-0.0001	0.00007	0.809
	5	-0.0004*	0.00007	0.000
2	1	0.0001	0.00005	0.414
	3	-0.0001	0.00005	0.544
	4	0.0000	0.00007	1.000
	5	-0.0004*	0.00007	0.000
3	1	0.0002*	0.00005	0.020
	2	0.0001	0.00005	0.544
	4	0.0001	0.00007	0.724
	5	-0.0003*	0.00007	0.005
4	1	0.0001	0.00007	0.809
	2	0.0000	0.00007	1.000
	3	-0.0001	0.00007	0.724
	5	-0.0004*	0.00009	0.002
5	1	0.0004*	0.00007	0.000
	2	0.0004*	0.00007	0.000
	3	0.0003*	0.00007	0.005
	4	0.0004*	0.00009	0.002

* The mean difference is significant at the 0.05 level.

Table 3. Post hoc (Tukey HSD) analysis of DNA extracted per crop using three different kits depicting a significant statistical differences amongst the kits

(I) Kit	(J) Method	Mean Difference (I-J)	Std. Error	Significance (p-value)
1	2	-0.3606*	0.02031	0.000
	3	-2.9039*	0.02031	0.000
2	1	0.3606*	0.02031	0.000
	3	-2.5433*	0.02224	0.000
3	1	2.9039*	0.02031	0.000
	2	2.5433*	0.02224	0.000

* The mean difference is significant at the 0.05 level.

Table 4. Post hoc (Tukey HSD) analysis of DNA extracted per crop using different methods revealed significant statistical differences amongst the methods used

(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Significance (p-value)
1	2	-2.7411*	0.10972	0.000
	3	-1.6178*	0.10972	0.000
	4	-0.0889	0.10972	0.927
	5	-1.3928*	0.10972	0.000
2	1	2.7411*	0.10972	0.000
	3	1.1233*	0.10972	0.000
	4	2.6522*	0.10972	0.000
	5	1.3483*	0.10972	0.000
3	1	1.6178*	0.10972	0.000
	2	-1.1233*	0.10972	0.000
	4	1.5289*	0.10972	0.000
	5	0.2250	0.10972	0.251
4	1	0.0889	0.10972	0.927
	2	-2.6522*	0.10972	0.000
	3	-1.5289*	0.10972	0.000
	5	-1.3039*	0.10972	0.000
5	1	1.3928*	0.10972	0.000
	2	-1.3483*	0.10972	0.000
	3	-0.2250	0.10972	0.251
	4	1.3039*	0.10972	0.000

* The mean difference is significant at the 0.05 level.

Appendix 2

Table 1. Quantification results obtained after extracting DNA from larval gut content using the QIAamp® DNA Mini Extraction Kit.

SAMPLE	AVERAGE OF TOTAL DNA (ng/µl)	SD OF TOTAL DNA	AVERAGE OF DNA PER SAMPLE (ng/µl)	SD OF DNA PER SAMPLE
1.1	0.43	0.03	0.43	0.03
1.2	0.35	0.01	0.35	0.01
1.3	0.33	0.03	0.33	0.03
1.4	0.22	0.02	0.22	0.02
1.5	0.57	0.02	0.57	0.02
1.6	0.50	0.02	0.50	0.02
1.7	0.35	0.05	0.35	0.05
1.8	0.42	0.02	0.42	0.02
1.9	0.46	0.01	0.46	0.01
3.1	1.90	0.06	0.63	0.02
3.2	1.67	0.03	0.56	0.01
3.3	1.62	0.04	0.54	0.01
3.4	1.49	0.04	0.50	0.01
3.5	1.63	0.08	0.54	0.03
3.6	1.52	0.04	0.51	0.01
3.7	1.55	0.06	0.52	0.02
3.8	1.56	0.13	0.52	0.04
3.9	1.69	0.02	0.56	0.01
5.1	3.95	0.04	0.79	0.01
5.2	3.62	0.04	0.72	0.01
5.3	3.84	0.04	0.77	0.01
5.4	3.60	0.03	0.72	0.01
5.5	3.98	0.06	0.80	0.01
5.6	3.95	0.06	0.79	0.01
5.7	3.89	0.06	0.78	0.01
5.8	3.83	0.05	0.77	0.01
5.9	3.85	0.01	0.77	0.00

Table 2. Quantification results obtained after extracting DNA from gut of larvae fixed by pouring hot water for 30s (QIAamp® DNA Investigator Kit).

Method - Hot Water for 30s				
SAMPLE	**AVERAGE OF TOTAL DNA** (ng/µl)	**SD OF TOTAL DNA**	**AVERAGE OF DNA PER SAMPLE** (ng/µl)	**SD OF DNA PER SAMPLE**
1.1	0.69	0.02	0.69	0.02
1.2	0.68	0.01	0.68	0.01
1.3	0.67	0.02	0.67	0.02
1.4	0.71	0.02	0.71	0.02
1.5	0.84	0.04	0.84	0.04
1.6	0.79	0.01	0.79	0.01
3.1	3.36	0.37	1.12	0.12
3.2	3.33	0.33	1.11	0.11
3.3	3.22	0.30	1.07	0.10
3.4	3.07	0.03	1.02	0.01
3.5	3.09	0.02	1.03	0.01
3.6	3.12	0.07	1.04	0.02
5.1	5.04	0.07	1.01	0.01
5.2	5.00	0.08	1.00	0.02
5.3	4.93	0.08	0.99	0.02
5.4	4.92	0.04	0.98	0.01
5.5	4.98	0.10	1.00	0.02
5.6	5.11	0.03	1.02	0.01

Table 3. Quantification results obtained after extracting DNA from gut of larvae fixed by only freezing at -20°C (QIAamp® DNA Investigator Kit).

Method - Frozen only (-20°C)				
SAMPLE	**AVERAGE OF TOTAL DNA** (ng/µl)	**SD OF TOTAL DNA**	**AVERAGE OF DNA PER SAMPLE** (ng/µl)	**SD OF DNA PER SAMPLE**
1.1	3.15	0.09	3.15	0.09
1.2	4.57	0.02	4.57	0.02
1.3	4.33	0.15	4.33	0.15
1.4	3.97	0.05	3.97	0.05
1.5	3.96	0.05	3.96	0.05
1.6	4.16	0.15	4.16	0.15
3.1	9.77	0.06	3.26	0.02
3.2	9.60	0.08	3.20	0.03
3.3	9.64	0.09	3.21	0.03
3.4	9.09	0.12	3.03	0.04
3.5	9.41	0.24	3.14	0.08
3.6	9.79	0.10	3.26	0.03
5.1	19.51	0.11	3.90	0.02
5.2	19.34	0.16	3.87	0.03
5.3	19.02	0.10	3.80	0.02
5.4	19.08	0.14	3.82	0.03
5.5	18.70	0.22	3.74	0.04
5.6	18.68	0.04	3.74	0.01

Table 4. Quantification results obtained after extracting DNA from gut of larvae fixed by first by pouring hot water for 30s and then placing in EtOH and storing at -20°C (QIAamp® DNA Investigator Kit).

Method - Hot Water for 30s then EtOH				
SAMPLE	**AVERAGE OF TOTAL DNA** (ng/µl)	**SD OF TOTAL DNA**	**AVERAGE OF DNA PER SAMPLE** (ng/µl)	**SD OF DNA PER SAMPLE**
1.1	0.88	0.00	0.88	0.00
1.2	0.82	0.02	0.82	0.02
1.3	0.84	0.05	0.84	0.05
1.4	0.82	0.03	0.82	0.03
1.5	0.86	0.03	0.86	0.03
1.6	0.86	0.02	0.86	0.02
3.1	3.71	0.13	1.24	0.04
3.2	3.62	0.04	1.21	0.01
3.3	3.47	0.06	1.16	0.02
3.4	3.30	0.03	1.10	0.01
3.5	3.68	0.09	1.23	0.03
3.6	3.60	0.14	1.20	0.05
5.1	5.26	0.06	1.05	0.01
5.2	5.14	0.05	1.03	0.01
5.3	5.17	0.06	1.03	0.01
5.4	5.06	0.04	1.01	0.01
5.5	5.16	0.03	1.03	0.01
5.6	5.02	0.05	1.00	0.01

Table 5. Quantification results obtained after extracting DNA from gut of larvae fixed first by freezing at -20°C then placing in EtOH and storing at -20°C (QIAamp® DNA Investigator Kit).

Method - Frozen (-20°C) then EtOH				
SAMPLE	**AVERAGE OF TOTAL DNA** (ng/µl)	**SD OF TOTAL DNA**	**AVERAGE OF DNA PER SAMPLE** (ng/µl)	**SD OF DNA PER SAMPLE**
1.1	2.56	0.11	2.56	0.11
1.2	2.37	0.12	2.37	0.12
1.3	2.45	0.05	2.45	0.05
1.4	2.54	0.05	2.54	0.05
1.5	2.61	0.09	2.61	0.09
1.6	2.51	0.07	2.51	0.07
3.1	7.50	0.03	2.50	0.01
3.2	7.69	0.19	2.56	0.06
3.3	7.43	0.17	2.48	0.06
3.4	7.91	0.09	2.64	0.03
3.5	7.58	0.06	2.53	0.02
3.6	6.97	0.05	2.32	0.02
5.1	9.86	0.09	1.97	0.02
5.2	9.73	0.04	1.95	0.01
5.3	9.60	0.11	1.92	0.02
5.4	10.06	0.04	2.01	0.01
5.5	10.00	0.08	2.00	0.02
5.6	9.62	0.10	1.92	0.02

Table 6. Quantification results obtained after extracting DNA from gut of larvae fixed by placing them directly in EtOH and then storing at -20°C (QIAamp® DNA Investigator Kit).

Method - EtOH only (-20°C)				
SAMPLE	**AVERAGE OF TOTAL DNA** (ng/µl)	**SD OF TOTAL DNA**	**AVERAGE OF DNA PER SAMPLE** (ng/µl)	**SD OF DNA PER SAMPLE**
1.1	3.12	0.14	3.12	0.14
1.2	2.77	0.09	2.77	0.09
1.3	2.63	0.16	2.63	0.16
1.4	2.86	0.09	2.86	0.09
1.5	3.21	0.23	3.21	0.23
1.6	3.38	0.07	3.38	0.07
3.1	7.95	0.07	2.65	0.02
3.2	7.78	0.16	2.59	0.05
3.3	7.68	0.17	2.56	0.06
3.4	8.15	0.20	2.72	0.07
3.5	8.28	0.16	2.76	0.05
3.6	8.12	0.12	2.71	0.04
5.1	9.96	0.14	1.99	0.03
5.2	9.90	0.07	1.98	0.01
5.3	9.77	0.09	1.95	0.02
5.4	10.09	0.04	2.02	0.01
5.5	10.13	0.07	2.03	0.01
5.6	9.82	0.06	1.96	0.01

Table 7. Quantification results obtained after extracting DNA from larval gut content using the PrepFiler® Forensic DNA extraction Kit (Applied Biosystems™).

SAMPLE	AVERAGE OF TOTAL DNA (ng/µl)	SD OF TOTAL DNA	AVERAGE OF DNA PER SAMPLE (ng/µl)	SD OF DNA PER SAMPLE
1.1	3.80	0.08	3.80	0.08
1.2	3.82	0.03	3.82	0.03
1.3	3.60	0.03	3.60	0.03
1.4	3.49	0.03	3.49	0.03
1.5	3.64	0.02	3.64	0.02
1.6	3.79	0.02	3.79	0.02
3.1	9.78	0.02	3.26	0.01
3.2	9.60	0.02	3.20	0.01
3.3	9.48	0.01	3.16	0.00
3.4	9.55	0.02	3.18	0.01
3.5	9.64	0.03	3.21	0.01
3.6	9.52	0.02	3.17	0.01
5.1	17.66	0.05	3.53	0.01
5.2	17.88	0.06	3.58	0.01
5.3	17.49	0.10	3.50	0.02
5.4	17.60	0.05	3.52	0.01
5.5	17.62	0.20	3.52	0.04
5.6	17.92	0.05	3.58	0.01

Appendix 3

Aligned sequences of the received from Eurofins Genomics, Germany. These were compared to sequences available in GenBank (MH319786.1, MG837550.1) to establish identity of the sample (1-10).

(a) cyt *b PORK*

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             ....|....| ....|....| ....|....| ....|....| ....|....|
                      10         20         30         40         50
MH319786.1   --GCCTTCAT AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
1            --GCCTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
2            ACGCCTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
3            -CGCCTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
4            -CGCCTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
5            ----CTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
6            -CGCCTTC-T AGGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
7            ---------- -AGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGAAGG
8            ------TCTA TAGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
9            --------TA TAGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
10           --------TA TAGCTACGTC CTGCCCTGAG GACAAATATC ATTCTGA-GG
Clustal Co                ******** ********** ********** ******* **


             ....|....| ....|....| ....|....| ....|....| ....|....|
                      60         70         80         90        100
MH319786.1   AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
1            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
2            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
3            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
4            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
5            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
6            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
7            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
8            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
9            AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
10           AGCTACGGTC ATCACAAATC TACTATCAGC TATCCCTTAT ATCGGAACAG
Clustal Co   ********** ********** ********** ********** **********


             ....|....| ....|..
                     110
MH319786.1   ACCTCGTAGA -------
1            ACCTCGTAGA TCGGTAC
2            ACCTCGTAGC CCTCTTT
3            ACCTCGTAGT AGTCGC-
4            ACCTCGTAGC AACTTCT
5            ACCTCGTAGA CTTG---
6            ACCTCGTAGC TTATGAC
7            ACCTCGTAGA TT-----
8            ACCTCGTAGA GGCTTCT
9            ACCTCGTAGA TGAAG--
10           ACCTCGTAGA ATGAC--
Clustal Co   *********
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(b) 16S rRNA

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               ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
MG837550.1     -----AGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
1              --CTCAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
2              GCCTAAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTCCA ACAAATTTC-
3              GATAGAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
4              -CCTAAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTCCA ACAAATTTCC
5              ACTAGAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
6              ---TCTGCAG CCATCAATTG AGCAAAGCGT TAAAGCTC-A ACAAATTC--
7              AGCTAAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
8              GATAGAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
9              ---AAAGCAG CCATCAATTG AGCAAAGCGT TAAAGCTC-A ACAAATTC--
10             GATAGAGCAG CCATCAATTG AG-AAAGCGT TAAAGCTC-A ACAAATTC--
Clustal Co           **** ********** ** ******* ******** * *******

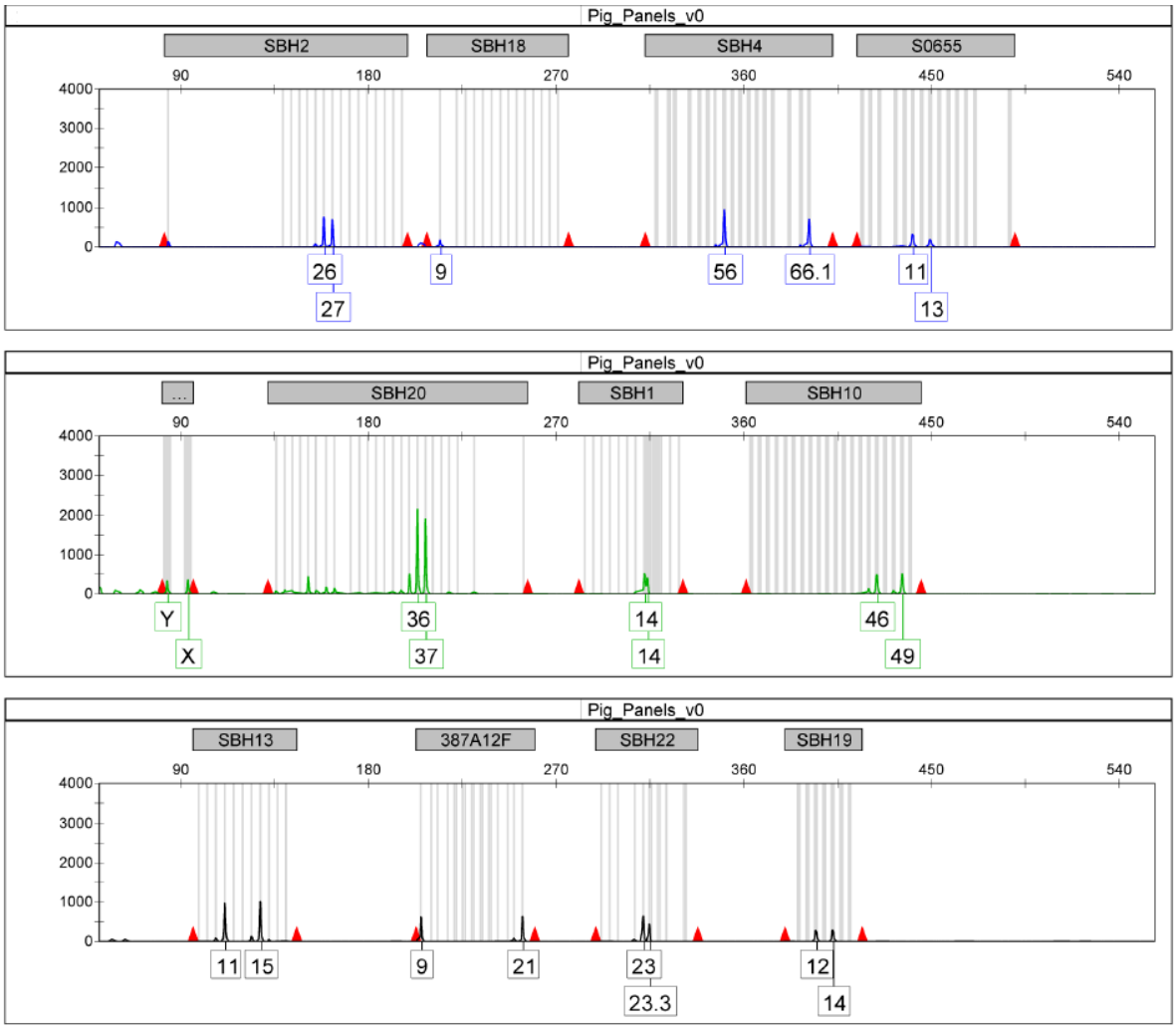

               ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
MG837550.1     ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACC---
1              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCGA
2              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC C---------
3              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCAA
4              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCTTAGCC C---------
5              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCAG
6              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCAA
7              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCAC
8              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCCGA
9              ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCAGA
10             ACCAACATAA TCCCAAAAAC TAATAACAAA CTCCT-AGCC CAATACCGGC
Clustal Co     ********** ********** ********** ***** **** *


               ..

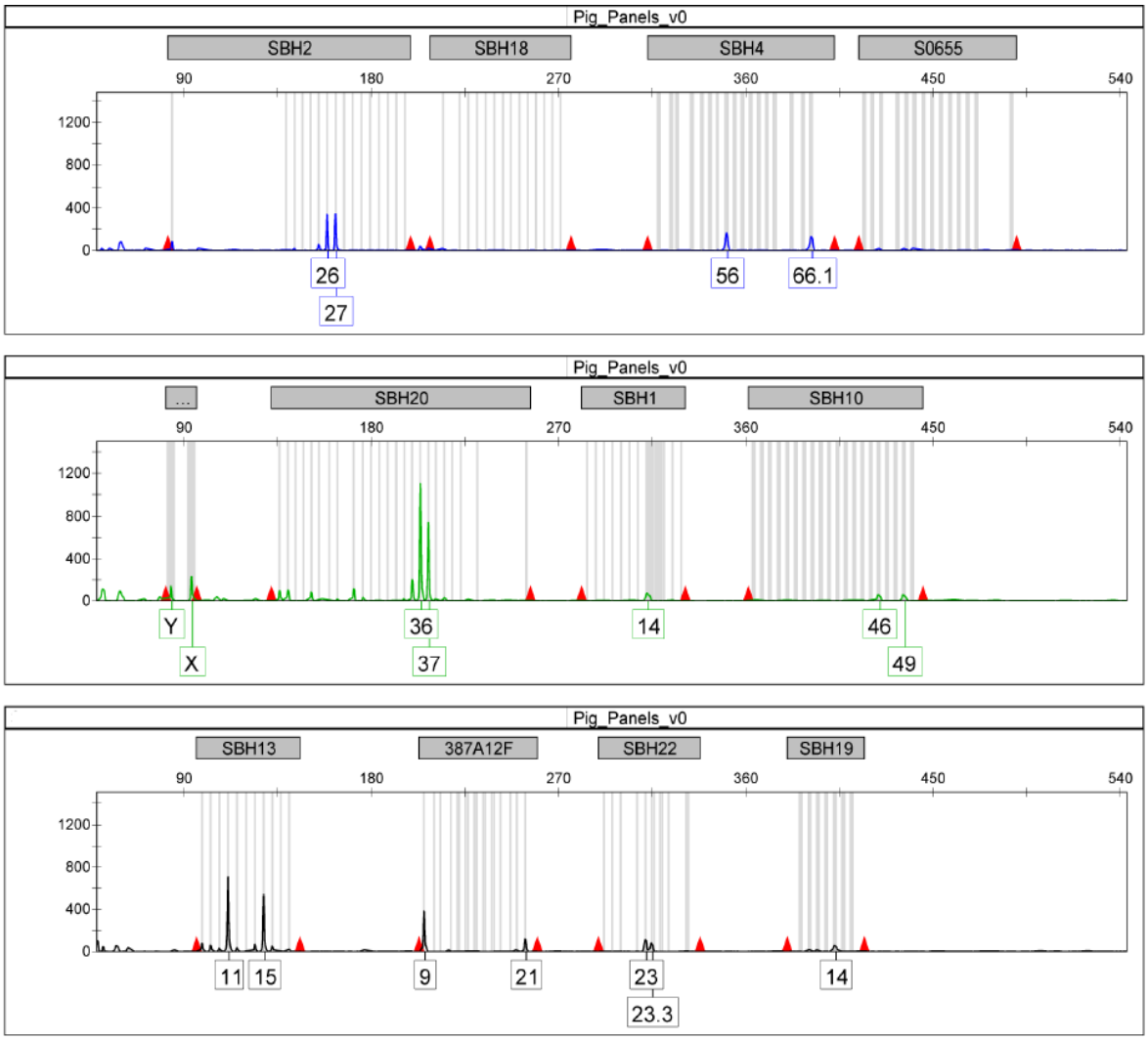
MG837550.1     --
1              AG
2              --
3              GG
4              --
5              A-
6              CG
7              AT
8              A-
9              C-
10             TG
Clustal Co
```

Appendix 4

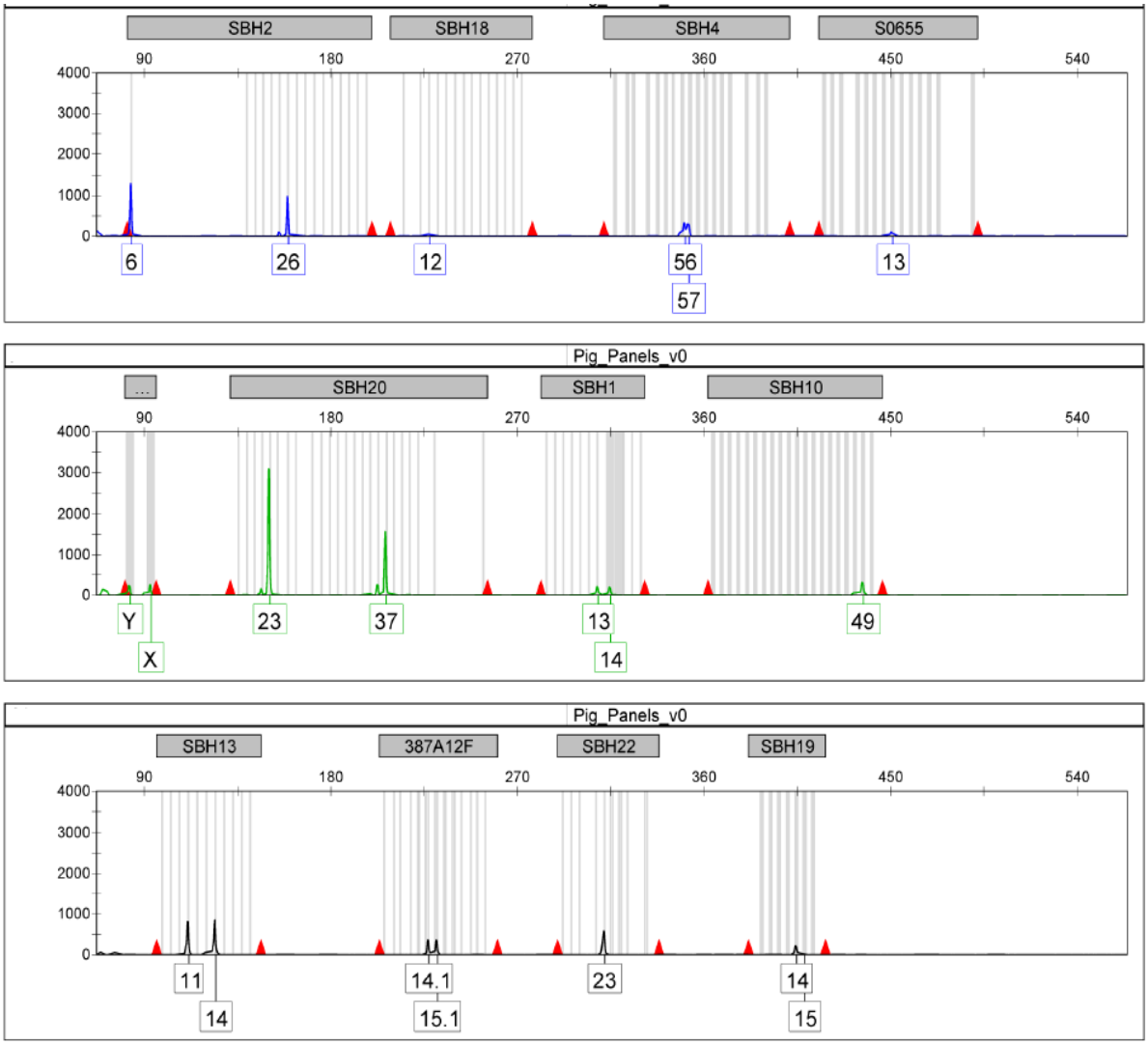
1. Electropherogram depicting a complete STR profile (12 loci) of *Sus scrofa* DNA extracted from the gut of one *M. scalaris* larva fixed with only EtOH (Sample 19).



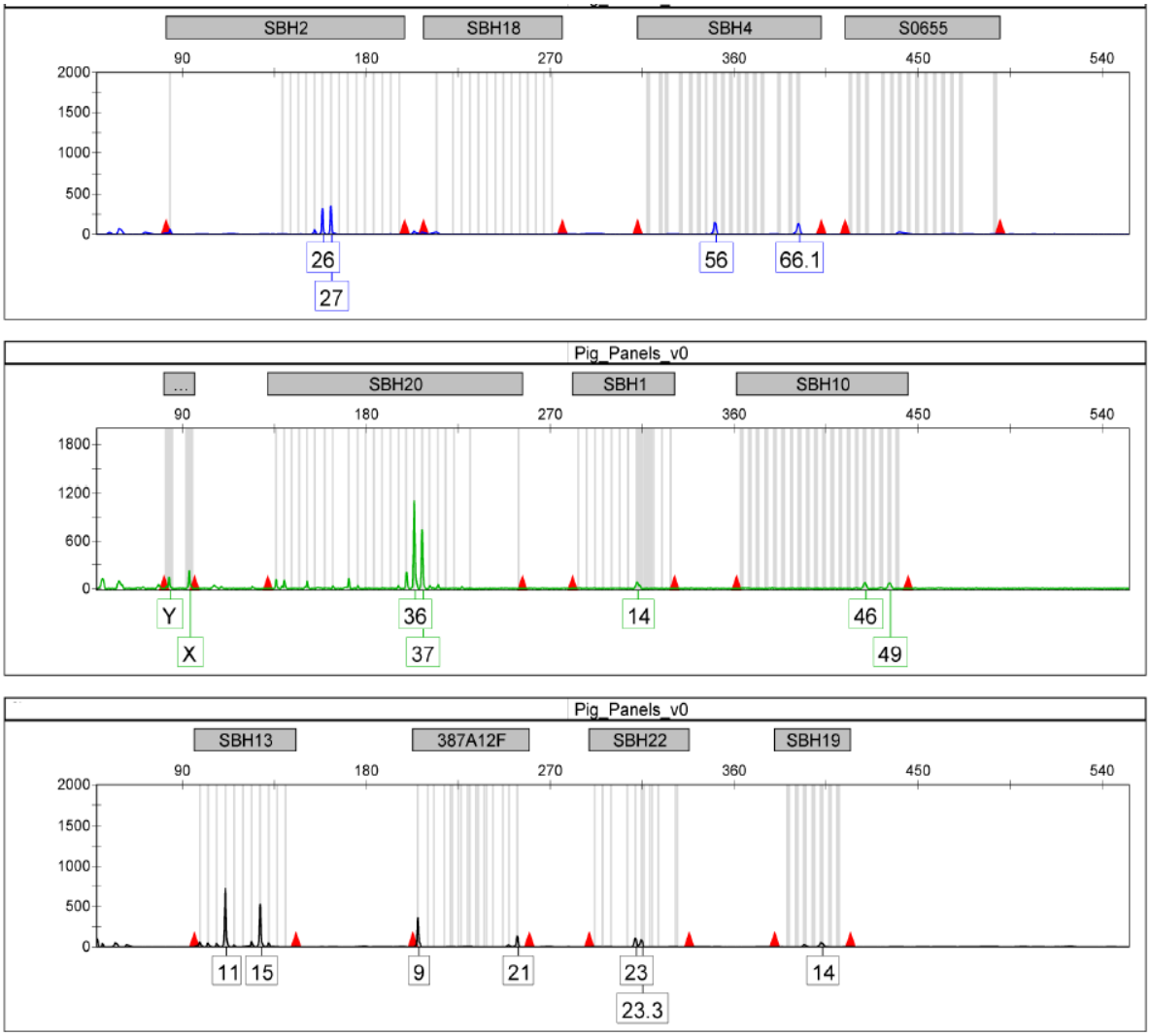
2. Electropherogram depicting a partial STR profile (10 loci) of *Sus scrofa* DNA extracted from the gut of three *M. scalaris* larvae fixed with only EtOH (Sample 20).



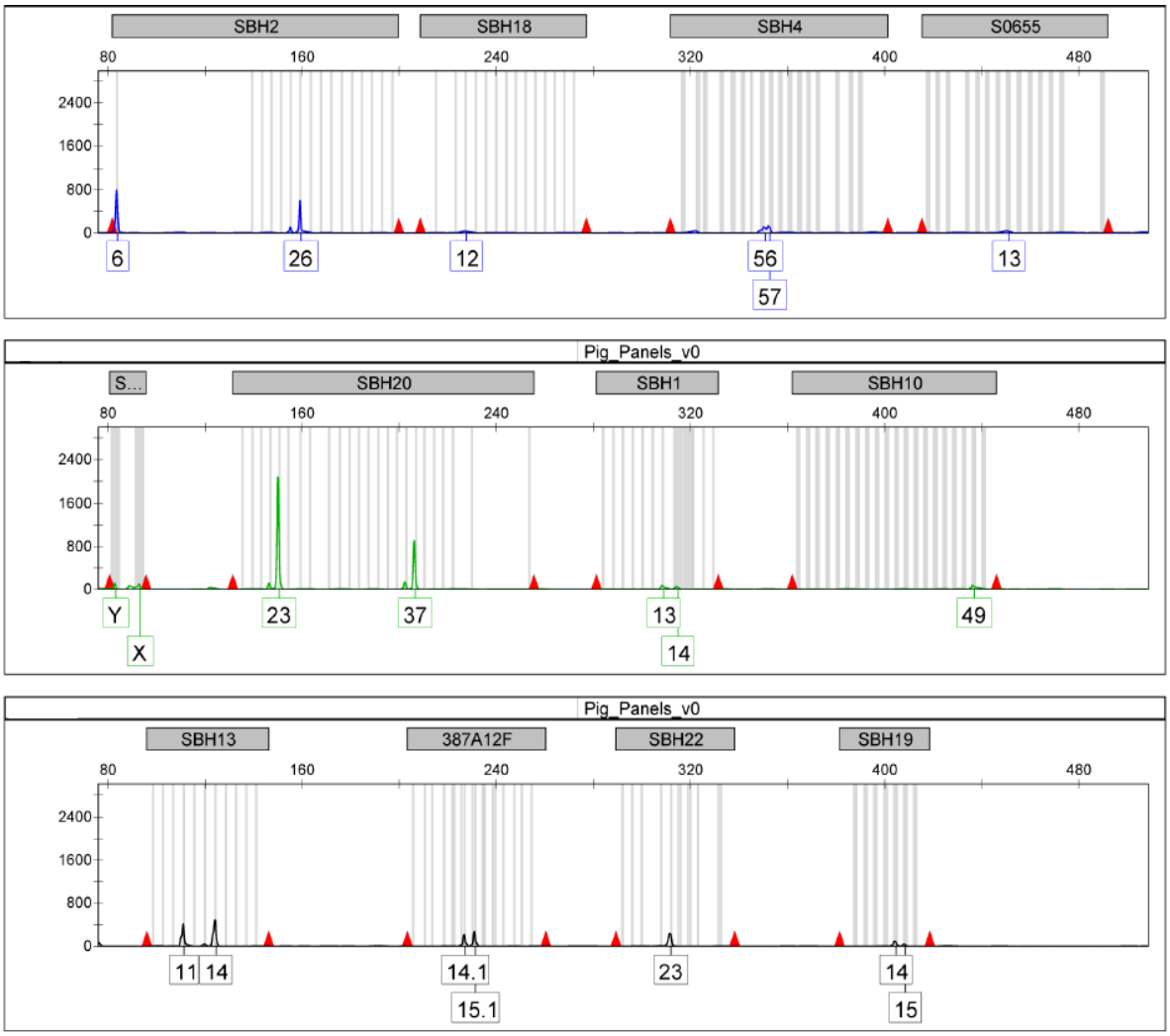
3. Electropherogram depicting a complete STR profile (12 loci) of *Sus scrofa* DNA extracted from the gut of five *M. scalaris* larvae fixed with only EtOH (Sample 21).



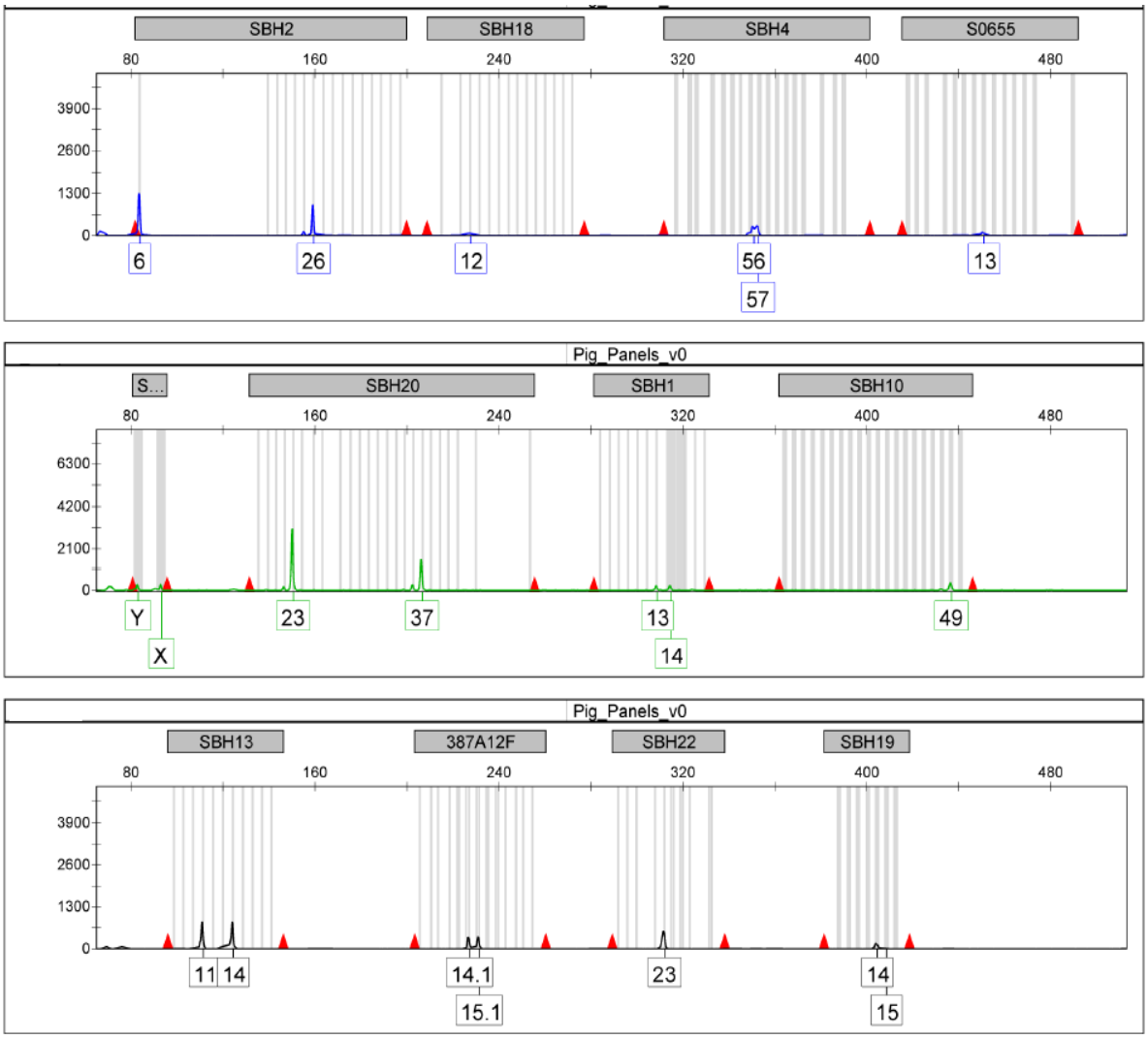
4. Electropherogram depicting a partial STR profile (10 loci) of *Sus scrofa* DNA extracted from the gut of one *M. scalaris* larva fixed with only freezing (-20°C) (Sample 16).



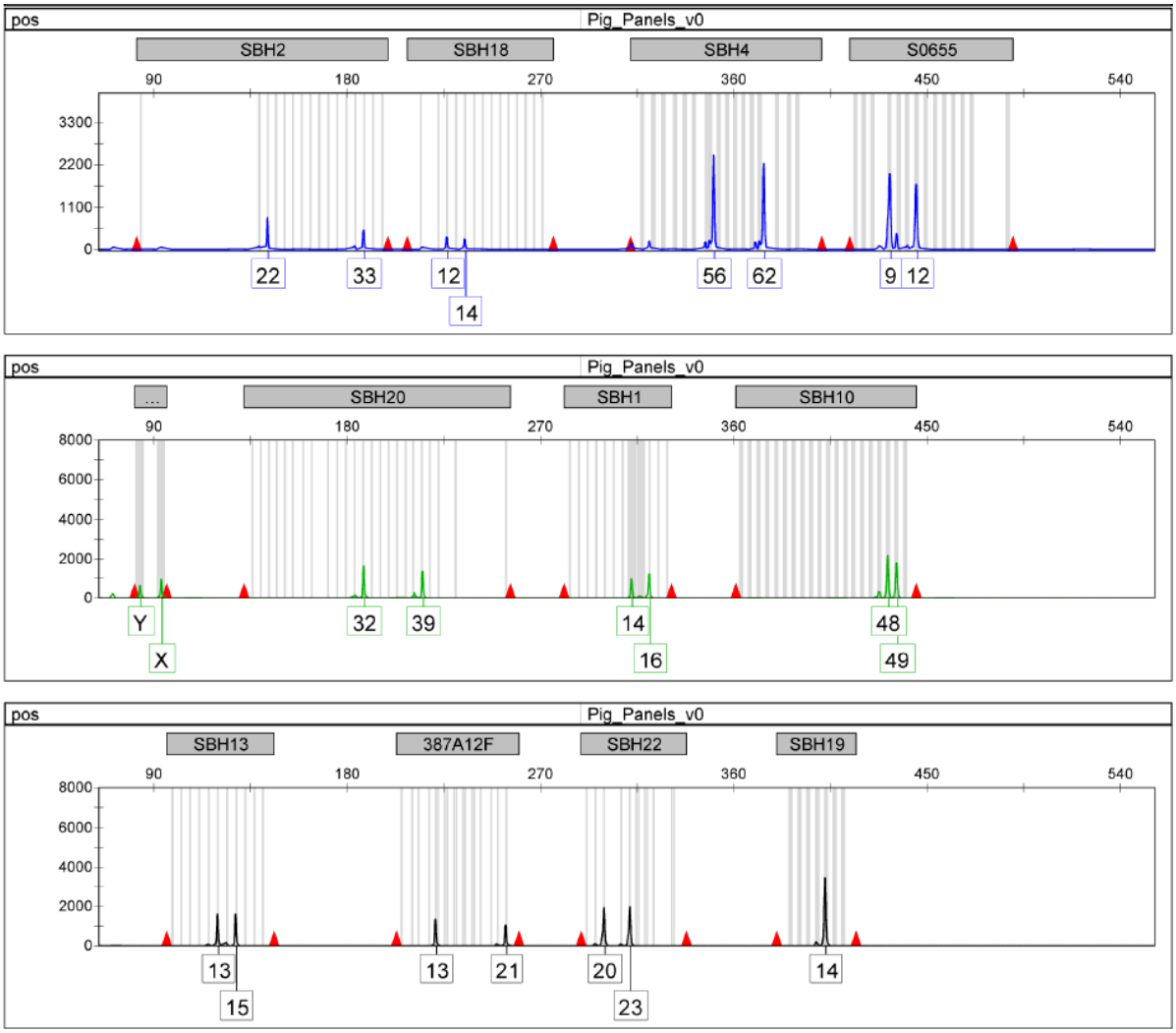
5. Electropherogram depicting a complete STR profile (12 loci) of *Sus scrofa* DNA extracted from the gut of three *M. scalaris* larvae fixed with only freezing (-20°C) (Sample 17).



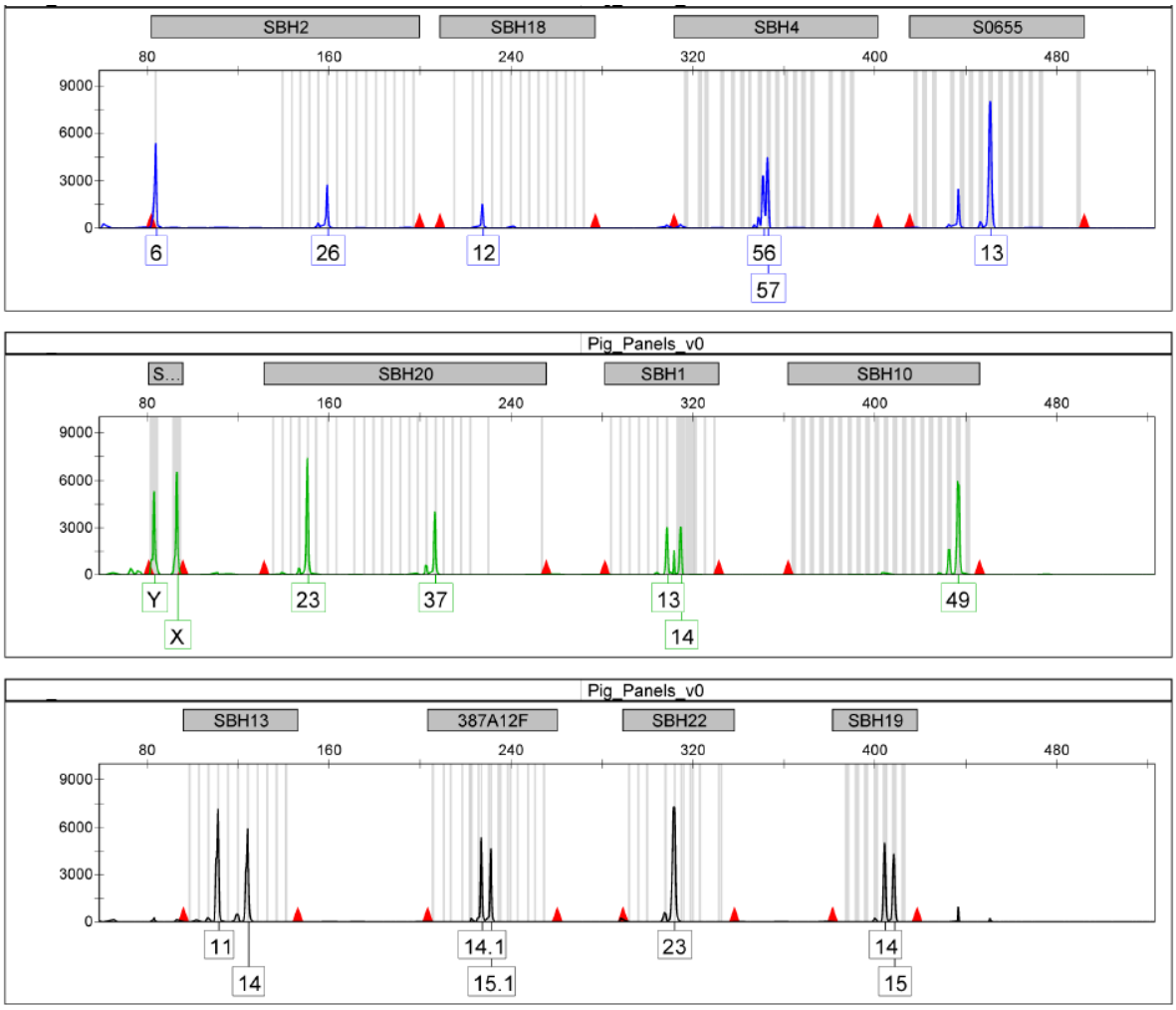
6. Electropherogram depicting a complete STR profile (12 loci) of *Sus scrofa* DNA extracted from the gut of three *M. scalaris* larvae fixed with only freezing (-20°C) (Sample 18).



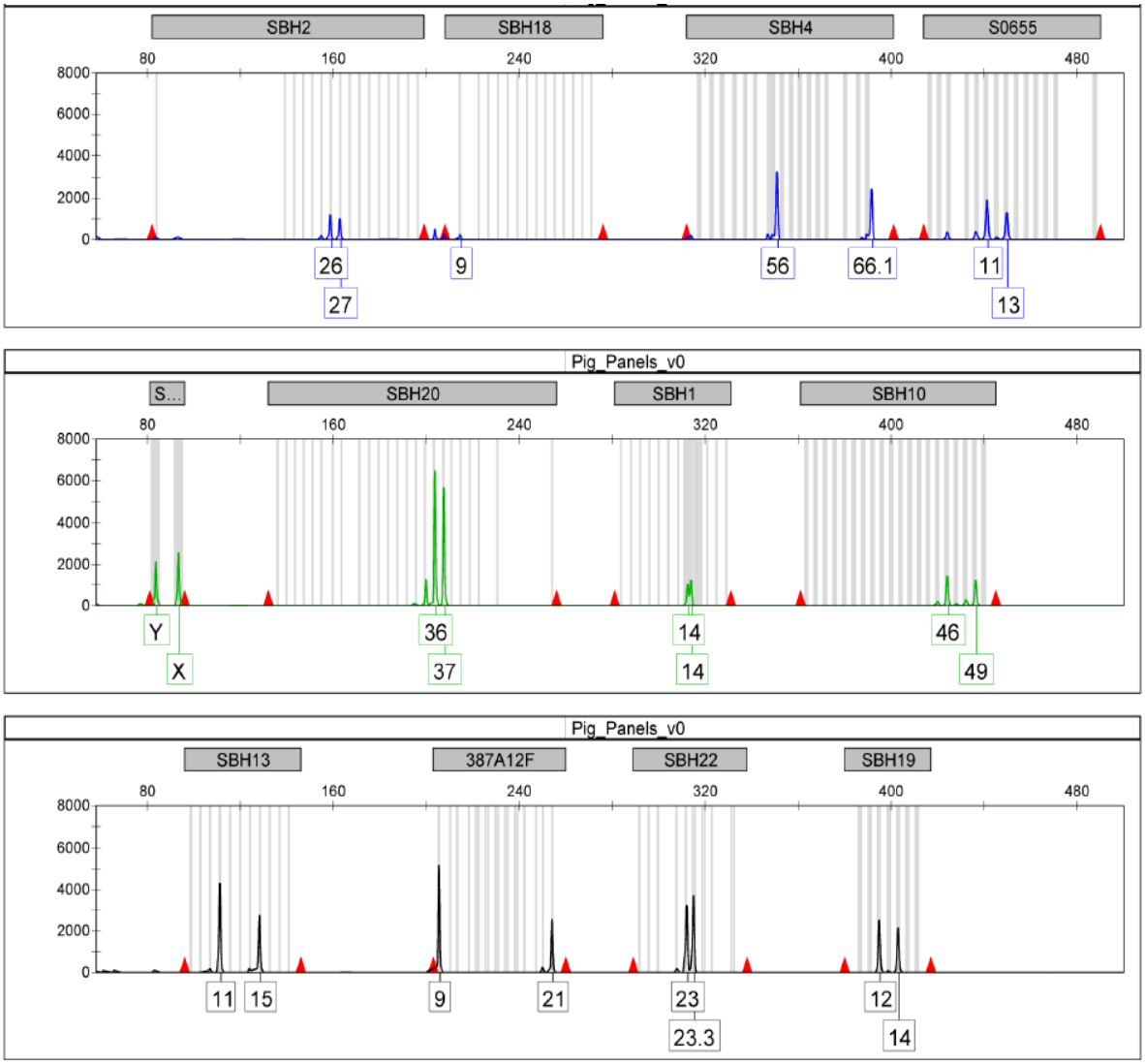
7. Electropherogram showing complete STR profile (12 loci) for the positive control, which was provided by the Animaltype Pig (Biotype) kit, to test proper functioning of the reaction.



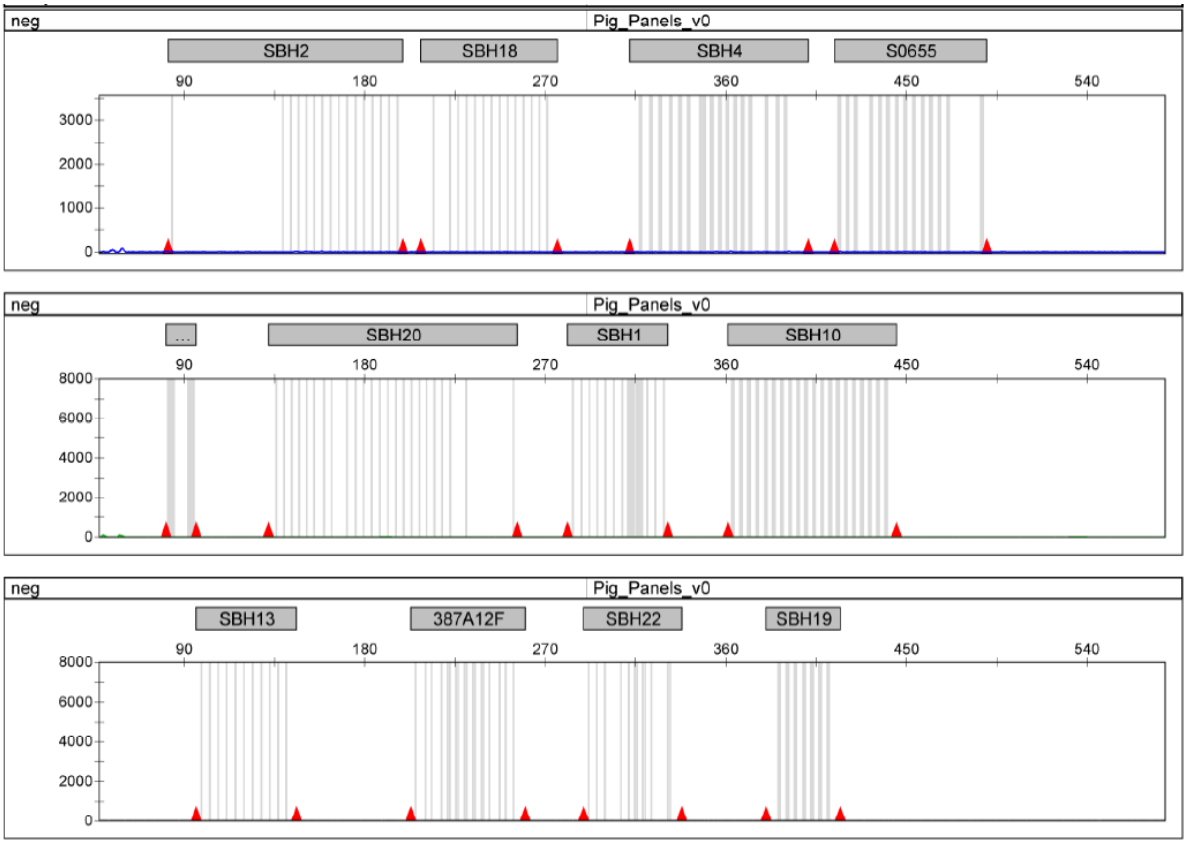
8. Electropherogram showing complete STR profile (12 loci) for DNA extracted from *Sus scrofa* meat (Standard 1), which was fed to *M. scalaris* larvae while rearing them. It was used as a reference to compare profiles obtained from DNA extracted from the larval gut content.



9. Electropherogram showing complete STR profile (12 loci) for DNA extracted from *Sus scrofa* meat (Standard 2), which was fed to *M. scalaris* larvae while rearing them. It was used as a reference to compare profiles obtained from DNA extracted from the larval gut content.



10. Electropherogram showing complete STR profile (12 loci) for the negative control, which was provided by the Animaltype Pig (Biotype) kit, to test proper functioning of the reaction.



11. Electropherogram showing complete STR profile (12 loci) for the Allelic Ladder, which was provided by the Animaltype Pig (Biotype) kit, to test proper functioning of the reaction.