University of HUDDERSFIELD

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

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A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Master by research.

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Abbreviations

°C	Degrees Celsius
A	Adenine
ABI	Applied Biosystems®
ANOVA	Analysis of variance
Bp	Base Pairs
C	Cytosine
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
COI I+II	Cytochrome c oxidase subunits I and II
C_T	Cycle Threshold
CytB	Cytochrome B
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide Triphosphate
dsDNA	Doube Stranded DNA
EDTA	Ethylenediaminetetraacetic acid
E-mail	Electronic mail
ESS	European Standard Set
et al.	And others
EtOH	Ethanol
FLEA	Forensic Laboratory of Entomology and Archaeology
FRET	Fluoresent Resonance Energy Transfer
FSS	Forensic Science Service
FTA	Flinders Technology Associates
G	Guanine

HCl	Hydrochloric Acid
H_o	Null Hypothesis
H-Strand	Heavy strand
HV1	Hypervariable region 1
HV2	Hypervariable region 2
ISAG	International Society of Animal Genetics
L-Strand	Light Strand
M.scalaris	*Megaselia scalaris*
mtDNA	Mitochondrial DNA
ng/µl	Nanograms per microliter
OL	Off Ladder
p	Significance
PBI	Post Burial Interval
PC	Personal Computer
PCR	Polymerase Chain Reaction
Pm	Match Probability
PMI	Post Mortem Interval
PSA	Prostate specific antigen
qPCR	Quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescent Units
RNA	Ribonucleic acid
rpm	Revolutions per Minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SGM	Second Generation Multiplex

STR	Short Tandem Repeats
T	Thymine
Taq	*Thermus aquaticus*
TBE	Tris/Borate/EDTA
V	Volt
VNTR	Variable Number Tandem Repeats
w/v	Weight over Volume

Abstract:

Over the years, after the advent of minisatellite and microsatellite DNA typing, investigators generally use conventional sources like body fluids and cadaver as a source of DNA evidence for establishing identification. But in cases of extreme body decomposition or in the absence of the body, removed from the crime scene, these conventional methods fail to obtain the identity of the victim, paving way for the use of non-conventional sources as source of DNA evidence. DNA extraction from larval gut content of *Megaselia scalaris* larvae (non-conventional source), can be used for victim identification. While most of the work focus on PMI estimation, DNA analysis of gut contents of larvae found at a crime scene and the determination of their food source is a relatively new field and is still in its initial periods of development and till now is only documented on larvae of big size, mainly belonging to species in the family Calliphoridae. In this study DNA was successfully extracted from the gut content of *M. scalaris* (Diptera, Phoridae) larvae fed on *Bos taurus* meat. The post feeding 3rd instar larvae were fixed using 5 different methods - hot water ($\geq$ 80˚C) for 30s on the larvae, only freezing the larvae (-20˚C), freezing (-20˚C) the larvae first and then placing them in EtOH, placing the larvae directly in EtOH (100%) and pouring hot water ($\geq$ 80˚C) for 30s on the larvae and then placing them in EtOH (100%). DNA extraction was performed using the Qiagen Investigator Kit. ANOVA test followed by a Post-Hoc analysis (Tukey HSD) revealed that there was statistical significant difference among all methods (p=0.000). Method 4 was seen to provide the maximum DNA yield (3.36 ± 0.14ng/µl). After this, amplification was done using the ABI PCR System of the mitochondrial gene Cytochrome B and ribosomal gene 16s rRNA. The sequences obtained showed positive results in almost all samples as well as in the controls. After a positive amplification result with both PCR and quantification analysis in qPCR, a successful STR analysis revealed an exact match of the STR profiles of the sample of DNA extracted from the gut and DNA extracted from the *Bos taurus* meat that was fed to the larvae. This proves that DNA can successfully be extracted and characterised from larval gut content irrespective of its size. The results obtained with *Megaselia scalaris* are particularly important since this species is found in both indoor and buried crime scenes. This study improves the ability to extract DNA from Dipteran larvae of forensic interest and it can also be used as an effective method to support forensic investigation

1. Introduction:

Forensic science is a multi-disciplinary field, which help in the reconstruction of a crime scene based on the collection, analysis and interpretation of scientific evidence. Each crime is unique in its own way. This is because the nature of the crime executed, the location of the scene of crime, the people involved in committing the crime, the items that have been used, all vary from one crime scene to the other (Houck & Siegel, 2010).

1.1 Crime Scene Investigation

Reconstruction of a crime scene begins with a primary response to the scene of crime, which includes securing and isolating the scene of incidence to avoid any disturbance to the possible evidence present. This is followed by documentation of the crime scene, recognition of the physical items (exhibits), their collection and then packaging and preservation. These are then sent to Forensic laboratories for examination which then results in the crime scene interpretation and reconstruction (Lee et al., 2001) (Fig.1.1).

Evidence can refer to any item either in the form of a personal testimony, a document, or material object, that is submitted in a court of law to aid a legal investigation. (Houck & Siegel, 2010).

Every crime scene has its own peculiar circumstances and problems and thus the kind of evidence that can be collected to aid in an investigation can vary . There are, however, some common kinds of physical evidence whose presence should be checked for at each crime scene (Saferstein, 2013). As a general outline they are classified with respect to the nature and manner of the evidence into the following types (Lee & Harris, 2011):

a. Transient evidence - This type of evidence can, according to its nature, be transient, i.e., it can be easily changed or lost. Some common transient evidence at crime scenes can be odours, temperatures, colour, occurrences such as rigidity or the drying of blood. Due to its temporary character, as soon as this form of evidence is encountered it must be documented.

b. Conditional evidence - This evidence is usually a result of a collection of activities or backdrops at the crime scene such as, settings of television and computer, state in which the victim's body is found, lighting conditions, window positions, smoke or fire, or precise locations of definite

evidence within the scene. This type of evidence is like transient evidence and if it is not spotted and documented immediately upon encounter, it cannot be recovered later.

c. Pattern evidence - Glass fracture patterns, blood spatter patterns, furniture position patterns, liquid burn patterns, track-trail patterns, clothing or article patterns, modus operandi patterns, skid or tire mark patterns, and gun powder residue patterns, are some of the chief pattern evidence encountered at a crime scene.

d. Transfer evidence/trace evidence - Following Locard's exchange principal, transfer evidence are produced due to the contact between individuals and objects. It can also happen due to the contact of individuals with objects. Fingerprint, hair, body fluids, fibre, soil and chemicals are some of the typically encountered trace evidence. This sort of evidence is the standard forensic evidence examined in forensic laboratories and more information about it has been discussed in this thesis.

e. Medical evidence – The manner and extent of injury, the number and size of the wounds along with the site and condition of a wound related to a victim's, suspect's, or witness's injuries is referred to as a medical evidence. It also includes the category of medical equipment on the person, house or car, medical history as well as the prescription history.

f. Electronic evidence - In this age of technology and the increased accessibility of electronic devices, most people possess a PC, mobile phone, iPod, tablet and additional personal devices. Several families and businesses install surveillance cameras and other recording equipment for security purposes which investigators may collect and preserve if it aids in the investigation. Examples of information that may offer investigative leads consist of call records of mobile phones, e-mails retrieved from a suspected PC, and security camera footage log from a bank.

g. Associative evidence - Evidence associating a victim or suspect with a crime or connecting them with each other are known as associative evidence. Examples of associative evidence consist of the personal belongings found in the scene of crime like wallet, rings, receipts, tickets or business cards.

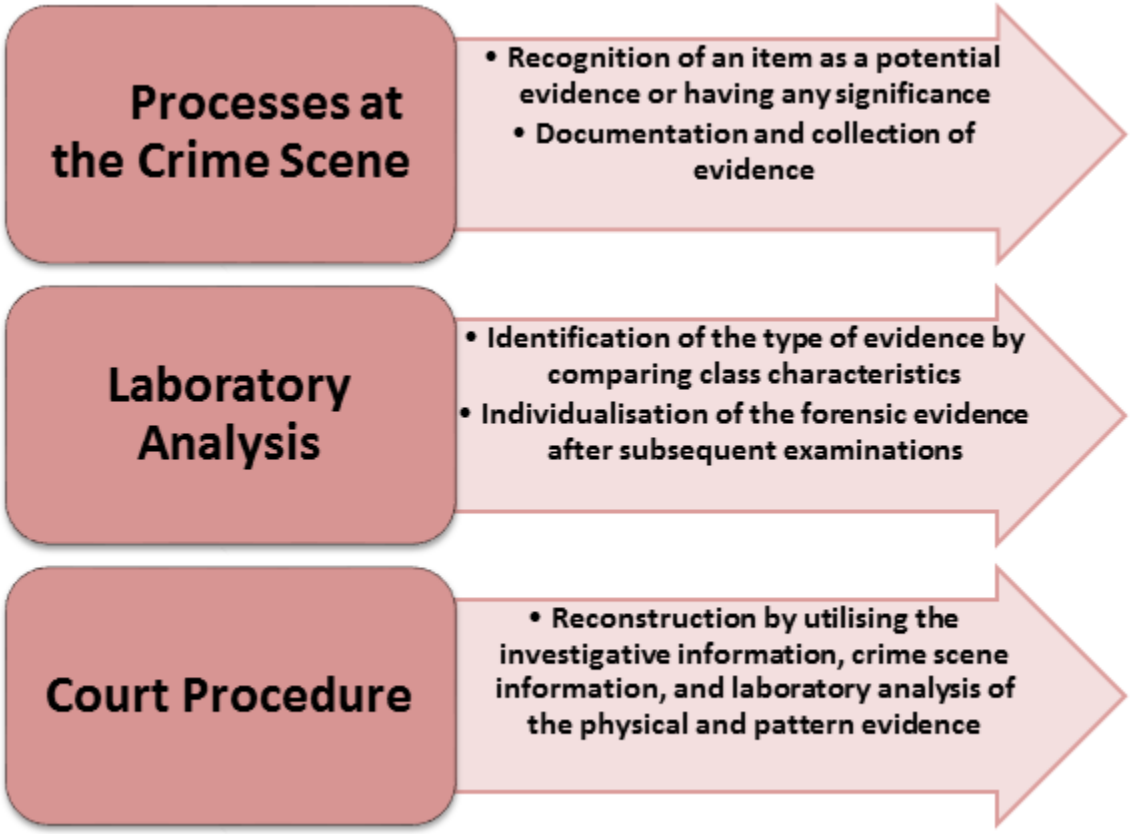


Figure 1.1 The course of a forensic examination of an evidence (Lee & Pagliaro, 2013).

1.2 Sample Characterization

After collection, the exhibits are classified as a biological or non-biological evidence and then processed accordingly. The former, due to its sensitivity and power of discrimination (Lee & Ladd, 2001), plays a fundamental role in the practice of identification of individuals involved in a crime (perpetrator, victim and witnesses). An evidence associated to a *corpus delicti* (body of the crime) is given the highest priority, for example the victim's blood, so as to establish victim-to-perpetrator or victim-to-scene or perpetrator-to-scene linkages (Li, 2008).

For collection of biological evidence the approach taken differs according to the state it is found in (Li, 2008).

a) Dry stains of biological fluids - These can be collected using swabs or by lifting or scraping. In a few cases, if present on a large object which cannot be transported, the stains have to be cut. In another type of case if an item contains blood stain pattern or the stain is difficult to swab or it requires multiple analysis, the entire item is collected.

b) Wet stains of biological fluids – These are usually collected by swabbing or using FTA (Flinders Technology Associates) cards. It is advisable to air dry a wet evidence before packaging to avert degradation.

c) Patent prints requiring multiple analysis – Sometimes a crime scene may contain a patent print such as a bloody shoe print which may require multiple analysis. In such cases a non-destructive procedure, for collecting the shoe ridge details, must be followed. Blood for DNA analysis can be collected after this.

d) Latent prints requiring multiple analysis – A fingerprint present can be used for DNA analysis along with linking it to a suspect. Hence in instances where latent prints may need multiple analysis.

e) Bones/teeth – These are frozen and placed in a container to preserve them.

f) Fingernails and scrapings – These can be collected by either clipping or scraping onto a clean paper and then wrapped using druggist's fold.

g) Hair – These are either lifted or transferred on a piece of paper that can be folded or vacuum is used to collect hair if none of the aforementioned procedures work.

h) Entomological evidence – For specimens already dead 70-90% ethanol is used to preserve them irrespective of whether they are adult or juvenile. For live specimens of eggs and larvae it is suggested to place them on dampened tissue paper inside vials and then transfer to a specialist for rearing within 24 hours. Pupae should be transferred for rearing under 24 hours and if not possible stored in vials at cool temperatures (2° – 6° C). Live adults should first be killed by leaving them inside a vial, which is then placed into a freezer (preferably set at −20°C) for 1 hour. The dead specimens are stored in 70–95% ethanol afterwards. For fixation, it is recommended that hot water ($\geq$ 80°C) be poured on larvae and after leaving them for 30s they should be stored in EtOH (70-85%) (Amendt et al., 2007).

i) Botanical Evidence – A common method to preserve botanical evidence is to press the plant material between sheets of paper and allow it to dry naturally. Other than the colour and shape of fleshy portions of the plant, nearly all other morphological characteristics are retained. Hence photographing any botanical evidence before collecting it is crucial. To evade disintegration of sample due to heat and moisture it is important to not store collected sample in plastic bags or non-porous containers (Hall & Byrd, 2012).

Once an exhibit has been ascertained as a biological substance, it is then collected and sent to an accredited and certified Forensic Biology laboratory for examination. Scientists there then (1) Identify the evidence using class characteristics (for example, distinguishing body fluids as either blood, saliva, urine, faecal material, vaginal secretions or semen) and (2) Compare individual characteristics (features that confer uniqueness to an object) of the evidence (Li, 2008).

Before the 1990s, blood factors such as A-B-O, were considered the best possible way for linking blood to an individual considering the fact that theoretically no two individuals, with the exception of identical twins, were anticipated to have a similar combination of blood factors (Saferstein, 2007). But after the introduction of DNA technology, DNA profiling is the definitive method of individualization and helps investigators immensely in the current forensic scenario. The individual characteristics of a questioned sample (evidence) can be compared to a known reference sample to establish their identity.

Before putting in an effort to establish a DNA profile, most laboratories prefer to perform preliminary or presumptive tests and confirmatory tests to confirm the exhibit as biological and containing DNA (Butler, 2012) (Table 1.1). Presumptive tests are simple, inexpensive, easy to perform and safe (Shaler, 2002). They require a minute amount of material and generally do not have any adverse effect on the downstream DNA processing (Tobe et al., 2007; Virkler & Lednev, 2009). There can however be some presumptive tests that are destructive in nature and every time a test is performed a part of the sample is lost. The Kastle Meyer (KM) and Leuchomalachite Green (LMG) tests for blood are an example. The confirmatory tests vary from one biological source to the other and some of them may cause problems in some downstream DNA isolation procedures as shown by Poon et al., (2009).

Sometimes apart from the preliminary and confirmatory tests it is also crucial to determine the species origin of the biological material. This is mostly done in cases involving body fluids or bones (Butler, 2015). For example, HemaTrace can be utilised for identification of species from blood if it is suspected that the blood sample found maybe non-human.

Table 1.1 Commonly performed presumptive and confirmatory tests in Forensic Laboratories (Butler, 2009)

Sample	Commonly used methods	
Blood	Presumptive	Phenolphthalein (KM), Luminol. Hemastix, LMG
	Confirmatory	Takayama and Teichmann Test
Semen	Presumptive	Fast Blue, Alternate Light Sources
	Confirmatory	Microscopic Examination with Christmas Tree staining, ABA card for p30
Vaginal Fluid	Presumptive	Alternate Light Sources
	Confirmatory	Fast Blue coupled with PSA and microscopic examination
Saliva	Presumptive	Alternate Light Sources, Phadebas
	Confirmatory	Phadebas Test and RSID Test for Human Saliva
Urine	Presumptive	Alternate Light Sources
	Confirmatory	BFID-Urine Kit
Faeces	Presumptive	Urobilinogen (Edelman's Test)
	Confirmatory	-
Hair	Microscopic examination for suitability/species	

1.3 DNA as Evidence

The introduction of Forensic DNA examination in the 1980s (Jeffreys et al., 1985), opened new frontiers in the criminal justice community by assisting condemnation of the guilty and exoneration of the innocent (Fig.1.2). Due to its unique capabilities, with respect to other Forensic disciplines, DNA can play many roles in criminal investigations (Butler, 2015):

1. In instances where brutal crimes such as rape and homicide occur, biological material is exchanged between the offender and the victim. DNA recovered from such crime scenes has power to possibly establish the identity of the perpetrator.

2. According to the principle of inheritance an individual shares half of the genetic code from his or her father and the other half from his or her mother, thus making close biological relatives as useful as points of reference. This is especially advantageous in cases that involve missing persons and disaster casualties, as they can be identified from kinship relations if no absolute reference sample is available for the purpose of comparison.

3. Before the introduction of DNA profiling, paternity testing involved identification of certain phenotypes such as the blood type of a child and use of this information to establish a relationship between the probable fathers. This was however very inconclusive and presented several problems. DNA profiling erased this drawback by providing 99.99% accuracy thus delivering concrete evidence when resolving parental disputes (Adams, 2008).

4. Acts of suspected wildlife crimes often require identification of the species involved. This is more crucial in cases where more than one species is concerned. With the recent developments in the field of DNA analysis, wildlife forensic investigators use mitochondrial DNA (mtDNA) as a standard method to identify a species. The sequences of the sample obtained are compared to ones available in the DNA database (Tobe & Linacre, 2010).

5. Food adulteration is an ever increasing widespread problem. DNA analysis can be used for identification of food constituents to prove conclusively that a fraud has occurred (Woolfe & Primrose, 2004).

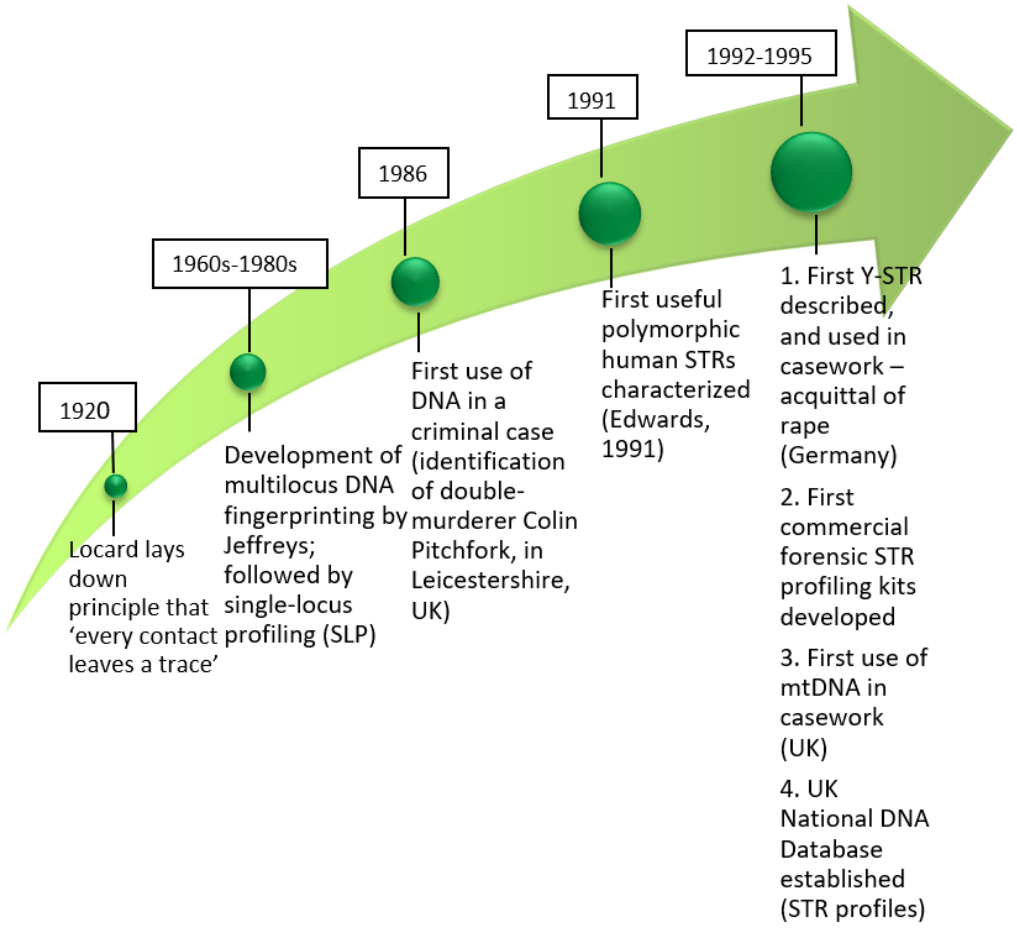


Fig.1.2 Timeline of Developments in Forensic Genetics (Jobling & Gill, 2004)

1.3.1 Structure of DNA

Deoxyribonucleic acid (DNA) is a polymer that is present in all the cells, except for red blood cells. It is found in two regions of the cell: nucleus and the mitochondria, and both can be useful in cases of DNA typing. The nuclear DNA molecule has a double helical shape with interchanging sugar molecules (deoxyribose) and phosphates. Attached to each sugar molecule is one of the four nitrogenous bases (nucleotides): adenine (A), guanine (G), cytosine (C), and thymine (T).

Thus, the DNA molecule comprises of the sugar-phosphate backbones which are coupled with linked base pairs and the linkages are governed by the concept known as complementary base pairing (Fig.1.3). According to this rule adenine at all times binds with thymine and vice-versa, and cytosine always binds with guanine and vice versa, i.e., the linkages are always A–T, T–A, G–C, or C–G. It is the sequence in which these pairs of bases are arranged which forms the principle of genetics and inheritance. The arrangement of these base pairs forms a genetic code which is responsible for the characteristics of a person (Houck & Siegel, 2010).

Mitochondrial DNA (mtDNA) is a circular, double stranded molecule inside the mitochondrion of a cell whose prime function is to produce cellular energy by oxidative phosphorylation. In addition to this the organelle also performs the very important function of programmed cell death also known as apoptosis (Andrews et al., 1999). As mtDNA is a multiple copy DNA, it is present in large numbers inside the cell (100-10000 copies in 1 cell) whereas the nuclear DNA is only present in 2 copies per cell (Chinnery & Hudson, 2013). The number of mtDNA varies depending upon an organism's cellular energy demand. It's length also differs from one species to another but in humans it is found to be a uniform length of ~16 569 bp. The two strands of mtDNA (heavy strand and light strand) are distinctly different due to their nucleotide contents. The light strand (L-Strand) is rich in cytosine whereas the heavy strand (H-Strand) is rich in guanine (Andrews et al., 1999). It is sometimes used in Forensic Genetics due to its high polymorphism among individuals. A noncoding region of mtDNA (1100 bp) however has only three hypervariable regions (HV1, HV2 and HV3) in humans (Fig 1.4a), hence making it less discriminating than nuclear DNA. mtDNA is inheritable only from mothers therefore functioning as a powerful tool to trace family lines from maternal side of an individual (Fig. 1.4b). Due to its presence in multiple copies in a cell and robust nature, it is very useful in DNA typing of samples that contain low quantity of DNA or are extensively degraded (Houck & Siegel, 2015).

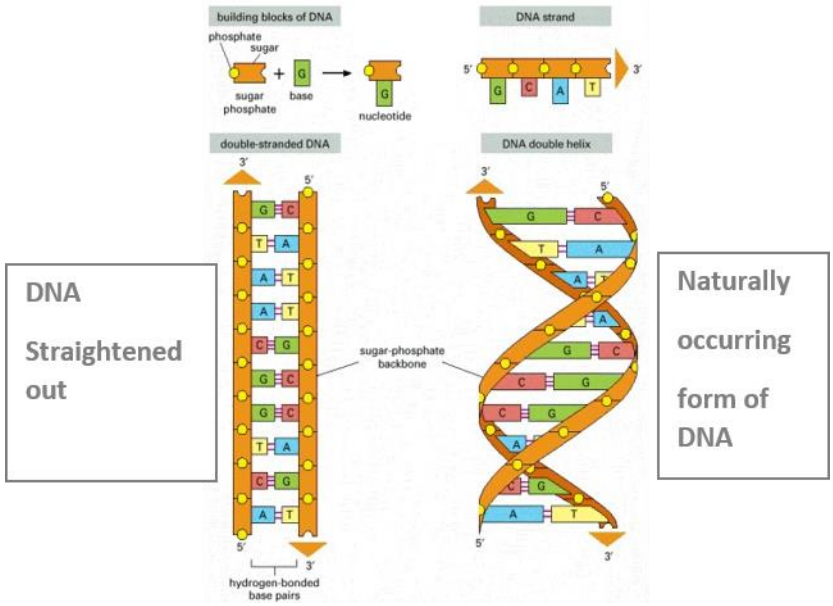


Fig.1.3 Structure of DNA with its four nucleotides (A, T, G, C), linked covalently into a polynucleotide chain which comprises a DNA strand. The bases extend from a sugar-phosphate backbone. The two DNA strands are joined by hydrogen bonds amongst the paired bases. The arrowheads at the ends of the strands denote their polarities, which run antiparallel to each other (Alberts et al., 2002)

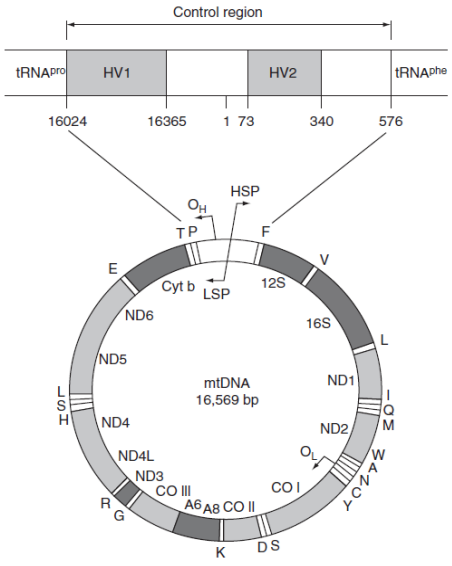


Fig. 1.4a Circular mtDNA containing 16,569 base pairs. The two hypervariable loci, HV1 and HV2 are used to generate mtDNA profiles (Houck & Siegel, 2015)

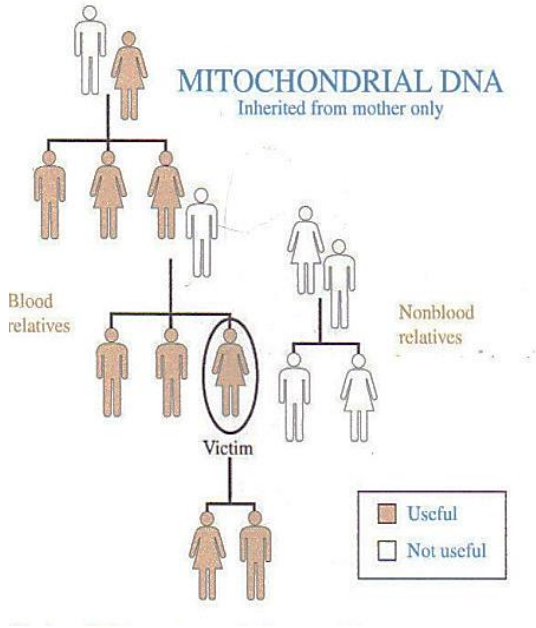


Fig. 1.4b mtDNA maternal inheritance pattern (National Institute of Justice)

1.3.2 Conventional and Non-Conventional Sources of DNA Evidence

Most common evidence to be submitted to crime laboratories for the purpose of DNA analysis, are those articles on which body fluids are thought to be present (Table 1.2). For example, evidence from cases that concern sexual assaults frequently include clothing of the complainant, bed linen and sometimes, suspect clothing. Similarly, another common evidence which is blood evidence, is abundantly found in many cases involving homicides, aggravated assaults and burglaries. Objects usually presented for blood testing consist of weapons, clothing, swabs from crime scenes or any amount of other objects that could have acquired bloodstains. When an object is small in size, it is commonly submitted to the laboratory in its entireness. For heftier items, stains can either be collected by a sterilised cotton swab or a cutting from the item can be taken for analysis. Other frequently collected items for DNA analysis are objects that may have come in contact with an individual's mouth such as drinking cans, cigarette butts, candy, cups, bottles, chewing gum, toothbrushes or masks. Usually these objects deliver adequate DNA for an STR profile to be developed. Other than this there are sometimes objects that have been touched or laid a hand on such as a steering wheel, phone, gun or a surface having a fingerprint. These may also enclose biological evidence, which can be collected for analysis but generation of a DNA profile cannot be guaranteed (Gefrides & Welch, 2011). All these evidence are typically easy to find and extract DNA from. Apart from these, cadaver as a whole also acts as a conventional source of DNA. Cadaver tissues, hair, or body fluids can be used to isolate DNA and ascertain the identity of the victim. If the corpse is found at the later stages of decomposition, bones, nails and teeth can provide adequate amount of DNA for the victim's genetic identification.

Table 1.2 Examples of Common Sources of Biological Evidence (National Institute of Justice, 2012)

Evidence	Possible Location of DNA on the Evidence	Source of DNA
baseball bat or similar weapon	handle, end	sweat, skin, blood, tissue
hat, bandanna, or mask	Inside	sweat, hair, dandruff
eyeglasses	nose or ear pieces, lens	sweat, skin
facial tissue, cotton swab	surface area	mucus, blood, sweat, semen, ear wax
dirty laundry	surface area	blood, sweat, semen
toothpick	Tips	saliva
used cigarette	cigarette butt	saliva
stamp or envelope	licked area	saliva
tape or ligature	inside/outside surface	skin, sweat
bottle, can, or glass	sides, mouthpiece	saliva, sweat
used condom	inside/outside surface	semen, vaginal or rectal cells
blanket, pillow, sheet	surface area	sweat, hair, semen, urine, saliva
"through and through" bullet	outside surface	blood, tissue
bite mark	person's skin or clothing	saliva
fingernail, partial fingernail	Scrapings	blood, sweat, tissue

As each crime scene is unique there can be cases where these common sources of DNA may not be present, and an alternate approach is to be used to extract DNA from what are called as “non-conventional sources”.

These non-conventional sources can either be in the form of:

1. Surfaces that have been touched directly or indirectly – This includes touch DNA (DNA that is recovered from epithelial cells of skin that is shed behind when a person undergoes contact with items such as a weapon, clothes, or other objects) (Williamson, 2012) and other Trace DNA transfers (Meakin & Jamieson, 2013). ‘Trace DNA’ has also come into light in recent years for its utility in cases where DNA profiling cannot be attained from identifiable body fluids. Cellular material containing DNA can be transported on a surface through either direct or indirect transmission. Direct transmission may occur due to contact or by accidental actions within the purlieu of an item such as speaking, coughing, and sneezing. Because of the acknowledged presence of DNA in saliva and nasal mucous, these actions result in the transfer of DNA to a nearby object (Meakin & Jamieson, 2013). Research regarding the same, with the help of a modelled crime scene, has shown that DNA could be detected if an individual is speaking or coughing on the floor in front of them, when the individual is approximately 1m away (Port et al., 2006; Rutty et al., 2003). Indirect transmission is when DNA from some individual transfers on an article via an intermediate surface. For example, DNA can be transmitted from one person to another person and eventually to an object. When there is one transitional step between an individual and the questioned sample it is known as a ‘secondary’ transfer, and if there are two intermediary steps involved it is called as a ‘tertiary’ transfer, and so on (Meakin & Jamieson, 2013). This was first demonstrated by van Oorschot and Jones in 1997. After several debates, it has come into recognition that indirect transmission does occur and can now be thought about during the assessment of trace DNA in forensic casework (van Oorschot et al., 2010).

2. Entomological evidence – This includes empty puparia found at crime scenes (Marchetti et al., 2013), maggot gut contents (Campobasso et al., 2005) , blood meal of mosquitoes and lice (Curic et al., 2014; Mumcuoglu et al., 2004), fly artifacts (Durdle et al., 2013). Sometimes, when

a corpse is missing from the primary crime scene or it has been moved to another location, which is referred to as the secondary crime scene, analysis of entomological evidence such as recovering DNA from gut content of larvae (Wells et al., 2001) or from empty puparia present on the crime scene (Marchetti et al., 2013) can be helpful. Mosquitoes present at a crime scene, dead or alive, can be used to recover human DNA and could be useful in revealing spatial and chronological relation of events that may have occurred at the crime scene (Curic et al., 2014).

1.3.3 Extraction of DNA

Any biological sample collected from a crime scene contains several impurities besides DNA. Also, within the cell, DNA is present along with other cellular materials like proteins. It is therefore essential to isolate DNA molecules from other cellular material and contaminations before commencing with the analysis. For example, histone proteins that are responsible for packaging and protecting, DNA act as inhibitors when analysing it. Due to this, many methods for extraction have been established to separate cellular proteins and other materials that may be present in the cell, from the DNA molecule. The procedure can be divided into 3 comprehensive steps: (a) lysis of the cell to release DNA, (b) separation of DNA from other cellular material, and (c) isolation of DNA to make it attuned to other downstream process of analysis such as PCR amplification (Butler, 2012).

Earlier the methods used for extraction were organic extraction (phenol-chloroform extraction), Chelex extraction (Boiling lysis and Chelation) and FTA paper extraction. Every biological material has a different biochemical composition, therefore, the DNA extraction technique also varies with respect to each biological substance. For example, if a biological evidence is a bloodstain the procedure for extracting DNA is different from the procedure for extraction of DNA from seminal stains (Li, 2008). With most steps of DNA analysis being automated, solid-phase extraction has emerged to be one of the most used techniques in today's world. In this method DNA is selectively bound to a substrate such as silica particles and after a few washing steps, DNA is retained while

proteins and other cellular constituents are discarded (Altayari, 2016). The most commonly used solid-phase extraction methods and their primary characteristics are summarised in Table 1.3 and Fig.1.5.

Table 1.3 Comparative description of commonly used extraction methods (Butler, 2012)

	Qiagen	Promega	Applied Biosystems
Products	QIAmp	DNA IQ	PrepFiler
Solid-Phase	Silica Beads	Magnetic silica beads	Magnetic silica beads
Wash Performed	Centrifuge or vacuum manifold	Magnet	Magnet

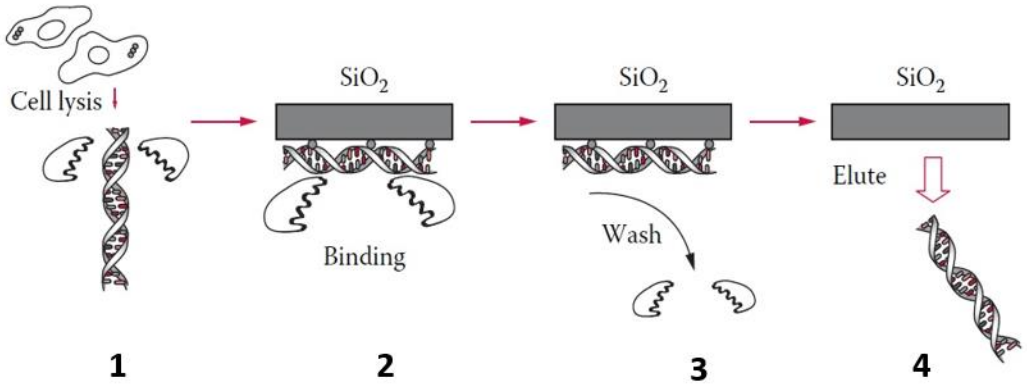


Figure 1.5 Process of Solid-Phase DNA extraction 1. Cell lysis in the presence of proteinase 2. Binding of DNA to the silica matrix 3. Washing step to remove unbound cellular materials and salts from the matrix 4. Elution of the purified DNA for use in downstream applications (Li, 2008)

DNA after elution is usually preserved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 4°C or –20°C. If the DNA sample is to be used within 1 week, it can be stored at 4°C and for long-term

storage –20°C or -80°C is preferred. It is advisable to avert recurrent freezing or thawing cycles as the fluctuations may result in breakage of single- and double-stranded DNA (Li, 2008).

1.3.4 Polymerase Chain Reaction

Alec Jeffrey's three publications in Nature in 1985 (Jeffreys et al., 1985; Jeffreys et al., 1985; Jeffreys et al., 1985) , showing that repetitive human DNA sequences can be useful for tracking genetic inheritance and distinguishing people, marked the beginning of routine restriction fragment length polymorphism (RFLP) DNA testing using variable number of tandem repeats (VNTRs) (Butler, 2012). VNTRs are a class of highly polymorphic DNA repeats that share a rich G-C or A-T core (Li, 2008). In RFLP, DNA after extraction is broken-down into smaller fragments of repeating units called minisatellites or VNTRs. The polymorphism present in four to six highly polymorphic loci is analysed, and the results help in generating a DNA profile that confers individuality. (Houck & Siegel, 2010). A basic RFLP procedure includes genomic DNA extraction, digestion of DNA into fragments using restriction endonucleases, agarose gel electrophoresis to separate the DNA fragments according to size, transferring the DNA fragments to a supporting matrix such as nylon membrane, hybridization with locus-specific probes using Southern transfer, and detecting the locus-specific bands by chemiluminescence or autoradiography (Li, 2008).

Although minisatellites became a powerful tool in criminal investigations, they suffered from quite a few drawbacks. The entire process was very time consuming and took more than a few weeks to complete. Moreover, a relatively large amount of DNA was required for accurate analysis and the procedure also failed to provide results in cases of degraded DNA (Goodwin et al., 2011).

Nonetheless, the use of minisatellites was common for many years until in 1985 Kary Mullis and members of the Human Genetics group at the Cetus Corporation (now Roche Molecular Systems) invented an automated process known as polymerase chain reaction (PCR) that revolutionized molecular biology(Butler, 2012). PCR helps in making copies by amplification of desired sequences of DNA using polymerase enzymes. These polymerase enzymes originated from the bacterium *Thermus aquaticus* (Taq) (Houck & Siegel, 2015). DNA in trace amounts is required for

PCR to generate sufficient copies to be analysed by means of conventional laboratory methods, hence making PCR is a very sensitive assay (Garibyan & Avashia, 2013). PCR was a better alternative to RFLP, and a boon for forensic scientists, because of its sensitivity, ability to make millions of copies in only a matter of hours and not being limited by the quality and quantity of the DNA sample (Butler, 2009).

Every PCR assay requires primers, deoxynucleotide triphosphates (dNTPs), DNA polymerase (Taq), magnesium ions (Mg^{2+}) and template DNA. The nucleotides include the four nitrogenous bases found in the DNA – adenine, thymine, cytosine, and guanine (A, T, C, G). The DNA polymerase acts on the nucleotides which act as the building blocks to create the resulting PCR product. Primers are short DNA fragments with a definite sequence complementary to the target DNA that is to be detected and amplified. (Garibyan & Avashia, 2013)

In order to amplify a DNA fragment, the reaction mixture is placed in a thermal cycler which essentially has a thermal block containing holes for holding the tubes containing PCR reaction mixture. The machine then elevates and lowers temperatures according to pre-programed steps, which results in DNA denaturation, primer annealing and extension and this is repeated in a cyclic manner collecting a large amount of identical copies of the DNA sequence (Weier & Gray, 1988). Under ideal circumstances, after about 32 cycles, a billion copies of the target sequences on the DNA template can be generated (Fig. 1.6). The product obtained after a complete PCR reaction is referred to as an amplicon (Butler, 2009). The equation

$$N_n = N_0 (1+ \varepsilon)^{n-2}$$

can be used to determine the relationship between the number of cycles of the PCR process and the product formed at the end of the reaction. Here N = number of copies of the target DNA, n = number of cycles, N_0 = initial copy number of the template and ε = efficiency of the PCR run.

The easiest and most widely used method for analysis and visualization of PCR products is the use of agarose gel electrophoresis, in which DNA products are separated based on their size and charge. It helps in determining the position and length of the PCR product using a prearranged set of DNA products of known sizes, known as allelic ladder. These standardized molecular

markers are run at the same time on the gel in order to conclude the size of the PCR product (Garibyan & Avashia, 2013).

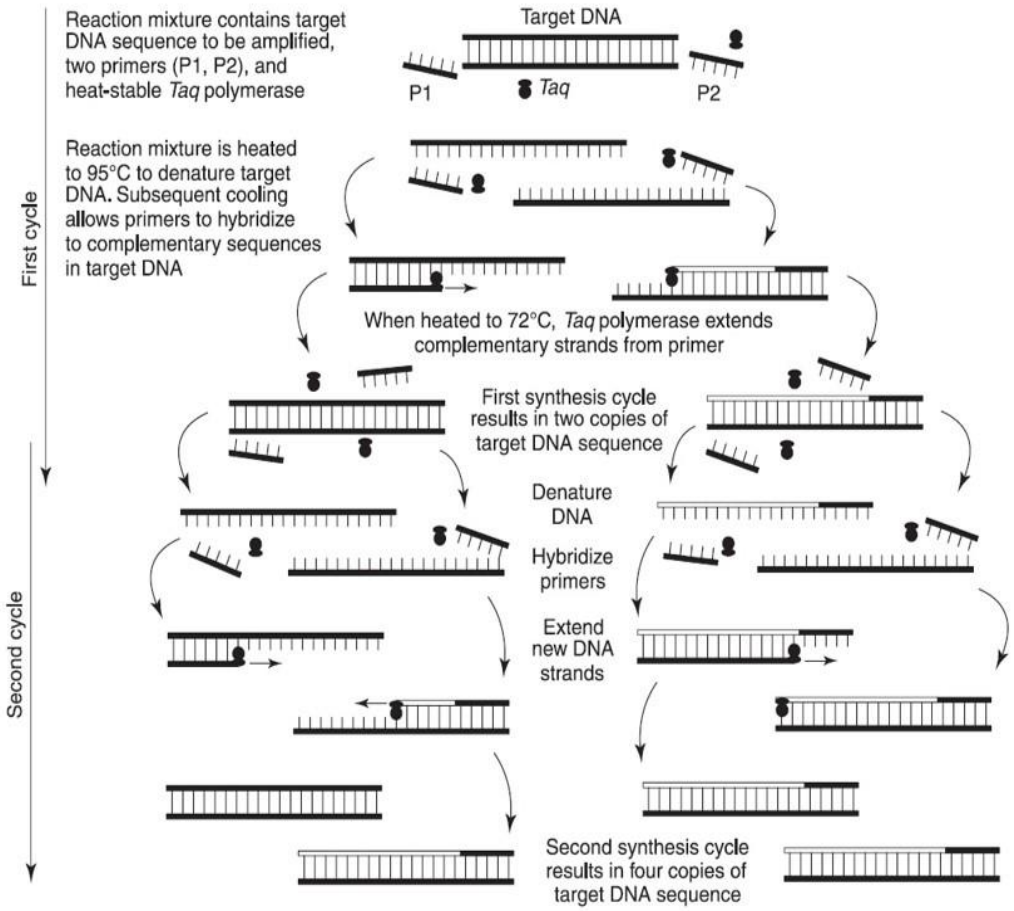


Fig.1.6 Process of PCR showing amplification of target DNA after two cycles through denaturation, annealing and extension (Siegel et al., 2000)

1.3.5 Quantitative PCR

Real time PCR (RT-PCR) or Quantitative PCR (qPCR) is an enhancement of the original PCR technique developed by Kary Bank Mullis and his co-workers. It provides a reliable quantification as well as the provision of monitoring the production of amplicons at the end of each PCR cycle (Kubista et al., 2006; Li, 2008). The process of real-time PCR requires a fluorescent reporter which interacts with the amplicon formed and then accounts its presence by generating a fluorescence signal that indicates the amount of product formed (Kubista et al., 2006). This means greater number of amplicons produced result in a stronger fluorescence signal. Two of most commonly used means of quantification are the insertion of a fluorescent dye (e.g. SYBR® Green) and the TaqMan® assay. When SYBR® Green intercalates to a ds-DNA, it results in an escalation in fluorescence, however, the dye attaches to any double stranded DNA molecule and hence the intercalation method is non - specific (i.e. it cannot distinguish between DNA molecules). This dye is very useful when samples do not contain any interfering DNA and are compared at the same level of fluorescence. On the other hand the TaqMan® assay utilises oligonucleotide probes (TaqMan® probes) that are specific to a particular region of DNA found between the forward and reverse PCR primers (Gunn, 2009). The probes possess a fluorescent reporter dye at their 5´ end and a quencher dye at their 3´ end. FRET (Fluoresent Resonance Energy Transfer) occurs while the probe is intact, and the fluorescence emission of the reporter dye is absorbed by the quenching dye (Arya et al., 2005). Taq Polymerase has a 5' nuclease activity, hence when it replicates a template on which a TaqMan ® probe is bound, it splits the probe which results in separation of the reporter dye and the quencher dye. This leads to reduction in the fluorescence of the quencher dye and an escalation in the fluorescence of the reporter dye. Due to its extreme sensitivity this method can detect as little as a twofold increase in the target DNA sequence. Custom designed primers are used in this process thus making it more specific than the intercalation method. However, in both cases with each cycle more amount of DNA is produced, and this is measured with the increase in fluorescence. Figure 1.7 summarizes the distinction between the two primary qPCR methods. In the end the target DNA is quantified and as it amplifies it is detected in 'real time' (Gunn, 2009).

A real time detection of the PCR reaction is possible using a computer software that constructs an S-shaped amplification plot using the fluorescence emission data that is collected (Arya et al., 2005). The amplification curve is divided into a lag phase, and exponential phase, a linear phase and a plateau (Li, 2008).

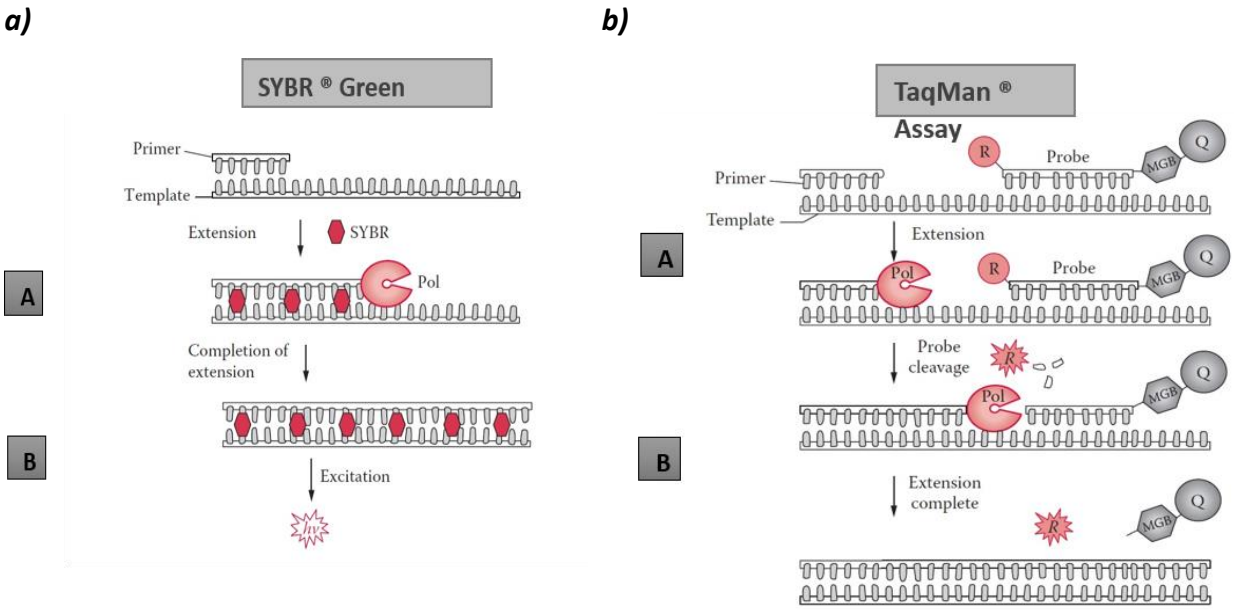


Fig. 1.7 The two most commonly used detection methods in qPCR (Li, 2008)

a) SYBR Green : A) Dye binds to amplicons at the extension stage of DNA synthesis B) Upon excitation emission intensity of the dye can be measured

b)) TaqMan : A) Taq Polymerase cleaves the probe during extionsion stage of DNA synthesis B) Reporter dye is released during the end of each cycle.

The exponential phase shows a consistent relationship between the input DNA and the quantity of product formed. The real time PCR software uses a cycle threshold (C_T) for these calculations. The C_T value is the point, with respect to PCR amplification cycles, where the level of fluorescence exceeds a subjective threshold which is determined by the software to avoid baseline noise that arises early during the reaction. The lesser cycles it takes for the level of fluorescence to traverse the threshold, higher is the initial concentration of DNA used for the PCR reaction. The baseline of the plot represents the PCR cycles in which the fluorescence signal of the reporter is accumulating but the instrument is unable to detect it. In the linear phase of amplification one or more components of the reaction, such as dNTPs or primers, may fall below a critical concentration and this results in the amplification efficiency slowing down when compared to the exponential phase. The PCR reagents can be used up at different rates and this varies from sample to sample, hence the linear phase is not consistent and not that useful for comparison purposes (Arya et al., 2005; Butler, 2012).

The y-axis of the plot denotes the fluorescence emission (ΔRn) which the software calculates using the equation **Rn = Rnf – Rnb**, where Rnf is the emission of fluorescence from the product at every time point and Rnb is the fluorescence emission of the baseline. The ΔRn is plotted against the number of cycles designated on the x-axis (Arya et al., 2005) (Fig.1.8).

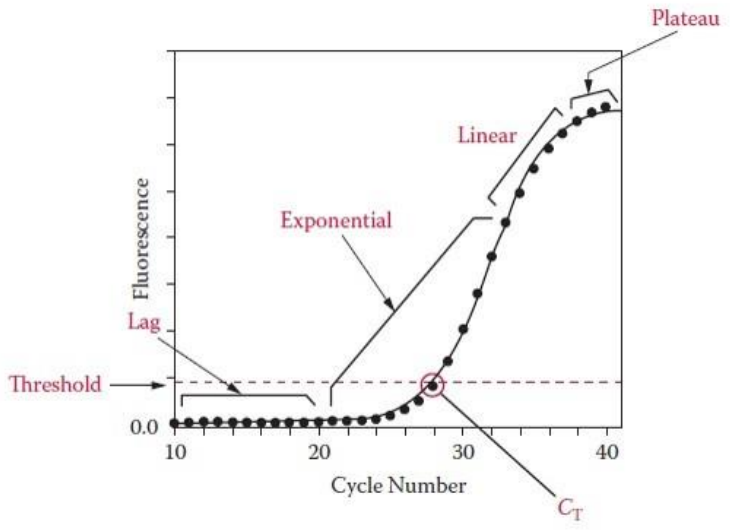


Fig.1.8 A typical PCR amplification curve and different stages of amplification (Li, 2008).

A standard curve is a linear curve, plotted on the basis of serial dilutions of a standard, which is used to assess the efficiency of a PCR assay. The standard could be a purified PCR product or any other DNA sample consisting of the desired sequence and the quantity of which is known (Rutledge, 2004). Because the standard curve is a plot of the log of initial target copy number for a series of known standards versus Ct, it also determines the quantity of an unknown DNA sample (Higuchi et al., 1993) (Fig.1.9).

Often there is formation of a primer–dimer complex when using SYBR® Green assay. It obstructs the development of PCR products, as both the reactions compete for reagents, which in turn results in erroneous readouts. Hence, it is beneficial to control the formation of primer-dimer with the help of melting curve analysis after completion of the PCR. Herein, temperature is gradually increased after the reaction completes and the fluorescence is assessed as function of temperature. When the melting temperature, Tm, (temperature at which the DNA strands separate) is reached the dye detaches and the fluorescence drops immediately (Nygren et al., 1998; Ririe et al., 1997). A distinctive melting peak at the Tm of the amplicon distinguishes it from primer-dimers that melt at lower temperatures and form broader peaks (Arya et al., 2005).

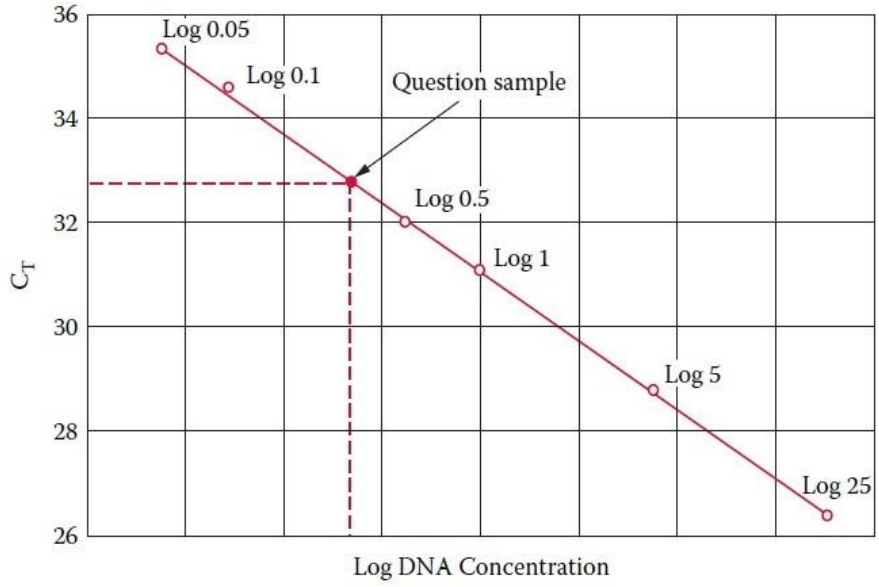


Fig.1.9 Quantity of an unknown DNA sample can be obtained from a standard curve (Li, 2008).

1.3.6 Genetic Profiling

DNA fingerprinting or DNA profiling made its way into forensic investigations in the year 1986 when molecular biologist Sir Alec Jeffreys was asked by the police in England to use DNA in order to validate the confession of a 17-year-old boy in two rape-murders which were committed in the English Midlands (Jeffreys et al., 1985; Meeker, 2004). The establishment of this procedure within the criminal justice systems lead to a revolutionary development in the discipline of forensic science.

After adopting several methods such as RFLP, recently, short tandem repeat (STR) analysis has arisen as the most successful and broadly used DNA-profiling procedure. STRs are loci on the chromosome comprising of short sequences of 3-7 bases which reoccur within the DNA molecule. Due to their abundant presence in the human genome, they function as helpful markers for personal identification. They are also preferred over other DNA typing procedures as the STR strands are considerably shorter in length (less than 450bp long) which makes them much less susceptible to degradation. Also, because they are short, STRs make an ideal candidate for amplification by PCR, thus helping forensic scientists to overcome the problem of limited-sample size often associated with crime-scene evidence (Saferstein, 2013).

After the discovery of STRs and automated sequencing technology together, a stronghold of individual characterisation of DNA was established. Currently forensic casework is carried out using STR multiplexes which have been commercially developed worldwide. STR multiplexes are single tube PCR reactions which are able to amplify multiple loci (Jobling & Gill, 2004). The STR loci currently in use were originally developed and characterized at either the laboratory of Baylor College of Medicine by Dr. Thomas Caskey (Edwards et al., 1991; Hammond et al., 1994) or at the Forensic Science Service (FSS) in England (Kimpton et al., 1993; Urquhart et al., 1994). The first multiplex developed had 4 simple STRs (F13A1, FES, TH01, and VWA), hence, it was also known as a quadruplex (Kimpton et al., 1993). It however had a match probability (Pm) of ~ 1 in 10,000 or 10^{-4}, which lead to subsequent addition of another two complex STRs in to the multiplex system (HUMD21S11 and HUMFIBRA/FGA) (Mills et al., 1992). These highly polymorphic STRs improved the previous Pm to ~1 in 50 million (Jobling & Gill, 2004). Sullivan et. al in 1993 (Sullivan et al., 1993) developed second generation multiplex (SGM) which introduced a PCR assay

targeted at the amelogenin genes (X and Y). This therefore also revealed the sex of the person whose DNA was being analysed. In 2000, SGM was renamed as SGM Plus after addition of 4 more loci (D3S1358, D19S433, D16S539 and D2S1338) which resulted in a reduction in Pm to 10^{-13} (Cotton et al., 2000). The first STR database was established in the United Kingdom in 1995, which was covered by the SGM, having 6 STR loci (D8S1179, D18S51, D21S11, FGA, TH01, VWA) and an amelogenin locus. It had a Pm of 10^{-7}. Although initially lagging behind from the Europeans, the United States established their database, sponsored by the FBI, in the year 1998. This database, known as the CODIS (Combined DNA Index System), contains 13 loci (CSFIPO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA) which were chosen out of 17 loci selected for evaluation during the STR Project between April 1996 and November 1997 (Budowle et al., 2001). The European Standard Set (ESS) of STR markers were selected in 1999 (Schneider, 2009) and most of the loci were those already employed by CODIS.

In forensic laboratories all over the world, capillary electrophoresis (CE) is the chief methodology used for detection and separation of STR alleles. Key elements in a capillary electrophoresis instrument comprise of narrow glass capillaries (4 or 16), a source of laser excitation, two vials containing buffer, a viscous polymer which creates a sieving environment for DNA molecules, a fluorescence detector, two electrodes coupled to a high voltage power supply, an autosampler which holds sample tubes and a computer having the software to control injection and detection of samples (Fig.1.10). A commercial STR kit contains a primer mix, a PCR buffer, DNA polymerase (may or may not be premixed with the buffer), an allelic ladder and a positive control DNA sample with a known quantity (Butler, 2012).

The primers are labelled with a fluorescent dye and are used to amplify STR loci. A multiplex STR system make use of several fluorescent dyes that can be resolved spectrally. This means that these fluorescent dye colours can be separated from one another so that the PCR products from different loci, labelled with different dyes, can be determined. Real time electrophoresis is used to detect and separate the amplified products. As soon as the fluorescent dyes are resolved by the detector, a specialised computer software identifies the DNA fragments. An internal size standard is used during electrophoresis to accurately size the PCR products. This internal size standard is composed of segments of DNA of known length and are marked with a fluorescent

dye in order to be detected along with the PCR products. The software after collection of data generates an electropherogram which shows each fragment of DNA as a peak. Hence a profile is generated consisting of peaks each representing an allele on a particular locus. The amplitude of a peak indicates the intensity of the fluorescence signal (Goodwin et al., 2011; Li, 2008).

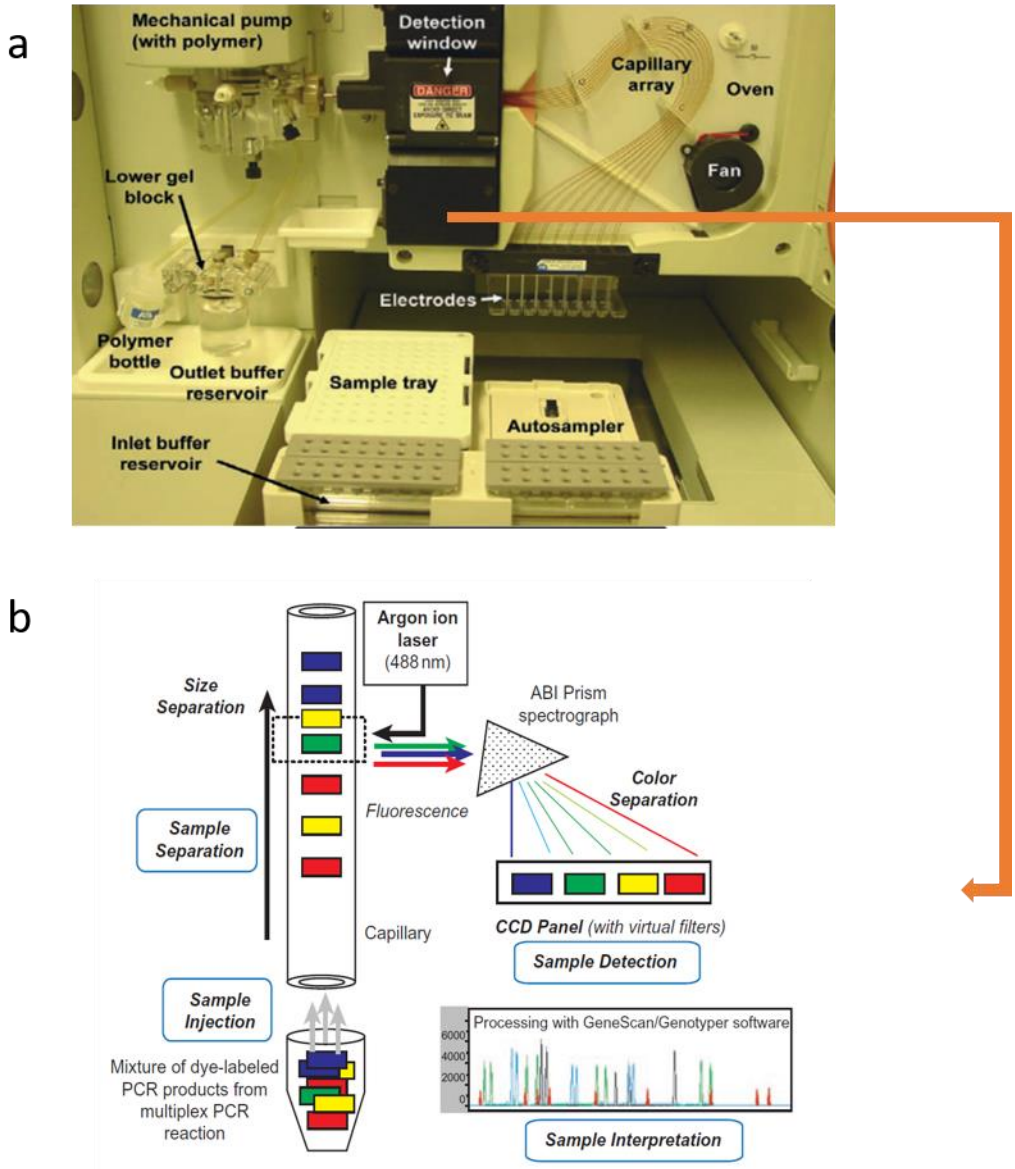


Fig.1.10 (a) Parts of a CE instrument (ABI 3130xl Genetic Analyser) (b) Diagrammatic representation of detection and separation of STR alleles in a CE instrument (Butler, 2012).

The step that finalises an STR profile is the designation of alleles to the fragments of DNA represented as peaks. A number is assigned to each peak which depicts the structure of that allele (Gill et al., 1997). While doing so each unknown peak is compared with the allelic ladder and if it falls within a 1-bp gap, that is ±0.5 bp of the allelic ladder size it is then allotted that particular allele. If not so a peak is categorized as 'Off Ladder' (OL) (Fig. 1.11) (Goodwin et al., 2011). If an STR profile of a suspect is found to be the exact same match of the questioned DNA sample, Forensic Scientists say that the two are genetic concordance to each other (Houck & Siegel, 2015).

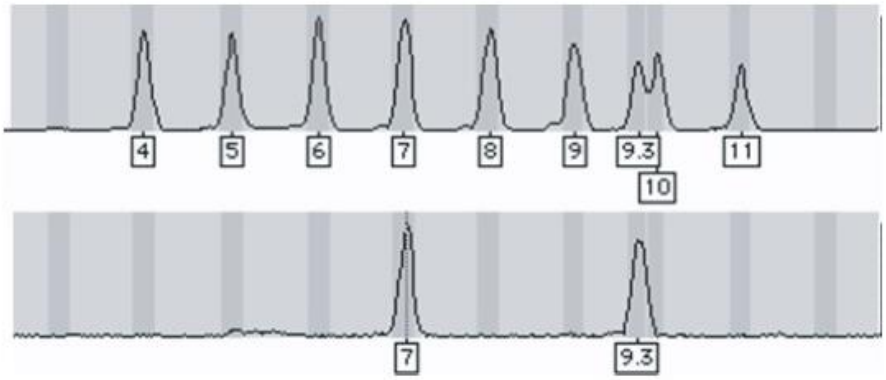


Fig.1.11 Comparison of the two unknown peaks with the allelic ladder above shows that the unknown peaks can be designated 7 and 9.3 as they both fall within the 0.5bp window (shaded area) of the peak in the allelic ladder at the same position (Goodwin et al., 2011)

1.4 Forensic entomology

Insecta is the biggest class in the phylum Arthropoda (Rivers & Dahlem, 2013) representing more than 75% of the described animals. Insects are prevalent throughout the ecosystem and they are the first animals to colonize a fresh corpse after death. In particular, flies are the first insects to lay eggs on the body after the death.

This relationship between fly larvae (maggots) present on a cadaver and the oviposition of adult flies was not recognised before the experiments done by Francesco Redi to confute the spontaneous generation. It was only in the XIX Century that the importance and utility of insects in investigating crime was proposed by Megnin and other French and German scientists (Benecke, 2001).

Right from the initial stages of decomposition insects are attracted to a decomposing body in a predictable sequence. Forensic entomology involves studying this insect population and the developing larval stages, and thus estimate the post-mortem interval, any changes that may have occurred in position of the corpse along with the cause of death (Amendt et al., 2011). The entomological samples can be in the form of larvae, puparia, adult insects such as flies (Diptera), beetles (Coleoptera) and various blood feeders [bed bugs (Heteroptera), lice (Anoplura), fleas (Silphonoptera) etc.] or insect artifacts. In addition to this the field of Forensic Entomology also has its application in identification of pests that may be found infesting food products (fruit, meat, etc.). Apart from infestations in urban locations may be observed such as at home, work location or commercial locations (Gennard, 2012).

There have been numerous techniques concocted recently allowing experts in this field to collect potent entomological evidence. These can deliver crucial information in a death investigation, answering questions relating to the movement or storage of the corpse, submersion interval, identifying specific sites of trauma, time of death, use of drugs using entomotoxicological techniques and connecting a suspect to the scene of crime and his identification (Amendt et al., 2011).

1.4.1 Applications of Forensic Entomology

The most common use of entomological evidence is in estimating PMI, wherein the species of a fly found on the corpse is first identified along with the stage of development of the fly and an assertion is made on how long it would take the flies to reach that stage of development depending upon the environmental conditions (Siegel et al., 2000). Apart from this, in a few cases, maggots and pupae are used for toxicological analysis to determine the presence of drugs in a decomposing corpse (Beyer et al., 1980; Pounder, 1991). A new frontier developing in the field of Forensic Entomology is the genotyping of insect gut contents. Blood meal of a mosquito can be analysed to reveal human DNA (Andrews et al., 1999), hence consigning a suspect at the site where the mosquito was found (Wells & Stevens, 2008). On similar grounds blood meal of a head louse, that could be transferred during a sexual assault, can be useful to identify the culprit (Lord et al., 1998; Mumcuoglu et al., 2004). Genetic analysis of maggot gut content can be used to answer several questions of investigators. When a corpse is absent from the scene of crime but maggots are present, it could be an indication that the corpse has been moved to another location. Sometimes maggots feeding on the dead body can be found several meters away from their food source when they are looking for a place to pupate (Wells et al., 2001). Furthermore, genotyping of gut content of larvae can also be useful in establishing the identity of the victim by comparing it to known samples of the victim or the victim's kin (Campobasso et al., 2005). Genetic analysis carried out can either be related to nuclear DNA or mitochondrial DNA. mtDNA has great benefit for forensic entomologists in determining the species of fly in question. Morphological identification, specially of larvae and eggs, is prone to errors and this in turn affects an ongoing investigation (Greenberg & Singh, 1995; Liu & Greenberg, 1989). This is more familiar in cases involving two or more morphologically similar species. Sperling et al. (1994) were the first to determine species of forensic interest using mitochondrial analysis and since then many have used this approach (Ames et al., 2006; Benecke, 1998; Chen et al., 2004; Harvey et al., 2003). Due to its haploidy, high copy number and ability to remain conserved for over a decade (Simon et al., 1994), mtDNA has been preferred for molecular identification of insects even in archaeoentomological cases (Vanin & Huchet, 2017). Cytochrome c oxidase subunits I and II (COI

I+II) are the commonly used segments of the mitochondrial gene as COI-I is also the site for the recommended worldwide animal DNA barcode (Wells & Stevens, 2008).

1.4.2 Post-Mortem Decomposition and Colonization of Insects

Following death, a corpse begins its process of decomposition during which it undergoes numerous biochemical events which are initiated by the deceased's own enzymes and by an array of microorganisms whose action promotes catabolism of all soft tissues (Rivers & Dahlem, 2013). This entire process has several distinct physical and chemical changes that are unique for each organism but the processes occurring in each case are sequential and relatively predictable (Vass, 2001). Environmental factors such as temperature, environment where the body is found for example terrestrial or aquatic, moisture levels, time of year (seasonality), geographic location, and if vertebrate scavengers and insects have access to the corpse, can all change the rate of decomposition of the corpse as well as other aspects of tissue decay (Goff, 2010).

Physical decay of human remains or any type of vertebrate carrion represents a series of events which are subject to a broad variation depending largely upon environmental influences (Kreitlow, 2010; Mann et al., 1990). Several authors, over the years, have proposed a range of sequential stages of decay to provide a point of reference for periods of insect activity on the carrion (Carter et al., 2007; Schoenly & Reid, 1987). An example is a model proposed by Payne (1965) in which six stages - fresh, bloated, active putrefaction, advanced putrefaction, dry putrefaction and remain, have been described by using pigs as examples for decomposition. But in order to be consistent with the majority of research in Forensic Entomology, the most commonly adopted method is the one described by both Goff (2010) and Kreitlow (2010), in which soft tissue decomposition occurs sequentially through the fresh, bloated, decay, postdecay, and skeletal or remains stages (Fig.1.12).

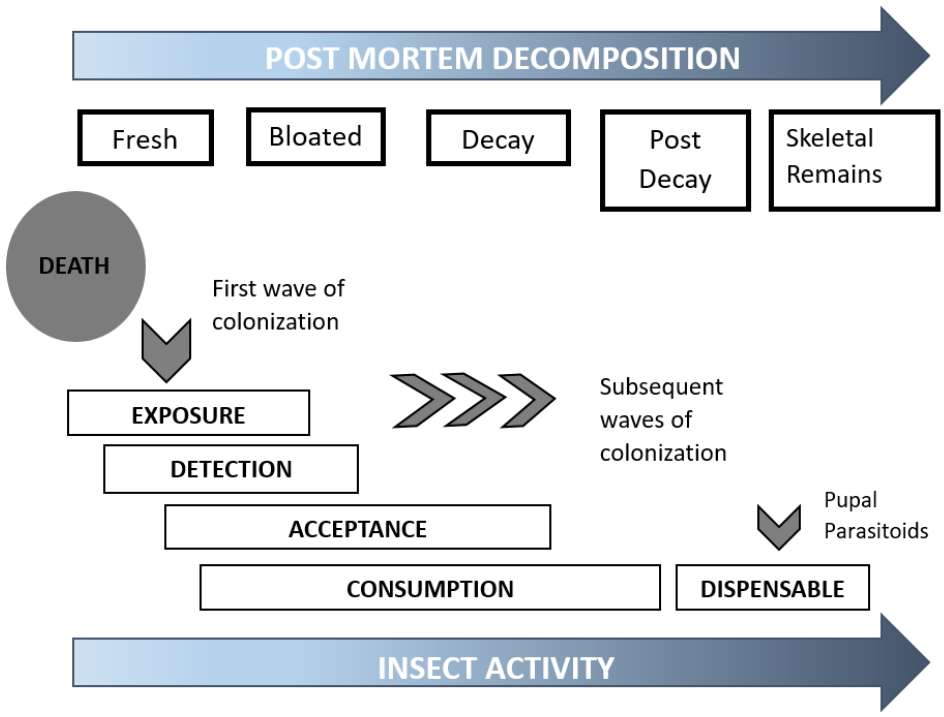


Fig. 1.12 Depiction of relationship between insect succession and post-mortem decomposition in case of carrion found in a terrestrial environment having warm ambient temperatures (Lefebvre & Gaudry, 2009; Tomberlin et al., 2011).

As a body decomposes the odours emanating from the corpse change, hence enhancing attraction for some species and becoming less attractive to others with progression of time. Although blow flies arrive very shortly after death and are among the first colonizers, they are no longer attracted when a carcass has crossed a certain stage of decomposition, or has mummified or become dry (Nuorteva, 1977). The insects colonising the corpse can either be necrophagous species (For example, Diptera : Calliphoridae, Sarcophagidae) or predators and parasites of necrophagous species (For example, Coleoptera : Silphidae, Staphylinidae), or omnivorous species (For example, Hymenoptera : Vespidae, Formicidae), or species that use the corpse as a part of their extended habitat (For example, Araneae, Chilopoda, Acaridae) (Goff, 1993). A typical

pattern of insect colonization on a decomposing body in a terrestrial environment is described in Fig.1.13.

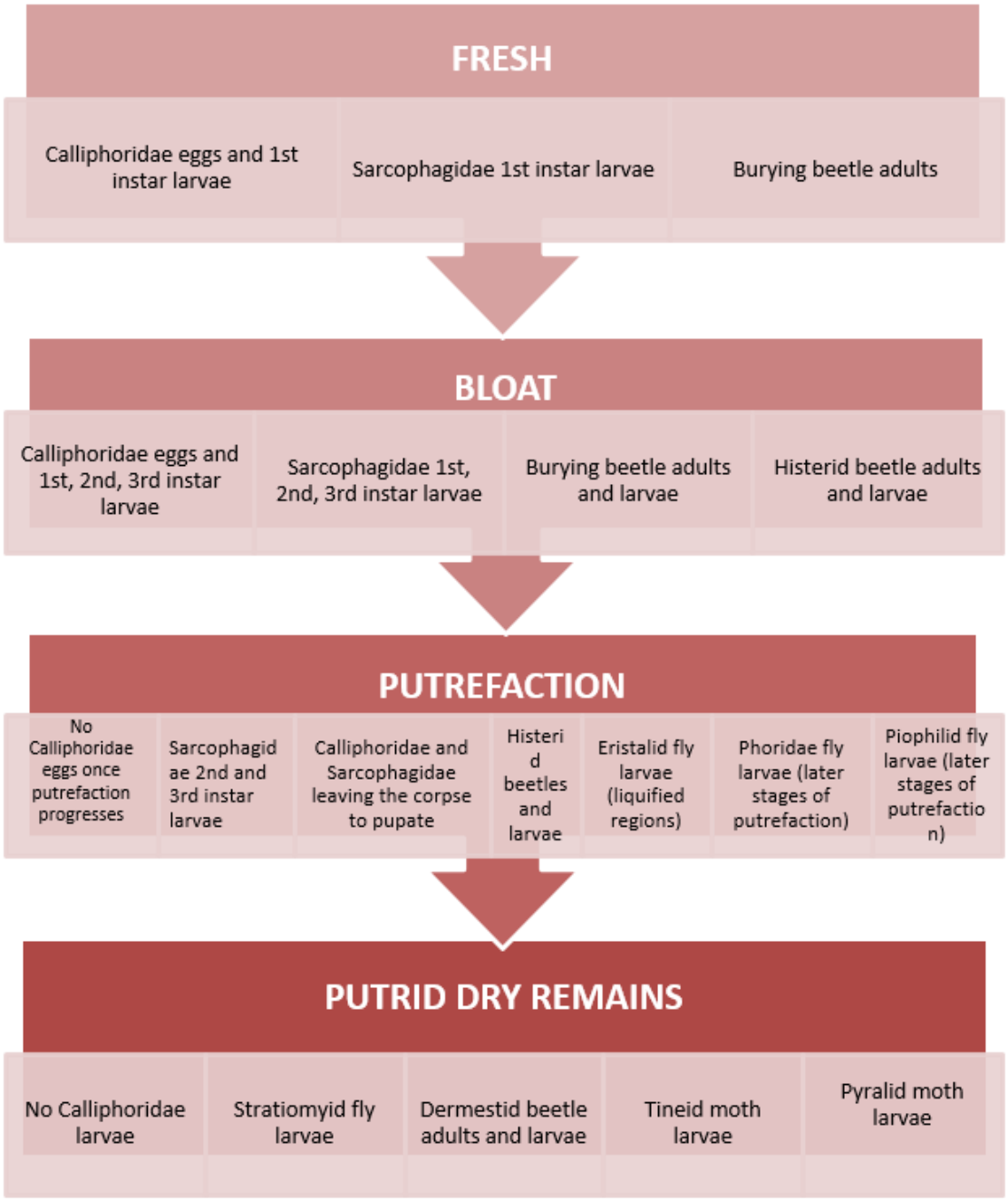


Fig.1.13. Insect colonization in different stages of corpse decomposition (Gunn, 2009)

External environmental factors as well as conditions relating to the physical structure of a corpse alter the pattern of insect colonization. The necrophagous development of an insect always differs according to the environment the corpse is found in (Table 1.4).

Table 1.4 Difference in fly colonization and development in assorted conditions in a typical environment (Rivers & Dahlem, 2013).

Stages of Development / ***Condition***	**DETECTION**	**OVIPOSITION**	**DEVELOPMENT**	**DISPERSAL**	**EMERGENCE**
AQUATIC	Delayed/ Obstructed	Reduced clutches	Standard	Drown if not close to shore	Do not reach pupation
BURNT	Accelerated	Standard	Standard to slower	Standard	Standard
INDOOR	Delayed	Reduced clutches	Extended	Inhibited	Delayed
BURIED	Delayed/ Obstructed	Reduced clutches	Slower	Obstructed depending upon depth of soil	Reduced if depth more than 6 inches
HANGED	Standard	Smaller and altered deposition areas	Altered during later larval stages	Earlier than usual	Reduced
VEHICLE	Delayed/ Obstructed	Reduced clutches	Accelerated	Inhibited	Reduced

Along with this, factors such as geographical location, seasons and temperatures also affect insect colonization. The times of arrival and the species involved in the chronological colonization of the remains vary from region to region. Perpetually, some particular groups will colonize in the beginning, such as blow flies (Calliphoridae) and flesh flies (Sarcophagidae), but the species involved will vary. This is because every region has species that are endemic to that particular geographical zone and hence data generated in one region or biogeoclimatic zone should not be used to determine time of death in another region. In addition, different insect species have different peaks of activity depending upon the season. Some species can be seen only during summers, while some only during winters. Hence, correct identification of insect species can also be helpful in determining season of death (Byrd & Castner, 2010). Even the degree of exposure of the sun on the corpse can alter the insect colonization pattern (Erincçlioglu, 1996; Shean et al., 1993; Smith, 1986). The species of insects also differ in urban and rural areas. Some species are specific to urban areas, while some to rural districts and there are others found in both regions (Haskell, 2000).

Keeping all these factors in mind, it is possible to assess the PMI of a corpse when found. With the knowledge of these aspects it can also be estimated if a corpse has been moved from the primary crime scene (Table 1.5).

Table.1.5 Summary of insect evidence that help in determining if a corpse has been moved (Gunn, 2009).

Movement of Body	Family of insects found	Evidence
Exposure followed by burial	Calliphoridae eggs and larvae	> 24 hours exposure
	Histerid beetle larvae	~ 2 – 3 days exposure
	Piophilid fly larvae	~ 7 days exposure
	Dermestid beetle adults and larvae	> 14 days exposure
Burial or positioned in a closed container followed by exposure	Calliphoridae eggs and larvae	Absent or stage of development younger than expected from ongoing decomposition
	Phoridae fly larvae	Abundant Phoridae fly larvae and adults but no evidence of Calliphoridae species
Inside to outside	Calliphoridae larvae and pupae	Carcass mummified or skeletonized but no evidence of preceding Calliphoridae larval activity on the body or pupae in the surrounding area
	Dermestid beetles and tineid and pyralid moths	Both present but no evidence of previous colonization by any other insect species
Outside to inside	Calliphoridae larvae and other detritus feeding species	Presence of species that are not normally found indoors.
	Any 'accidentally associated' species	Woodland, moorland etc. species accidentally trapped in Clothing
Geographical (between different regions or countries)	Calliphoridae larvae / other detritus feeding species or any 'accidentally associated' species	Species found outside their typical geographical distribution

1.4.3 Insect Colonization of Buried Corpses

A common approach used by culprits to dispose of a victim's body is to bury it. Studies by Lundt (1964); Payne (1965); Payne et al. (1968) have shown that when bodies are buried, their decomposition rate is more protracted as compared to the bodies exposed to air. Therefore, as proposed by VanLaerhoven and Anderson (1999), in such cases post-burial interval (PBI) is used in order to estimate the time elapsed since death by observing the insect succession patterns on the carrion. This is similar to estimating PMI but considering the different environment the species colonising the corpse are different and hence a different approach has to be used. A study by Manhein (1997) and Gunn and Bird (2011), showed that most corpses buried are found between 30cm-90cm depth as burying any deeper than this requires more time and labour and the culprit fears getting caught with the corpse. An experiment conducted by Pastula and Merritt (2013) revealed the insect colonization pattern on carcasses buried at 30cm and 60cm depth and to compare there were exposed control carcasses used (Fig 1.14).

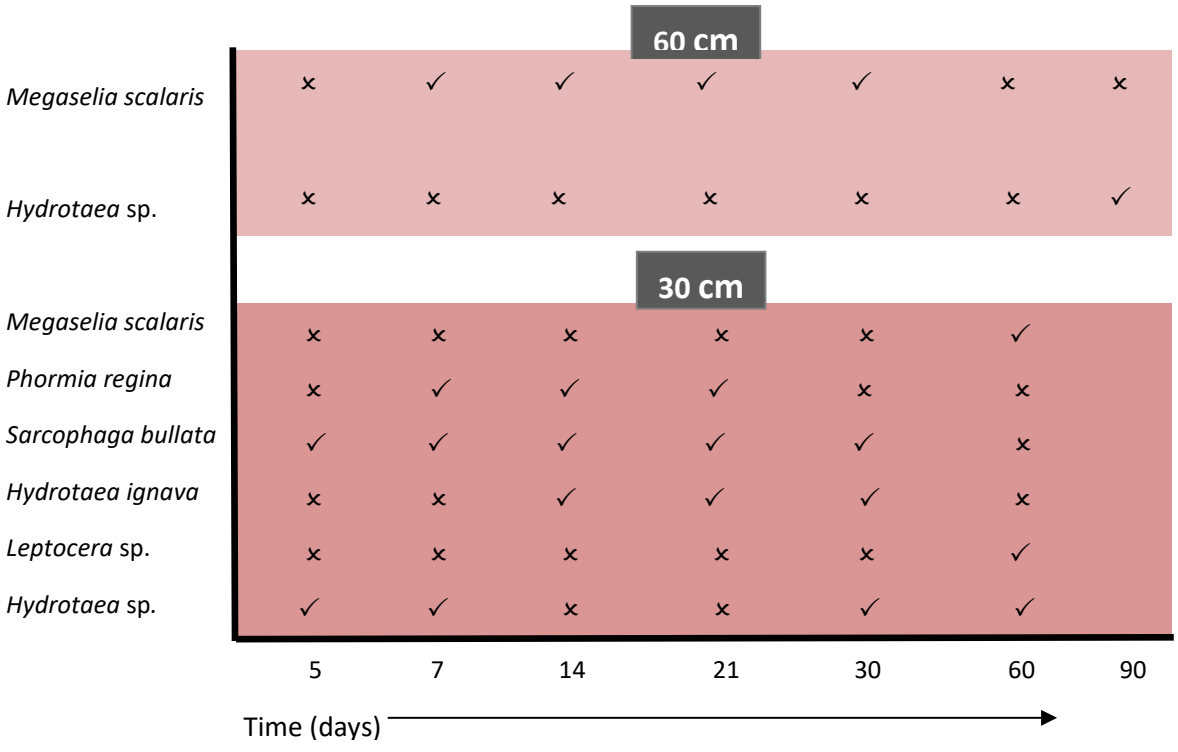


Fig.1.14 Presence (✓) or absence (×) of various species of flies at 30cm and 60cm depth (Pastula & Merritt, 2013)

It can be observed in both cases that *Megaselia scalaris* (Loew, 1866) of family Phoridae is the most prominent species present in situations regarding buried corpses. Staphylinid beetles (rove beetles) are commonly found on buried corpses as well but these are predaceous species preferring fly eggs and larvae as food rather than the dead body itself (Lundt, 1964; Rodriguez & Bass, 1985; VanLaerhoven & Anderson, 1999). Hence *M.scalaris* is an important fly of forensic interest in cases involving buried corpses. Apart from this, the species is also of prime importance in cases of indoor crime scenes as demonstrated by Bugelli et al. (2015) and Reibe and Madea (2010). As described by Disney (2008), it is the small size of this fly which allows it to infiltrate or escape from closed environments hence being more beneficial than blow-flies in estimating time since death accurately in indoor crime scenes.

1.5 *Megaselia scalaris*

Megaselia is the biggest genus of the Phoridae family, having a cosmopolitan distribution of approximately 1400 species. The species *Megaselia scalaris* is found to be endemic to warmer environments however its worldwide dispersal is due to unwary activities caused by humans. It can survive even in colder climates as it moves indoors where it is warmer to breed (Disney, 2008; Turchetto & Vanin, 2004).

The life cycle of *M. scalaris* is typically completed in 3 weeks at optimum room temperature and consists of 4 discrete stages – egg, larva, pupa and adult. The larval development occurs over 3 instars – first, second and third (Fig.1.15).

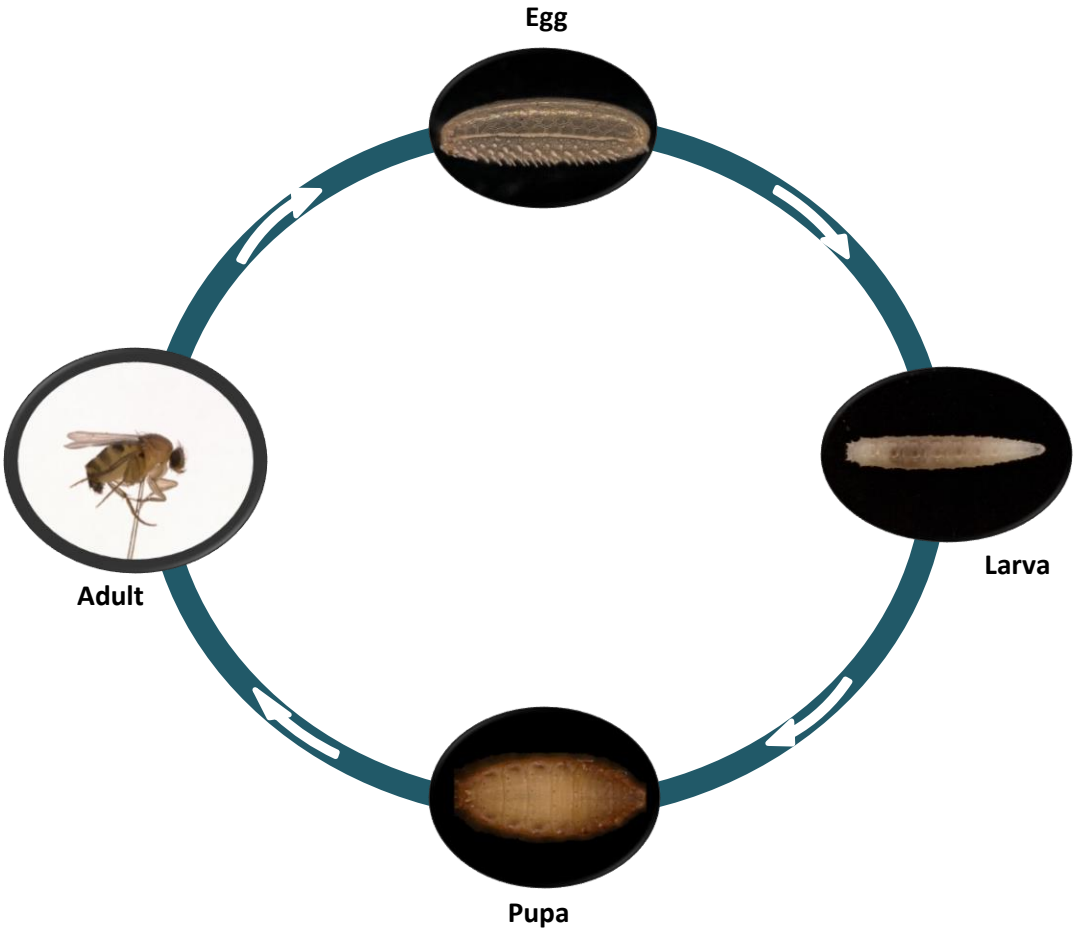


Fig.1.15 Life cycle showing different stages of development in *Megaselia scalaris*

1.5.1 Morphology

Megaselia is similar to the fruit fly (*Drosophila*) in size. It has a yellow to yellowish brown thorax and the above section of the abdomen is black with yellow to white sections present both laterally and ventrally. The abdomen also has brown bands clearly visible against its yellow surface. The thorax has a humpbacked appearance which is characteristic of the family Phoridae. It has yellow legs which are generally well developed having a plump, broadened, laterally compressed hind

femur (Fig 1.16). The wings are large and have the typical Phorid heavy venation at the base of the anterior wing margin (Fig.1.17) (Byrd & Castner, 2010).

Fig.1.16 Lateral view of an adult *M. scalaris* fly with 50x magnification and scale (200µm)

Fig.1.17 Characteristic venation on the wing of an adult Phoridae (MAF Plant Health & Environment Laboratory, 2011)

Larvae of *M. scalaris* are spindle-shaped and yellowish to whitish in colour. Their size ranges from 0.5-10mm depending upon the instar (first, second and third). The third instar of the larval development lasts the longest and larvae at this stage are approximately 4mm in length. The body is tubular and tapers in the direction of the head (Fig 1.18). Figure 1.19 shows comparison of size of a 3rd instar *M. scalaris* larva to a 3rd instar larva of Calliphora sp. The anterior spiracles can be seen as oval shaped and have two spriracular slits (Fig.1.20) (Sukontason et al., 2002). Morphological features peculiar this larva have been described in more detail by Zumpt (1965), Kaneko et al. (1978), Liu and Greenberg (1989) and Sukontason et al. (2002).

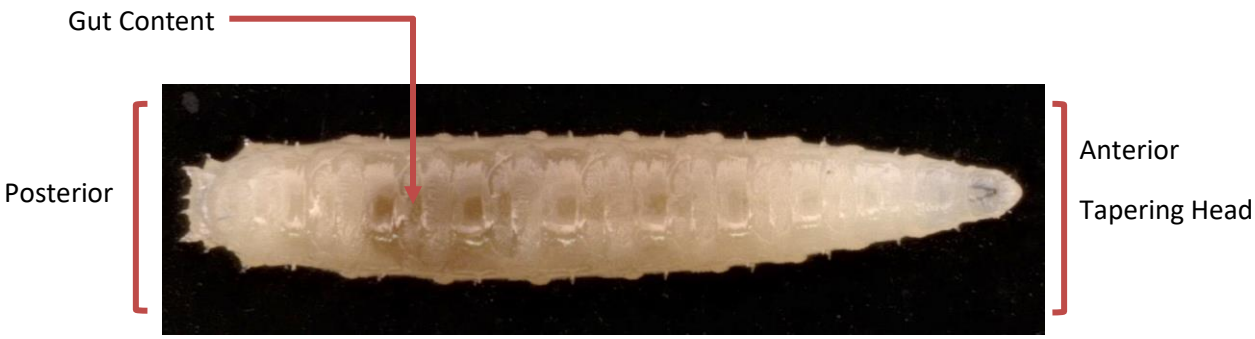


Fig. 1.18 Cylindrical third instar *M. scalaris* larva with a tapering head and visible gut content. Larval size about 2mm (See Fig.1.19).

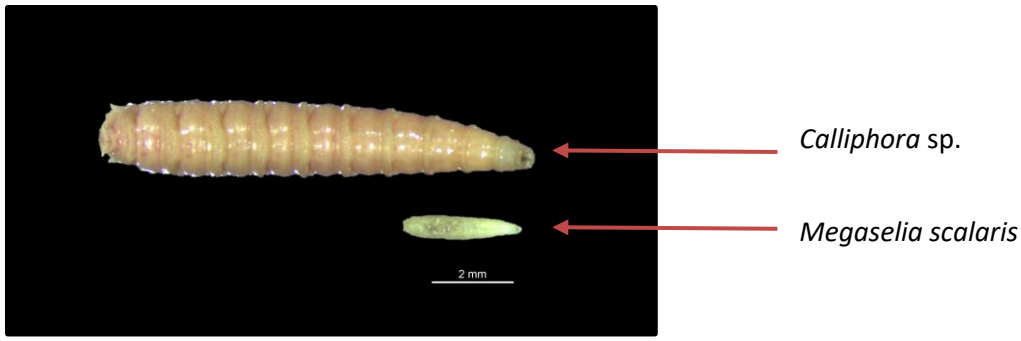


Fig. 1.19 Comparison of size of 3rd instar larva of *Calliphora* sp. (Blow-fly) with 3rd instar larva of *Megaselia scalaris* (1x magnification, scale – 2mm).

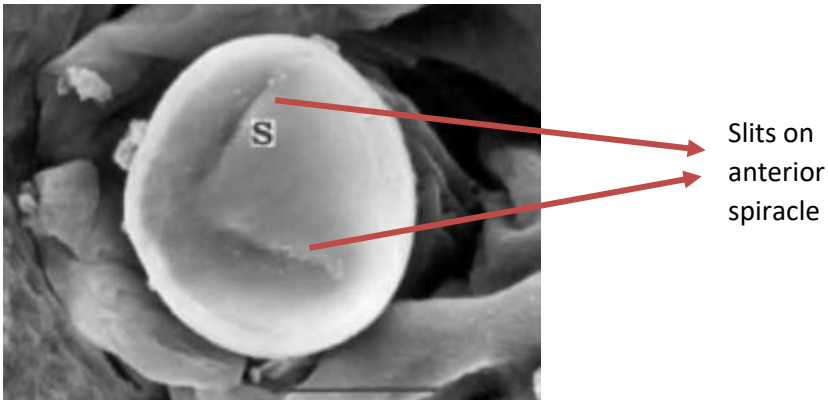


Fig.1.20 Scanning electron micrograph showing 2 slits on the anterior spiracle of third instar larva (Sukontason et al., 2002)

2. Aims:

In the recent years, some research has shown that extracting human DNA from larval gut can be used for personal identification in order to aid an ongoing criminal investigation. However these studies focus only on flies belonging to families Calliphoridae or Sarcophagidae (Di Luise et al., 2008; Li et al., 2011; Njau et al., 2016; Wells et al., 2001; Zehner et al., 2004) due to their bigger size and ease of DNA extraction from gut. Belonging to family Phoridae, *Megaselia scalaris* is also an important fly of forensic interest and is found in a lot of indoor and outdoor crime scenes. In cases involving buried corpses however, *M. scalaris* is the most dominant species present on the corpse as mentioned in section 1.4.3. Due its small size this species is able to penetrate deep into the soil to reach carcasses and colonise them. This ability also makes it colonise corpses before blow-flies in the indoor crime scenes and is therefore used for accurate PMI estimation in such cases (Bugelli et al., 2015; Reibe & Madea, 2010). No research has yet been done on extraction and characterization DNA from *M. scalaris* larvae gut content using STR analysis, due to small size of the fly. This research aims to demonstrate the possibility to -

- Extract DNA from gut content of larvae of *Megaselia scalaris* fed on Bovine meat,
- Determine the preservation technique which helps in performing a clean dissection and also gives best results in extraction of DNA,
- Ascertain the best suited method for specimen collection from the crime scene which is uncomplicated and quick for crime scene officers and also doesn't affect further analysis.

  Characterise the extracted DNA from the gut content using STR analysis. This approach is typically used in forensic cases and is widely accepted in the court of law for criminal investigations. This study uses an animal model but has its implications for human identification.

3. Materials and Methods:

3.1 Breeding of flies

From the descendants of flies bred in the Forensic Laboratory of Entomology and Archaeology (FLEA), University of Huddersfield, since 2011, a few adults of *Megaselia scalaris* were taken. These were placed in glass jars in order to be reared (Byrd & Castner, 2010). Bovine meat (*Bos taurus*, Linneaus 1758), obtained from a local butcher in Huddersfield (UK), was used as the rearing medium. Insect development then occurred in a temperature-controlled incubator (Panasonic MIR-I54) at 25°C in a dark setting.

3.2 Preparing the larvae

Third instar larvae were removed from the food source (Bovine meat) and were fixed using 5 distinct techniques:

a) Larvae were kept in hot water (>80°C) for 30 seconds, as described by Amendt et al. (2007)(Fig.3.1 a).

Fig. 3.1 a

b) A vial was taken and half filled with 98% ethanol and then a few larvae were placed inside it. The vial was then kept at room temperature (Linville et al., 2004) (Fig.3.1 b).

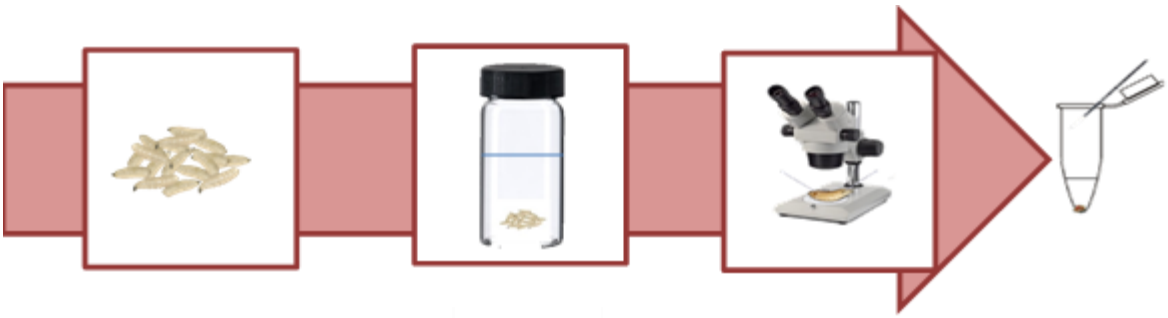


Fig. 3.1 b

c) After transferring a few larvae in a small glass vial, they were kept at -20°C for approximately 4 hours. The bottle was then half filled with 98% ethanol and was again stored at -20°C overnight (Linville et al., 2004) (Fig.3.1 c).

Fig. 3.1 c

d) Larvae were moved directly into vials and were stored in -20°C overnight (Li et al., 2011) (Fig.3.1 d).

Fig. 3.1 d

e) Following the standard procedure defined by Amendt et al. (2007), the larvae were placed in hot water (>80°C) for 30 seconds and then transferred into a vial containing 98% ethanol. It was then stored at -20°C (Fig.3.1 e).

Fig. 3.1 e

The dissection of larvae, using sterilised needles, was performed as per the protocol described by (Tuccia et al., 2016) under a stereo microscope (Leica MRZ) . The larval guts dissected were then weighed using an electronic balance (Fisherbrand™ Precision Series Balances – SG-402). After this the guts were placed in 1.5 ml tubes containing a tissue lysis buffer (section 4.3) and were kept at room temperature prior to the DNA extraction process.

3.3 DNA Extraction

The working area was first cleaned with Thermo Scientific™ DNA AWAY™ Surface Decontaminant and then with EtOH (98%), in order to minimise the risk of contamination. Powder free sterilised rubber gloves were worn in the entire process of DNA extraction. Aerosol free micropipettes and filter tips were used to perform DNA extraction. Three commercially available kits, based on solid phase DNA extraction (section 1.3.2), were used to extract DNA from larval gut content:

a) QIAamp® DNA Mini Extraction Kit (Qiagen, Netherlands)

b) QIAamp® DNA Investigator Kit (Qiagen, Netherlands)

c) PrepFiler® Forensic DNA extraction Kit (Applied Biosystems, USA)

The QIAamp® DNA Mini Extraction Kit and QIAamp® DNA Investigator Kit are based on DNA extraction with the help of spin columns, which have a silica membrane onto which DNA molecules are adsorbed (Greenspoon et al., 1998). The PrepFiler® Forensic DNA extraction Kit uses polymer-embedded magnetic particles to bind DNA and isolate it (Brevnov et al., 2009).

The protocols provided by the kit manufacturers (Qiagen and Applied Biosystems) were followed in order to extract DNA, and a few volumetric adjustments were made to increase the yield of extracted DNA. The steps followed in each case are described in Appendix A.

Along with the gut contents, control samples were also extracted using each of the aforementioned DNA extraction kits following the manufacturer's protocol. For a control to be used with the gut contents DNA was extracted from the bovine meat fed to the flies while breeding them. To be used with the larvae sample, DNA was extracted from the larval tissues.

3.4 DNA Quantification

The quantity of DNA extracted was assessed using the Invitrogen™Qubit® 3.0 (Life technologies, USA). The protocol recommended by the manufacturers was followed (Qubit® fluorometer - dsDNA assay user guide, 2015). Using 199µL of Qubit® dsDNA HS Buffer and 1µL of Qubit® Fluorophore per sample a reading solution was prepared. Qubit® assay tubes, with respect to the number of samples to be quantified, were taken and 199µl of the reading solution was added to each tube. To this 1µl of extracted DNA sample was added. The tubes were placed in the Qubit® 3.0 Fluorometer to be analysed. The readings for each sample [in ng/µl (nanograms per microliter] were taken in triplicates and their average and standard deviation was evaluated.

3.5 Statistical Analysis

After weighing the dissected larval guts and quantifying the extracted DNA samples, statistical analysis was carried out in order to evaluate the results, using the IBM® SPSS Statistics 24 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, H_o, of no difference between the samples). Values obtained

below the threshold of 0.05 denoted significant difference (rejection of the H_o). In this thesis all of the descriptive values are reported as mean ± standard deviation (SD).

3.6 Polymerase Chain Reaction

As prior to DNA extraction, the working area was first cleaned with Thermo Scientific™ DNA AWAY™ Surface Decontaminant and then with EtOH (98%). Powder free sterilised rubber gloves were worn in the entire process of PCR amplification. Each of the extracted DNA sample was amplified using PCR and depending upon the sample type (Gut content/larval tissue), each PCR assay was designed separately. Bio-Rad C1000 thermo cycler (Bio-Rad laboratories, USA) was used to carry out the PCR process.

3.6.1 Primers for PCR Analysis

After going through literature, primers that were species specific were selected for the PCR assay. Universal insect specific primer COI-I (Cytochrome c oxidase subunit I), commonly used for invertebrate barcoding (Hebert et al., 2003) was used to amplify DNA extracted from larval tissue samples (Folmer et al., 1994). To distinguish larval tissue samples from the gut content samples a nested PCR (Fig.3.2) was performed first with the 'Cytochrome B Long' primer (Naidu et al., 2012) specific to mammals and then with primers mcb398 (forward) and mcb869 (reverse) ('Cytochrome B Short') which amplify shorter nucleotide sequences within the Cytochrome B region (Verma & Singh, 2003). Cytochrome B primers specific for Bovine (Drummond et al., 2013; Zhang et al., 2007) were used to amplify the DNA extracted from Bovine meat. To amplify the Bovine ribosomal RNA gene 16S rRNA primers were used as described by Lee et al. (2016). Table 3.1 summarizes the primary features of the primers used to perform specific PCR assays.

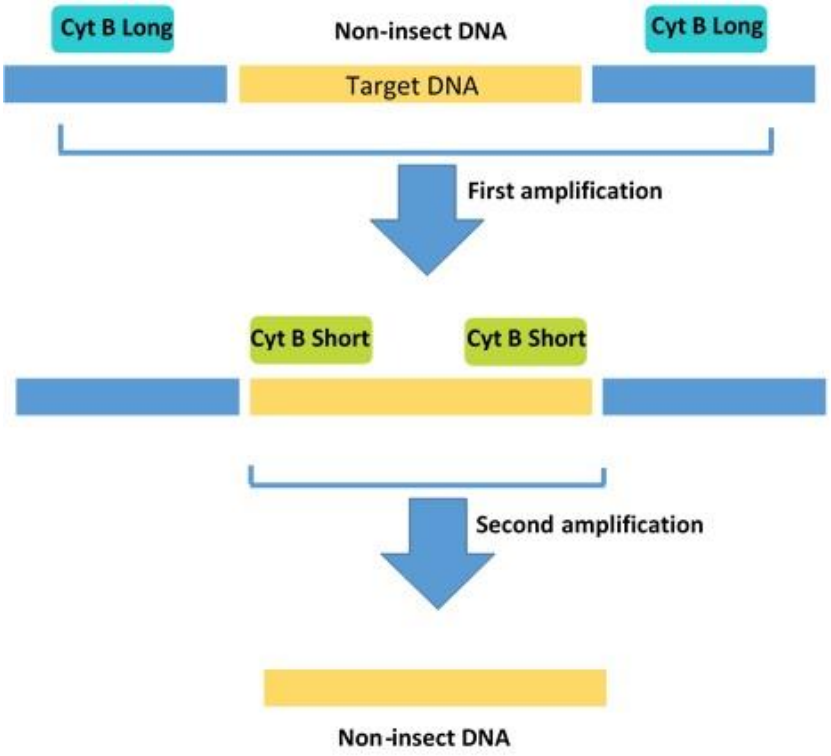


Fig. 3.2 Nested PCR to distinguish larval tissue samples from the non-insect DNA in the larval gut content**3.6.2 Amplification of Target DNA**

A standard end-point PCR procedure was carried out to amplify target DNA in gut content of the larvae and Nested PCR was performed as well to distinguish mammalian DNA from the insect tissue.

a) End-Point PCR :

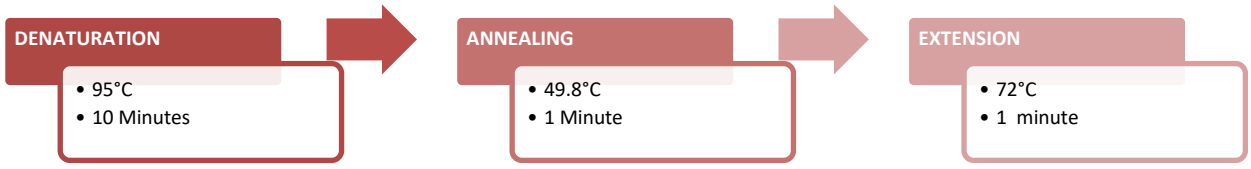
A reaction mix was prepared in PCR assay tubes (Star Lab, UK) of 200µl. 16µl of master mix and 4µl of DNA template was mixed to have a final volume of 20µl following the Promega GoTaq® Flexi DNA Polymerase Protocol. For the master mix, per sample, 4µL of 5x GoTaq Flexi® Buffer (Promega, USA), 4µL $MgCl_2$ (25mM) (Promega, USA), 0.5µL of forward and reverse primers (10pmol/µl), 0.5µL of PCR Nucleotide Mix (10mM), 0.25µL GoTaqG2 polymerase (5u/µl) and 6.25µL of Ultrapure™ PCR grade water (Invitrogen, USA) were added. Before the start of the amplification process there was an initial denaturation step at 95°C for 10 minutes. This was similar for all the PCR programs which used different primers. The duration and temperature

settings for the denaturation, annealing and extension steps for different primer sets has been summarized in Fig.3.3. The process was repeated for 30 cycles and at the end of each cycle there was a final elongation step of 72°C for 10 minutes.

Table 3.1 Summary of all the primers used for specific PCR assays. Primers 'Cyt B Long', 'Cyt B short', Cyt B Bovine and 16S rRNA Bovine were used to amplify bovine DNA from larval gut content. Primer COX1 was used to amplify larval tissue.

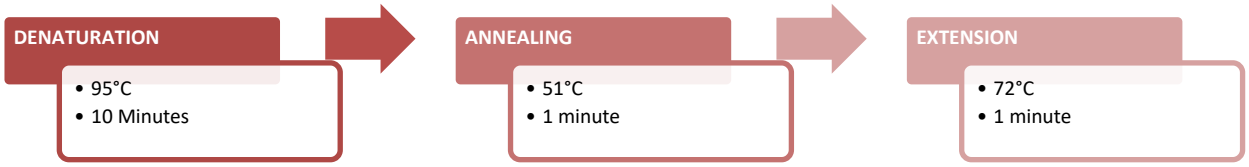
PRIMER		PRIMER NAME	PRIMER SEQUENCE (5' – 3')	FRAGMENT SIZE (bp)
COI-I (Folmer et al., 1994)	Forward	LCO	GGTCAACAAATCATAAAGATATTGG	
	Reverse	HCO	TAAACTTCAGGGTGACCAAAAAATCA	658
'Cyt B (long)' Mammals (Naidu et al., 2012)	Forward	MTCB-F	CCHCCATAAATAGGNGAAGG	
	Reverse	MTCB-R	WAGAAYTTCAGCTTTGGG	1140
'Cyt B (short)' Mammals (Verma & Singh, 2003)	Forward	mcb398	TACCATGAGGACAAATATCATTCTG	
	Reverse	mcb869	CCTCCTAGTTTGTTAGGGATTGATCG	472
Cyt B Bovine (Drummond et al., 2013; Zhang et al., 2007)	Forward	Cyt B Bos F	CGGAGTAATCCTTCTGCTCACAGT	116
	Reverse	Cyt B Bos R	GGATTGCTGATAGGTTGGTG	
16S rRNA Bovine (Lee et al., 2016)	Forward	SFI11-Cow F	TATCTTGAACTAGACCTAGCCCAATG	131
	Reverse	SFI11-Cow R	GGTACTTTCTCTATAGCGCCGTAC	

(a) Primer COI-I on larval tissues

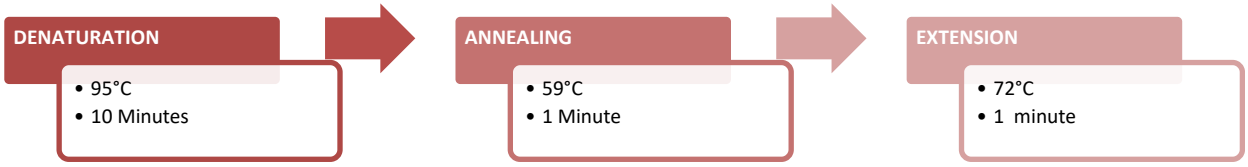


(b) Primer 'Cyt B Long' on larval gut content

(c) Primer 'Cyt B Short' on larval gut content



(d) Primer Cyt B Bovine on larval gut content



(e) Primer 16S rRNA Bovine on larval gut content.

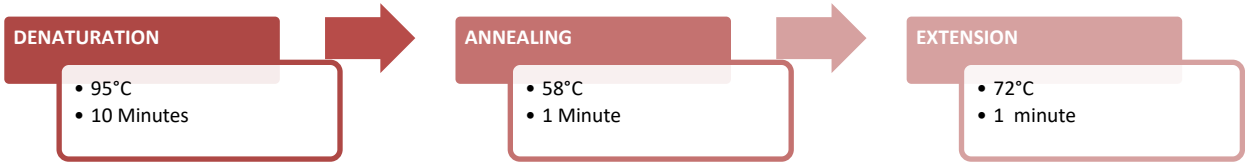


Fig.3.3 Temperature and duration settings of PCRs set for different targets and substrates, for 30 cycles.

b) Nested PCR:

This is a variation of the standard PCR procedure in which two primer SETS are used in order to increase specificity of the target region. The first step utilises primers MTCB-F AND MTCB-R (Fig.3.2) for amplification of target region in the gut content. A master mix was prepared as described above and the final PCR tubes contained 16µl of the former and 4µl of template DNA. There was an initial denaturation step of 10 minutes at 95°C followed by 30 cycles each consisting of three steps – first step at 95°C for 30s (denaturation), second step at 55°C for 30s (annealing) and third step at 72°C for 45s (extension). Subsequently there was the final elongation step at 72°C for 10 minutes. The PCR product was kept at 4°C for further use. Afterwards this was purified using the QIAquick PCR Purification Kit (Qiagen®, Netherlands) and the protocol provided by the manufacturer was followed (QIAquick®, 2015). The PCR products purified were used as a DNA template for the second step of Nested PCR. In this step primers mcb398 and mcb869 (Fig.3.2) were used. A reaction mix of 20µl volume was prepared having 16µl of master mix and 4µl of DNA template. Similar to above, there was a key denaturation step of 10 minutes at 95°C and 30 cycles were set. As described above every cycle had 3 steps - 95°C for 30s (denaturation), 51°C for 30s (annealing) and 72°C for 45s (extension), with a final elongation step of 10 minutes at 72°C. The PCR product was afterwards stored at 4°C till further use in analysis. As mentioned previously, this PCR was performed to optimize the amplification of non-insect DNA, targeting the mammalian DNA.

3.7 Agarose Gel Electrophoresis

1.5g Agarose was dissolved in 100µl of 1x TBE Buffer to prepare a 1.5% w/v agarose gel, in order to visualise the amplified PCR product obtained. 3µl of Advanced DNA Stain Midori Green (Nippon Genetics) was then added as a visualisation dye. The gel was then allowed to solidify for 30-45 minutes at room temperature. In the meantime, samples to be loaded in the wells were prepared by mixing 5µL of the PCR product and 2µL of 6x Blue/orange dye (Promega, USA). After the gel was set it was positioned in an electrophoresis unit (Bio-Rad) having 1X TBE running buffer. In

this the samples prepared were loaded along with a 100bp DNA ladder (Promega, USA). The gel was run at 100V for 45 minutes.

The gel was visualised in a UV gel doc system (InGenuis Syngene Bio Imaging System) using the Gene Snap software from Syngene. The size of the DNA fragments was compared with respect to the DNA ladder.

3.8 Purification of DNA

QIAquick PCR Purification Kit (Qiagen®, Netherlands) was used to purify the PCR products. Steps according to the manufacturer's protocol were followed (QIAquick®, 2015). The samples were eluted in 30µl of AE Elution Buffer and were stored at room temperature and then sent for Sanger sequencing to Eurofins Genomics (Germany).

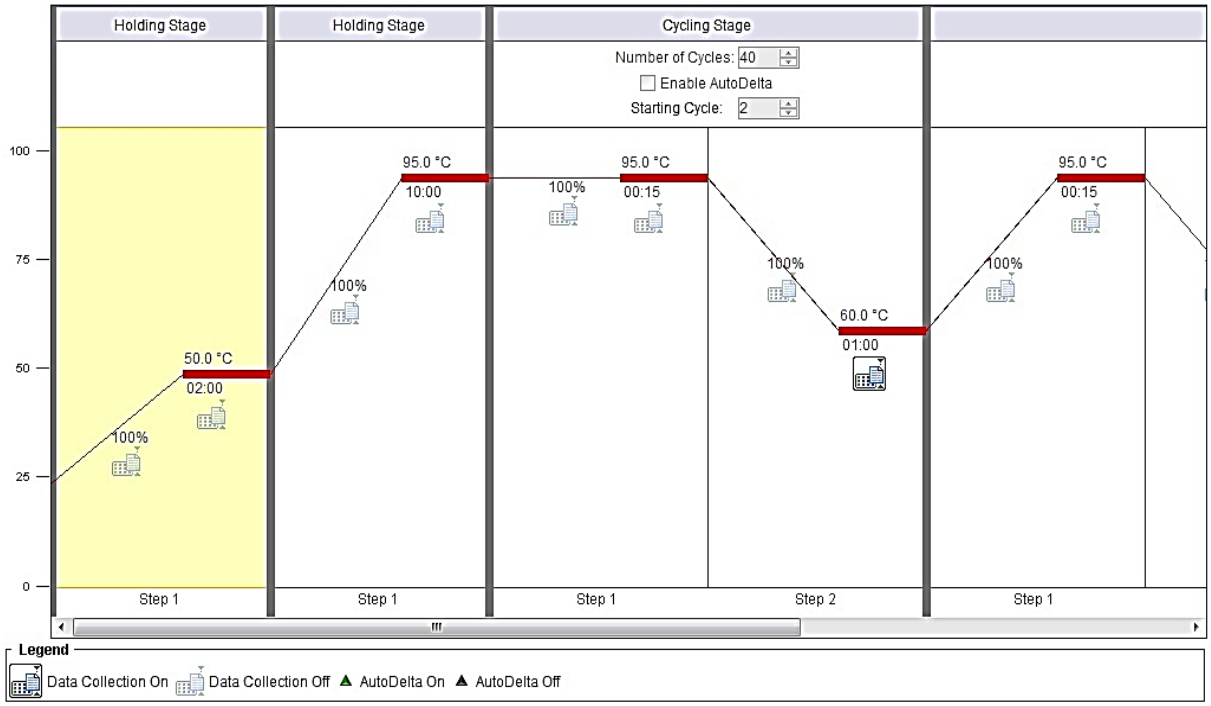
3.9 Quantitative PCR

As described in section 1.3.5, a Quantitative PCR (qPCR) or Real-Time PCR (RT-PCR) is performed in order to accurately assess of quantity of DNA obtained. It was performed on 7500 Fast Real Time PCR instrument (Applied Biosystems, USA). The primers used were Cyt B Bovine (Drummond et al., 2013; Zhang et al., 2007) and 16S rRNA (Lee et al., 2016). These are specific to the *Bos* genus.

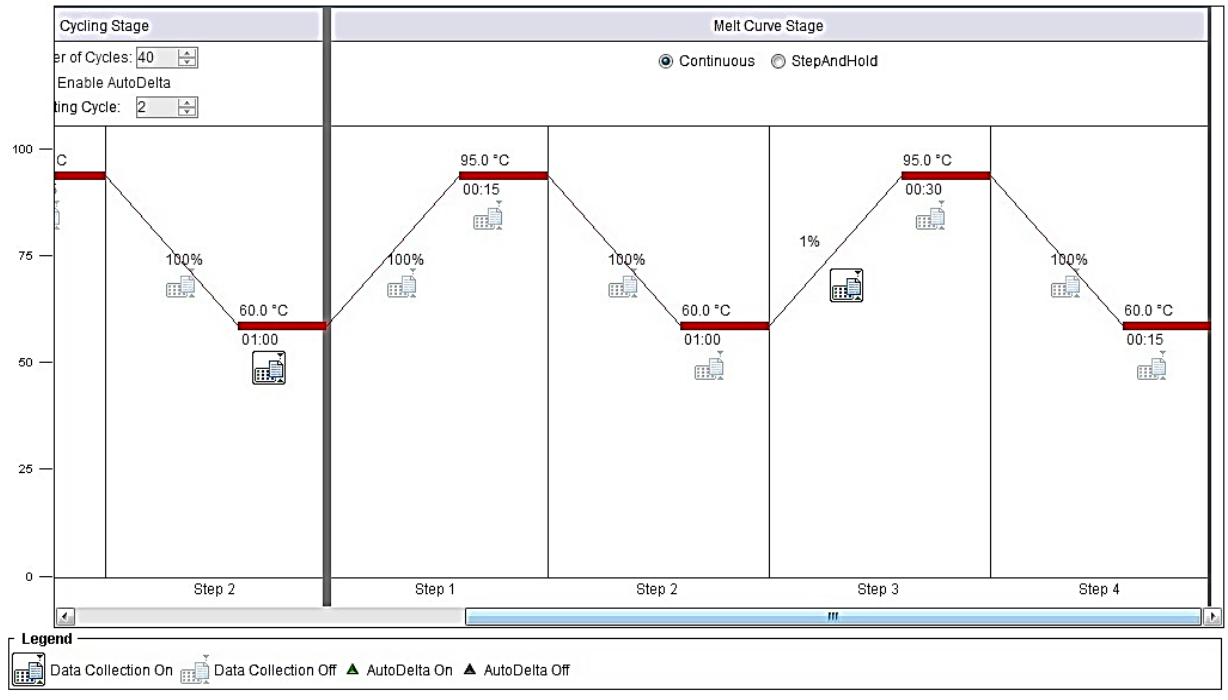
3.9.1 Amplification by qPCR

A reaction mixture of 20µl was prepared in the MicroAmp® Fast Optical Reaction Plate (Life Technologies, USA). This contained 15µl of the master mix and 5µl of template DNA. The master mix consisted of 10µL of SYBR® Green (Applied Bio-System, USA), 0.5µL of each forward (CytB Bos F/ SFI11-Cow F) and reverse primers (CytB Bos R/ SFI11-Cow R) and 4µL of Ultrapure™ PCR grade water (Invitrogen, USA). A MicroAmp™ Optical Adhesive Film (Applied Biosystems, USA) was used to seal the reaction plate. After this the plate was centrifuged at 12,000 rpm briefly using the Axygen® Mini Plate Spinner Centrifuge (Thomas Scientific, USA) and was placed on the

sample block. The 7500 software v2.3 was used to set up the amplification reaction. A standard run method with standard (~2hrs) ramp speed for quantification was selected. The option for melt curve analysis was selected when choosing the dye used in the reaction mix. The duration and temperature of different stages of the run for 40 cycles were set. There was an initial holding stage for 10 minutes at 95°C followed by two steps of 95°C for 15s and 60°C for 60s. After this there was a melt curve analysis stage having an initial holding period of 15s at 95°C. This was proceeded by two steps of 1min at 60°C, and 30s at 95°C. It ended with a final stage of 15s at 60°C (Fig. 3.4).



(a)



(b)

Fig.3.4(a) Stages of the standard run method on the 7500 software v2.3 (Applied Biosystems, USA) **(b)** Stages involved in the melt curve analysis of samples

After completion of the run, an amplification curve, a standard curve and a quantification of each sample was obtained. The standard curve was plotted was in accordance to the standards loaded along with the samples in the reaction plate. The quantity of unknown samples of DNA was ascertained with respect to these standards. They were prepared manually by selecting a DNA sample of known quantity and then serially diluting it in the ratio 1:5.

3.10 STR Analysis

After the above quantification, samples having the maximum amount were selected and their STR analysis was carried out for the 18 microsatellites which cover all the 12 STR loci recommended by the International Society of Animal Genetics (ISAG) for bovine ancestry testing and identification (TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824 and BM1818) (van de Goor et al., 2011) (Table 3.2). The Thermo Scientific Bovine Genotypes Panel 1.2 Kit was used to amplify the 12 STR loci and the 3130 Genetic Analyser (Applied Biosystems, USA) was used to perform capillary electrophoresis.

3.10.1 PCR Amplification

The process was followed as per the instructions in the Bovine Genotypes Panel 1.3 User Guide (Thermo Fisher Scientific©, 2012). A reaction mix for the process was prepared in a 1.5ml microcentrifuge tube, having 10µl of Bovine Genotypes Master Mix and 10µl of Bovine Genotypes Panel 3.1 Primer Mix per sample. 18µl of this mix was then transferred to 200µl PCR tubes and 2µl of template DNA was then added in each respective tube. Along with the samples, two tubes one for a positive control (Bovine Genotypes Control DNA001) and one for a negative control (nuclease free water) were allotted as well. After briefly centrifuging them to mix all the reagents, the tubes were places in the Bio-Rad C1000 thermal cycler (Bio-Rad laboratories, USA) for amplification for 30 cycles. There was an initial activation stage for 60s at 98°C, following which three stages were repeated for all cycles. The first lasts for 20s at 98°C, the second at 60°C for 75s and the third at 72°C for 30s. Proceeding this was the concluding elongation stage for 5 minutes at 72°C. The obtained product was stored at 4°C until further use in analysis.

Table 3.2 Description of the loci for the Bovine Genotypes Panel 1.2 microsatellites which include all the 12 STR loci recommended by ISAG for bovine identification and parentage (Bovine Genotypes Panel 1.2 User Guide, Thermo Fisher Scientific©, 2012)

LOCUS NAME	CHROMOSOME	REPEAT MOTIF	SIZE RANGE (bp)	DYE COLOR
TGLA227 (D18S1)	18	di	63–115	Blue
BM2113 (D2S26)	2	di	116–146	Blue
TGLA53 (D16S3)	16	di	147–197	Blue
ETH10 (D5S3)	5	di	198–234	Blue
SPS115 (D15)	15	di	240–270	Blue
TGLA126 (D20S1)	20	di	104–132	Green
TGLA122 (D21S6)	21	di	133–193	Green
INRA23 (D3S10)	3	di	194–236	Green
BM1818 (D23S21)	23	di	248–276	Green
ETH3 (D19S2)	19	di	89–131	Black
ETH225 (D9S1)	9	di	132–166	Black
BM1824 (D1S34)	1	di	170–218	Black

3.10.2 Capillary Electrophoresis

Capillary electrophoresis was performed on the 3130 Genetic Analyzer (Applied Biosystems, USA) which had the POP-4 polymer (Applied Bio-Systems, USA) set up. A reaction mix for electrophoresis was prepared in a 1.5ml microcentrifuge tube containing 11µl of deionised Hi-Fi™ formamide (Applied Biosystems, USA) and 0.3µL of GeneScan™ 500 LIZ® Size Standard (Applied Biosystems™, USA). 10µl of this mix was pipetted into the MicroAmp® Fast Optical Reaction plate (Life Technologies, USA) and 1.5µl of the PCR product was added to each respective well. A 96 well plate septa (Life Technologies, USA) was used to seal the reaction plate. It was then briefly

centrifuged using Axygen® Mini Plate Spinner Centrifuge (Thomas Scientific, USA). Using the Bio-Rad C1000 thermal cycler (Bio-Rad laboratories, USA), the plate was heated at 95°C for 3 minutes to denature the samples and was then cooled at 4°C for 3 minutes using the same. The reaction plate was pooled with a retainer and a plate base (Life Technologies, USA) and was mounted on the plate bay of the Genetic Analyzer. A set up for the run was created following the steps described in the Bovine Genotypes Panel 1.2 User Guide (Thermo Fisher Scientific©, 2012) and injection list parameters were set up (Table 3.3) in the Data Collection v2.3 software.

Table 3.3 Injection parameters setup for injection with 36cm capillaries, prior to electrophoresis

Injection Seconds	22
Injection Voltage (kV)	1.0
Run Voltage (kV)	15.0
Run Temperature (°C)	60
Run Time (s)	1200

Electrophoresis was commenced according to the Applied Biosystems 3130 Genetic Analyzers Getting Started Guide (Applied Biosystems, USA).

3.10.3 Analysis and Interpretation of Data Collected

Once a run is completed, data is collected in the Data Collection v2.3 software and electropherograms are generated and analysed using the Genemapper v 3.2 software (Applied Biosystems, USA). 50 RFU (Relative Fluorescent Units) was set as a threshold for peak detection for every sample during analysis, as established by the laboratory practice where these analyses were performed.

4. Results:

4.1 Weight of Larval Gut

Ten larval crops were removed after dissecting larvae, fixed using 5 different methods as described in Materials and Methods (section 3.2) and their wet weight was recorded. After this they were dried in an oven for 45s so that the water content could be reduced and then dry weight of the crops was recorded. The readings were taken in triplicates (same sample measured three times). A one-way ANOVA showed that there wasn't any statistical significant difference between or within the triplicate readings of wet weight of gut of larvae obtained using various techniques ($F=2.925$, $df=4$, $p=0.77$). A two-way ANOVA test also showed no statistically significant difference, with respect to temperature and EtOH on the wet weight of the gut of larvae as well as between the interaction of temperature ($F=3.328$, $df=2$, $p=0.79$) and EtOH ($F=0.274$, $df=1$, $p=0.612$) (Fig.4.1a). Similar to wet weight, readings in triplicates (same sample measured three times) were taken for dry weight as well and in this case statistical analysis revealed a significant difference between methods ($F=121{,}800$, $df=4$, $p=0.000$). A statistical significant difference was observed between in dry weight of larvae pertaining to temperature ($F=117.208$, $df=4$, $p=0.000$) and the interaction between temperature and EtOH also resulted in a statistically significant difference ($F=182{,}250$, $df=4$, $p=0.000$). EtOH, however, as a variable alone did not affect the dry weight ($F=2.250$, $df=4$, $p=0.165$). A Post-Hoc analysis (Tukey HSD) revealed that method 5 (EtOH only) of fixation is statistically different from all other methods ($p=0$) Fig.4.1 illustrates the weight of crops when wet (Fig. 4.1a) and dry (Fig. 4.1b) extracted with different methods.

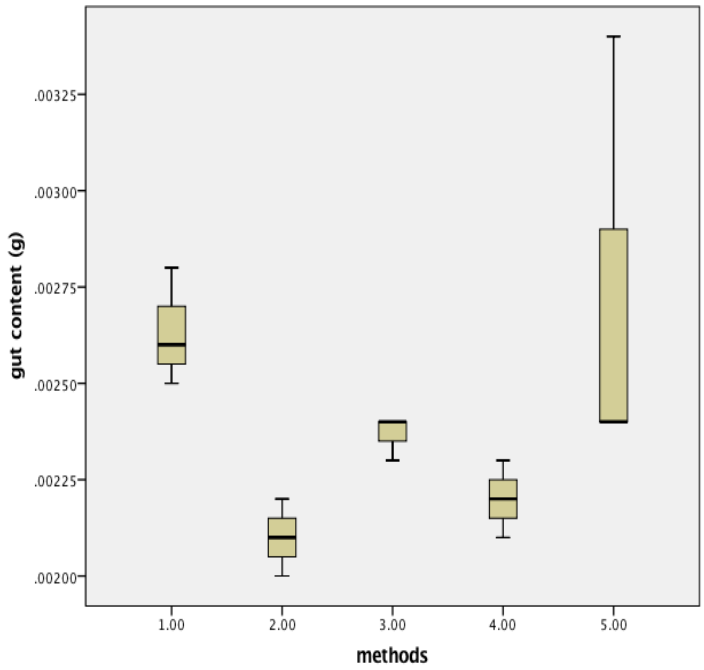


Fig. 4.1a. Wet weight of 10 larval crops extracted with different methods (1= Hot water (≥90˚C), 2= Frozen Only, 3= Frozen + EtOH, 4= Hot water + EtOH and 5 = EtOH only).

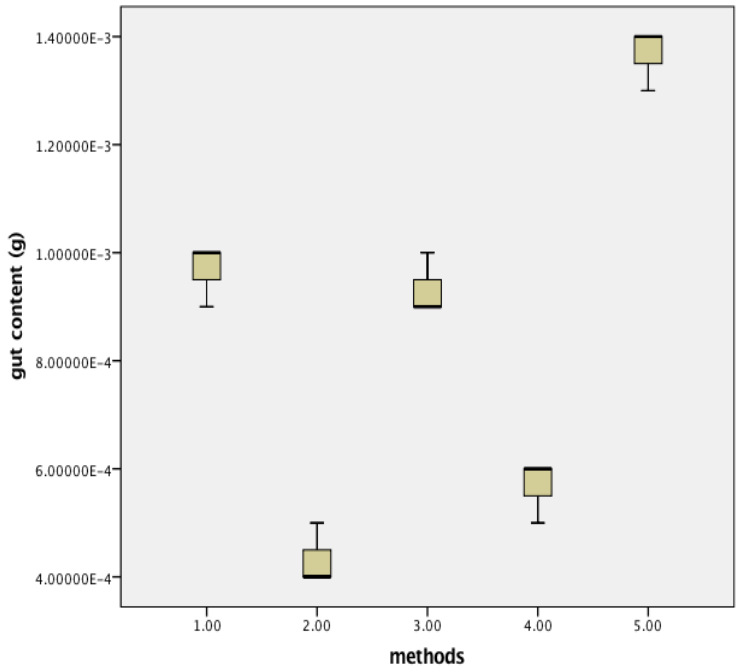


Fig. 4.1b. Dry weight of 10 larval crops extracted with different methods (1= Hot water (≥90˚C) , 2= Frozen Only, 3= Frozen + EtOH, 4= Hot water + EtOH and 5 = EtOH only). Method 5 shows statistically significant difference from all.

4.2 Quantification of DNA

The Invitrogen™Qubit® 3.0 (Life technologies, USA) was used to quantify the extracted, as described in Materials and Methods (section 3.4) and the readings for each sample were taken in triplicates (same sample measured three times) (Appendix C). Three different kits were used to extract DNA (section 3.3) from 1 crop, 3 crops, 5 crops and 10 crops. A one-way ANOVA test followed by a Post-Hoc (Tukey) between DNA per specimen and the extraction kits revealed statistically significant differences ($F = 112.731$, $dF = 6$, $p = 0.000$). Table having details of the Post-Hoc analysis has been attached in Appendix B. Following this one-way ANOVA test was performed to assess the effect of the methods adopted for extraction on the DNA per specimen. Method 1 refers to DNA extraction by PrepFiler Kit, method 2 refers to DNA extraction after fixing larvae with Hot water for 30s (Investigator Kit), method 3 refers to DNA extraction after fixing larvae by only freezing (Investigator Kit), method 4 refers to DNA extraction with Mini Kit, method 5 refers to DNA extraction after fixing larvae by freezing then placing in Ethanol (Investigator Kit), method 6 refers to DNA extraction after fixing larvae with only ethanol (Investigator Kit); method 7 refers to DNA extraction after fixing larvae with hot water (≥80˚C) then placing in ethanol (Investigator Kit). Significant statistical difference was found between the methods of DNA extraction ($F=112.731$, $df=6$, $p=0.000$) and a Post-Hoc (Tukey HSD) analysis revealed that method 4 was statistically different from all the other methods ($p=0.000$) in comparison to all methods) (Fig. 4.2). Keeping these results in mind, the QIAamp® DNA Investigator Kit (Qiagen, Netherlands) was selected for further extractions of DNA as it provided a good DNA yield. From the control samples (Bovine meat) DNA of adequate quality and quantity was obtained as well (76 ± 5ng/µl). Invitrogen™Qubit® 3.0 (Life technologies, USA) does not give species specific quantifications, therefore, utmost attention was paid to avoid preventing addition of any larval DNA while dissecting. However, it is possible that DNA from some larval tissue surrounding the crop may be present along with the extracted DNA from the gut content.

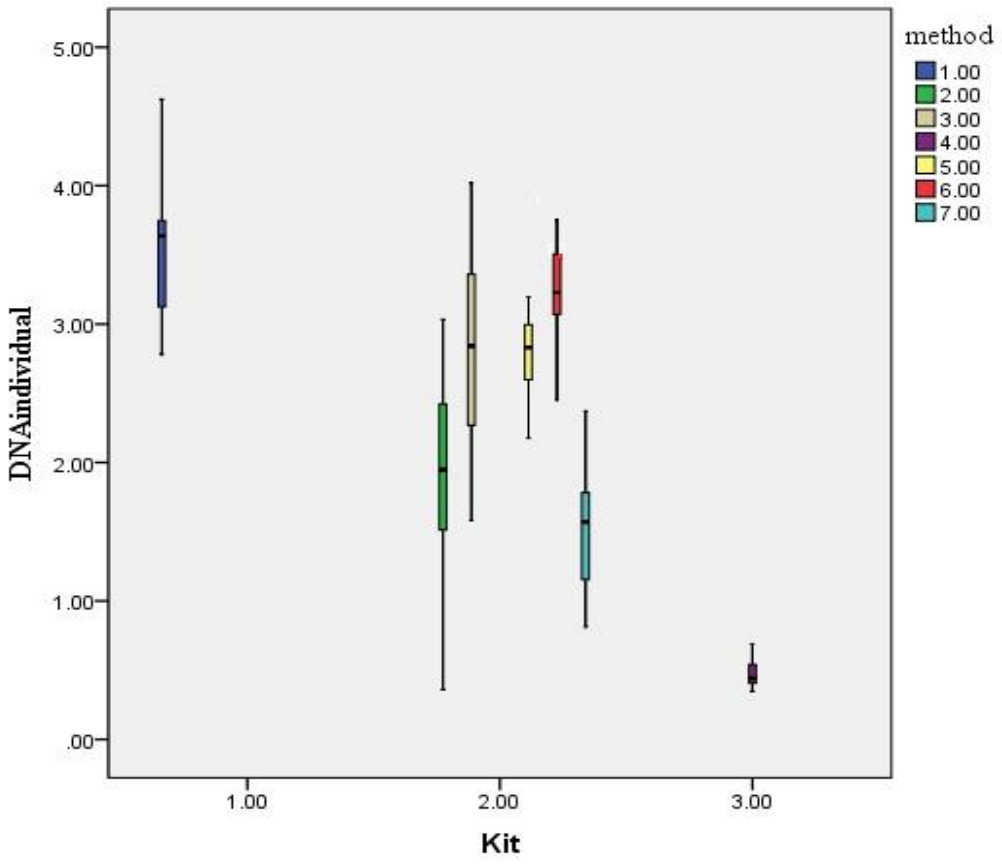


Fig.4.2 Post-Hoc Analysis shows that method 4 differs statistically from all other methods of DNA extraction, in terms of quantity of DNA (in ng/µl) attained per specimen. It gives the minimum quantity of DNA. [method 1 – PrepFiler Kit; method 2 – Hot water for 30s (Investigator Kit); method 3 – Only Frozen (Investigator Kit); method 4 – Mini Kit; method 5- Frozen + Ethanol (Investigator Kit); method 6 – Only ethanol (Investigator Kit); method 7 - Hot Water (≥90˚C) + Ethanol (Investigator Kit)]. Each box summarizes the results obtained after analysing the normalised data generated from each of the methods mentioned above.

Analysis of total DNA obtained (in ng/µl), in all methods, also revealed that there was a gradual increase in the total amount of DNA as the number of crops were increasing in each case (Fig.4.3).

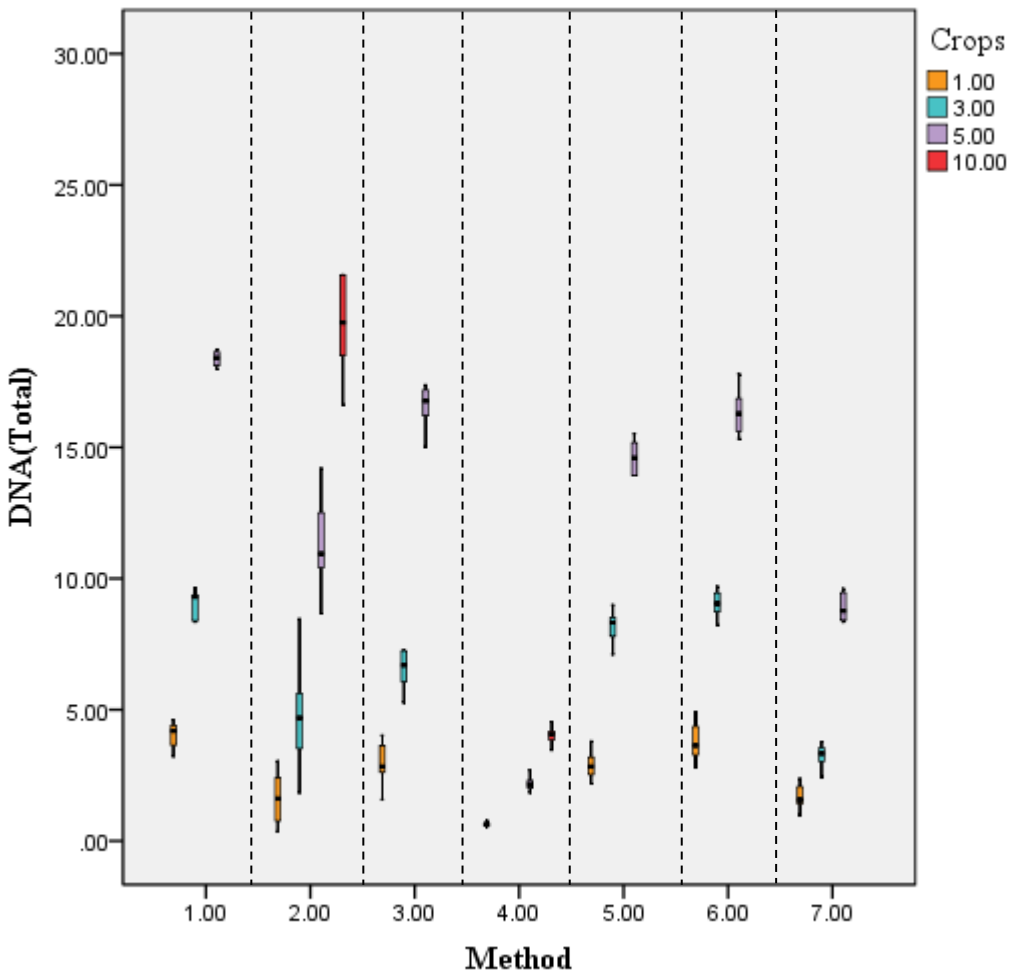


Fig.4.3 An increase in total amount of DNA (in ng/µl) extracted is observed with increase in number of crops for all methods of DNA extraction [method 1 – PrepFiler Kit; method 2 – Hot water for 30s (Investigator Kit); method 3 – Only Frozen (Investigator Kit); method 4 – Mini Kit; method 5- Frozen + Ethanol (Investigator Kit); method 6 – Only ethanol (Investigator Kit); method 7 - Hot Water (≥90˚C) + Ethanol (Investigator Kit)].

An ANOVA test was also performed to assess the effect of temperature, EtOH and number of crops on the quantity of DNA extracted per specimen. It was observed that EtOH as an individual variable does not affect the DNA quantifications ($F=2.280$, $df=1$, $p= 0.133$) while temperature ($F=46.065$, $df=2$, $p= 0.000$) and number of crops ($F=5.386$, $df=3$, $p= 0.001$) does. The interaction among the variables, temperature, EtOH and number of crops, also affects the results ($F=4.300$, $df=2$, $p= 0.015$).

4.3 PCR Results

First the gel to distinguish larval samples from mammalian samples was visualised. The primers used (COI: LCO and HCO) were successful in amplification of *M. scalaris* DNA in all the samples extracted using QIAamp® DNA Mini Extraction Kit (Qiagen, Netherlands) (Fig.4.4).

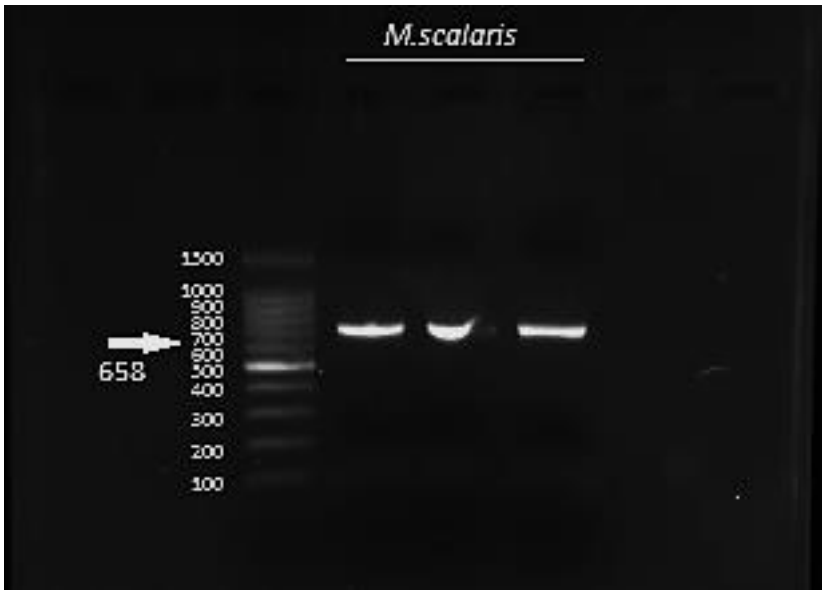


Fig. 4.4 PCR performed using primer COI-I (LCO and HCO) on the DNA extracted from *Megaselia scalaris* larva tissues. White arrow indicates the expected size (in bp) of the amplification bands

To identify mammalian samples, the primer set of MTCB (Cytochrome B Long) was used. This however failed to show any results so a nested PCR was carried out, as described in section 3.6.2(b), by taking DNA amplified with Cytochrome B Long as the template. The 2nd PCR successfully amplified the template DNA using primers Cytochrome B Short (mcb398 and mcb869) (Fig. 4.5, 4.6).

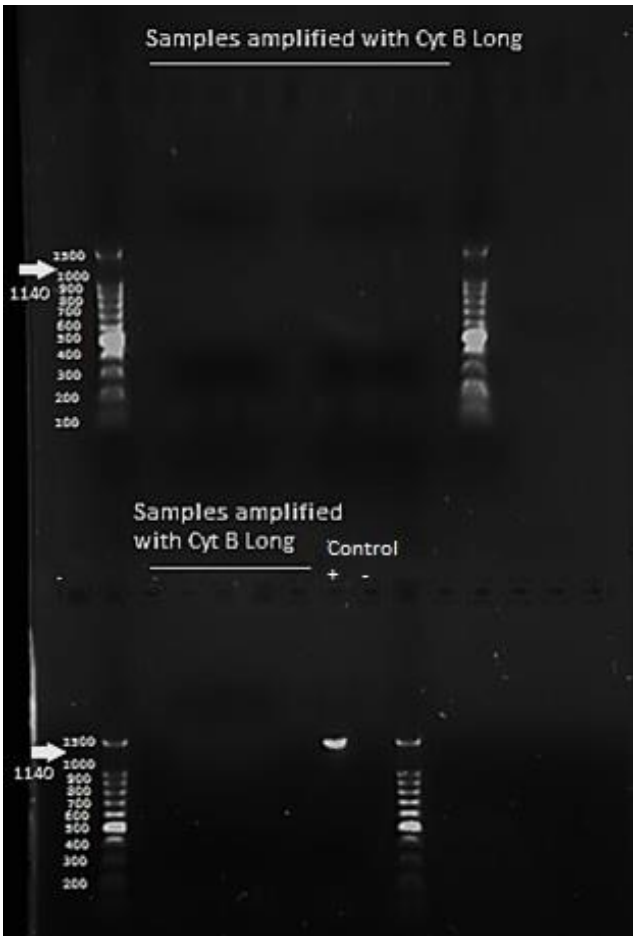
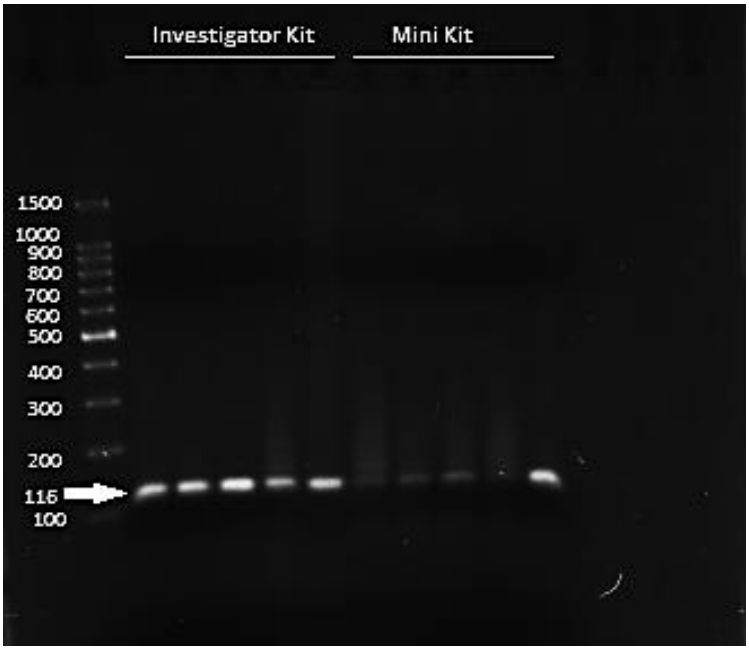


Fig.4.5 Visualisation of 1.5% agarose gel of DNA extracted from *M.scalaris* gut, amplified using primer Cyt B Long (MTCB-F,R). White arrow indicates the expected size (in bp) of the amplification bands.

Fig.4.6 1.5% Agarose gel after nested PCR in which DNA amplified using Cyt B Long was the template and was re-amplified using primer Cyt B Short (mcb398 and mcb869). White arrow indicates the expected size (in bp) of the amplification bands.

After the above discrimination of larval and mammalian DNA, larvae were again dissected and DNA from the gut content was extracted using three kits QIAamp® DNA Mini Extraction Kit (Qiagen, Netherlands), QIAamp® DNA Investigator Kit (Qiagen, Netherlands) and PrepFiler® Forensic DNA extraction Kit (Applied Biosystems, USA). Upon comparison it was found that QIAamp® DNA Investigator Kit gave the best results in terms of DNA yield without the inclusion of reagents that hamper downstream analysis of the sample (Fig. 4.7 a & b).

a)



b)

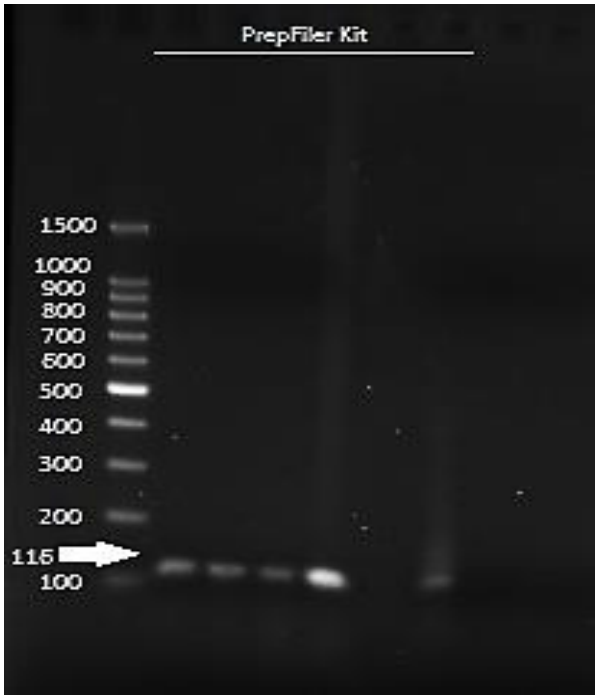


Fig. 4.7 (a)(b) Comparison of PCR products of DNA samples extracted using three different kits - QIAamp® DNA Mini Extraction Kit (Qiagen, Netherlands), QIAamp® DNA Investigator Kit (Qiagen, Netherlands) and PrepFiler® Forensic DNA extraction Kit (Applied Biosystems, USA) for larvae fixed using hot water ($\geq$ 80°C). White arrow indicates the expected size (in bp) of the amplification bands.

Further DNA extractions were carried out using QIAamp® DNA Investigator Kit for larvae fixed with different practices, as maximum DNA yield was obtained from this kit. It can also be seen in Fig.4.6 that upon comparison the brightest amplification bands are seen in samples extracted with the Investigator Kit. A positive result was obtained in all cases upon gel visualisation of the PCR products with both CytB Bos and 16S rRNA primers. However, CytB Bos showed better results among the two (Fig. 4.8 – 4.11). A comparative description of the three DNA extraction kits used and the overall results obtained from them has been depicted in Fig.4.12.

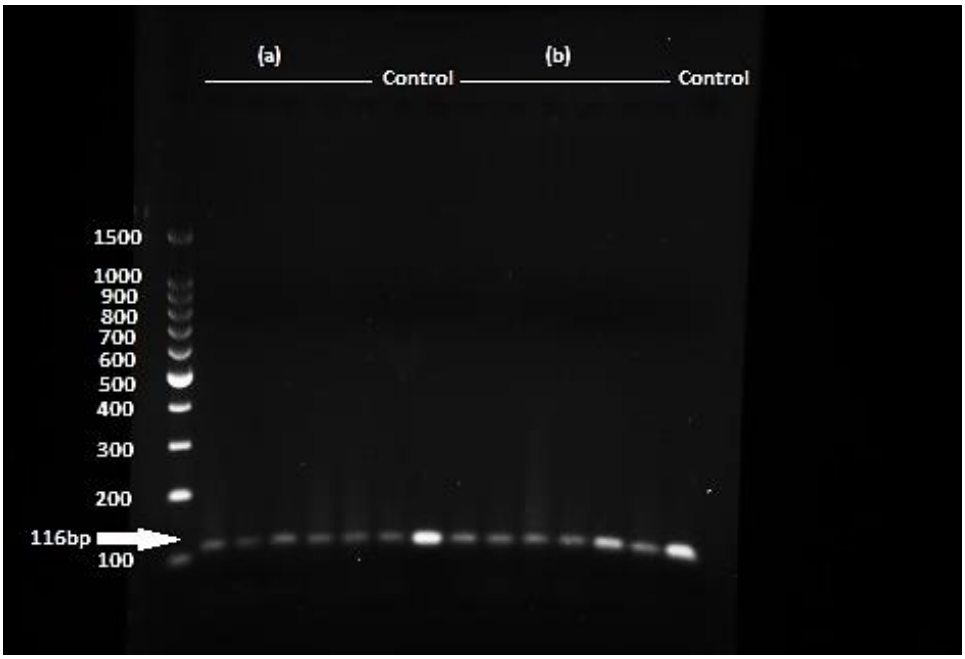


Fig.4.8 1.5% Agarose gel visualisation of DNA extracted from gut of larvae (a) fixed using hot water (≥ 80°C) and then placed in EtOH (b) fixed by freezing larvae, amplified with primer CytB Bos. DNA extracted from Bovine meat used as control

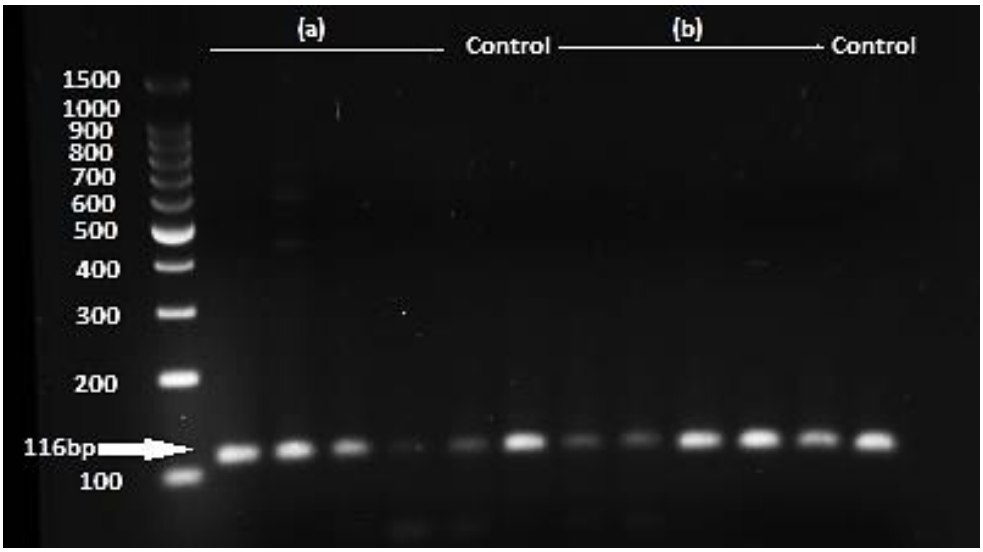


Fig.4.9 1.5% Agarose gel visualisation of PCR products of DNA extracted from gut of larvae (a) fixed by freezing them then placing in EtOH (b) fixed by placing them directly in EtOh, amplified with primer CytB Bos. DNA extracted from Bovine meat was used as control.

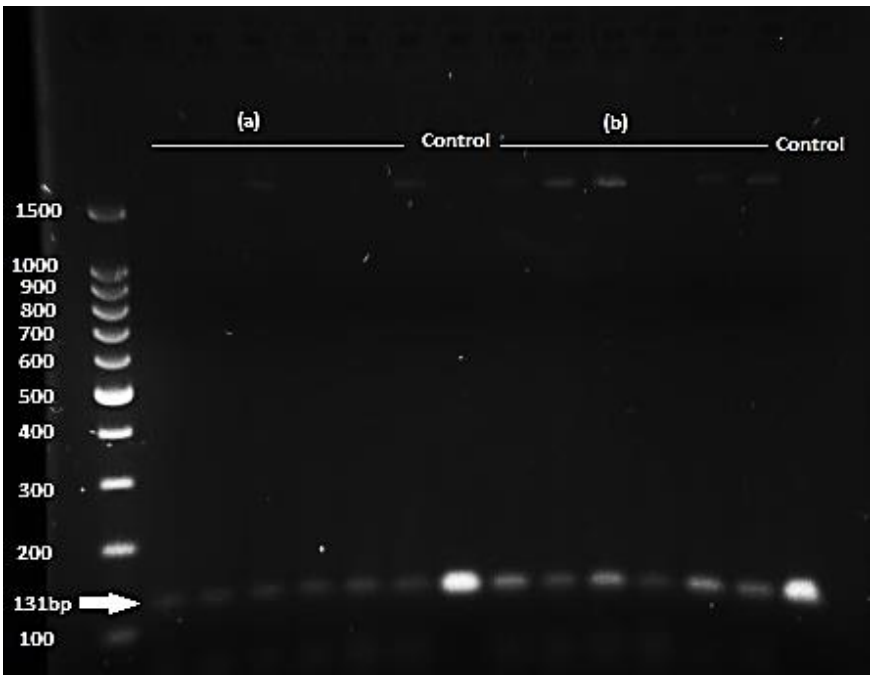


Fig.4.10 1.5% Agarose gel visualisation of PCR products of DNA extracted from gut of larvae (a) fixed using hot water (≥ 80°C) and then placed in EtOH (b) fixed by freezing larvae, amplified with primer 16S rRNA. DNA extracted from Bovine meat used as control

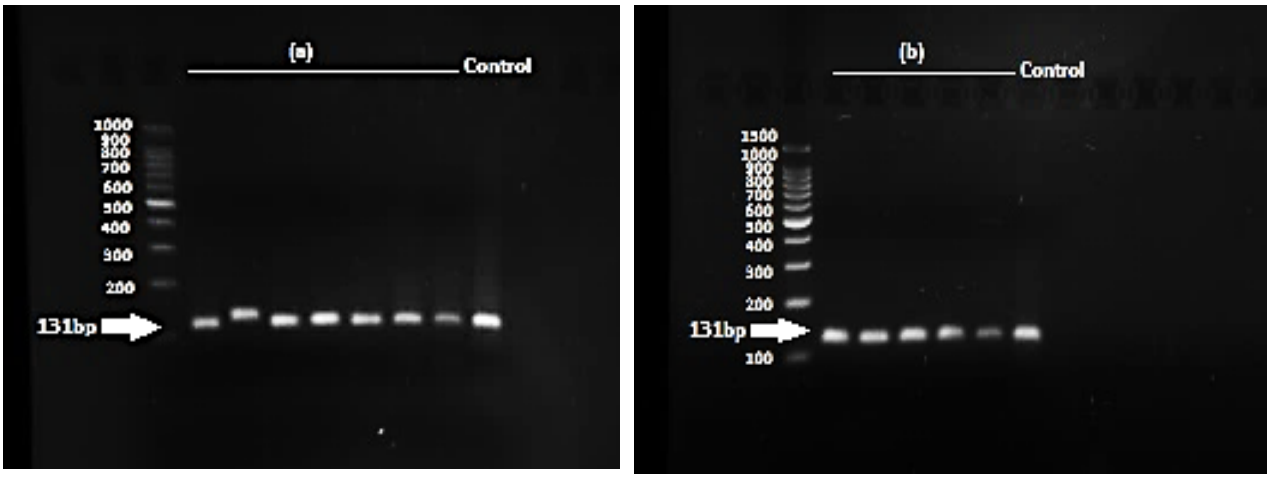


Fig.4.11 1.5% Agarose gel visualisation of PCR products of DNA extracted from gut of larvae (a) fixed by freezing them then placing in EtOH (b) fixed by placing them directly in EtOh, amplified with primer 16S rRNA. DNA extracted from Bovine meat used as control.

QIAamp® DNA Mini Extraction Kit	QIAamp® DNA Investigator Kit	PrepFiler® Forensic DNA Extraction Kit
•***Lysis Time*** - Overnight	•***Lysis Time*** - Overnight	•***Lysis Time*** - 30 min
•***Extraction time excluding lysis*** - 45 min - 1 hr	•***Extraction time excluding lysis*** - 35 min - 40 min	•***Extraction time excluding lysis*** - 40 min - 50 min
•***Average amount of DNA (ng/µl) per specimen*** - 0.14 ± 0.08	•***Average amount of DNA (ng/µL) per specimen*** - 2.6 ± 1.8	•***Average amount of DNA (ng/µL) per specimen*** - 3.6 ± 0.9
•***PCR amplification for Cyt-B Bos*** - Positive with faint amplification bands	•***PCR amplification for Cyt-B Bos*** - Positive with bright amplification bands	•***PCR amplification for Cyt-B Bos*** - Positive with faint amplification bands
•***qPCR Quantification for Cyt-B Bos and 16s rRNA*** - Negative	•***qPCR Quantification for Cyt-B Bos and 16s rRNA*** - Positive	•***qPCR Quantification for Cyt-B Bos and 16s rRNA*** - Negative
•***STR Analysis*** - Not conducted	•***STR Analysis*** - Positive	•***STR Analysis*** - Not conducted

Fig. 4.12 Parameters taken into account to evaluate the extraction kit best fit to carry out further DNA xtractions. The values are reported as mean ± SD.

4.4 Sequencing

All the samples which showed positive results were purified as mentioned in section 3.7 of Materials and Methods, and then were sent for Sanger sequencing to Eurofins Genomics. The obtained sequences were analysed with BlastN to find the highest identity with the sequences already available in Genebank. The results obtained after BlastN showed most of the samples belonging to *Bos taurus* (Table 4.1).

Table 4.1 Results obtained after BlastN analysis of the sequences showed most of the samples were of *Bos Taurus.* Complete sequences for each sample have been attached in Appendix D.

Sample Name	No.of crops	Primer Used	Sequencing result	Max Score	Total Score	Query Cover	E-Value	Indent	Accession Number
1	1	MTCB-F,R	*Bos taurus* (65bp)	121	121	100%	1e-24	100%	MG820631.1
2	5	MTCB-F,R	*Bos taurus* (68bp)	126	126	100%	3e-26	100%	AY521038.1
3	1	Nested PCR (Template – mcb398 ; 2nd PCR - MTCB	*Bos taurus* (123bp)	215	215	100%	6e-53	98%	JX472267.1
4	5	Nested PCR (Template – mcb398 ; 2nd PCR - MTCB	*Bos taurus* (140bp)	220	220	97%	1e-54	96%	JX472267.1
5	5	Nested PCR (Template – mcb398 ; 2nd PCR - MTCB	*Bos indicus* (204bp)	372	372	100%	3e-100	99%	HM209289.1
6	10	Nested PCR (Template – mcb398 ; 2nd PCR - MTCB	*Bos taurus* (139bp)	224	224	97%	5e-55	96%	JX472267.1

7	10	Nested PCR (Template – mcb398 ; 2nd PCR - MTCB	*Bos taurus* (108bp)	172	172	100%	2e-39	97%	JX472267.1
8	1	16S rRNA	*Bos taurus* (77bp)	124	124	92%	2e-25	99%	MG820631.1
8	1	Cyt B Bos	*Bos taurus* (76bp)	135	135	96	1e-28	100%	MG820631.1
9	1	16S rRNA	*Bos taurus* (78bp)	122	122	88%	8e-25	99%	MG820631.1
9	1	Cyt B Bos	*Bos taurus* (75bp)	124	124	89%	2e-25	100%	MG820631.1
10	3	16S rRNA	*Bos taurus* (77bp)	124	124	90%	2e-25	99%	MG820631.1
10	3	Cyt B Bos	*Bos taurus* (76bp)	122	122	92%	8e-25	99%	MG820631.1
11	3	16S rRNA	*Bos taurus* (82bp)	137	137	93%	3e-29	99%	MG820631.1
11	3	Cyt B Bos	*Bos taurus* (80bp)	139	139	93%	8e-30	100%	MG820631.1
12	5	16S rRNA	*Bos taurus* (77bp)	122	122	85%	8e-25	100%	MG820631.1
12	5	Cyt B Bos	*Bos taurus* (78bp)	135	135	93%	1e-28	100%	MG820631.1
13	5	16S rRNA	*Bos taurus* (78bp)	122	122	88%	8e-25	99%	MG820631.1
13	5	Cyt B Bos	*Bos taurus* (75bp)	122	122	92%	8e-25	99%	MG820631.1
14	10	16S rRNA	*Bos taurus* (77bp)	122	122	89%	8e-25	99%	MG820631.1
14	10	Cyt B Bos	*Bos taurus* (74bp)	119	119	86%	1e-23	100%	MG820631.1

4.5 Quantitative PCR

A standard qPCR run using SYBR Green, on DNA obtained from larval gut content as discussed in section 3.2, showed successful amplification of target region (CytB Bos) in all samples (Fig. 4.13). The standard curve plotted (Fig.4.14) determined the quantity of investigated samples. Melt curve analysis showed one peak in most of the samples hence confirming the amplification of only the target region. Table 4.2 summarizes the quantification results and from those, samples suitable for STR analysis were selected (quantity $\geq$ 0.05ng/µl). All the samples showed a Tm (melting temperature) of 77.23 $\pm$ 0.34°C (Fig.4.15) and C_T was minimum for sample with the highest quantity (13.45 for Standard 1) and maximum of sample with the lowest quantity (23.71 for Standard 6).

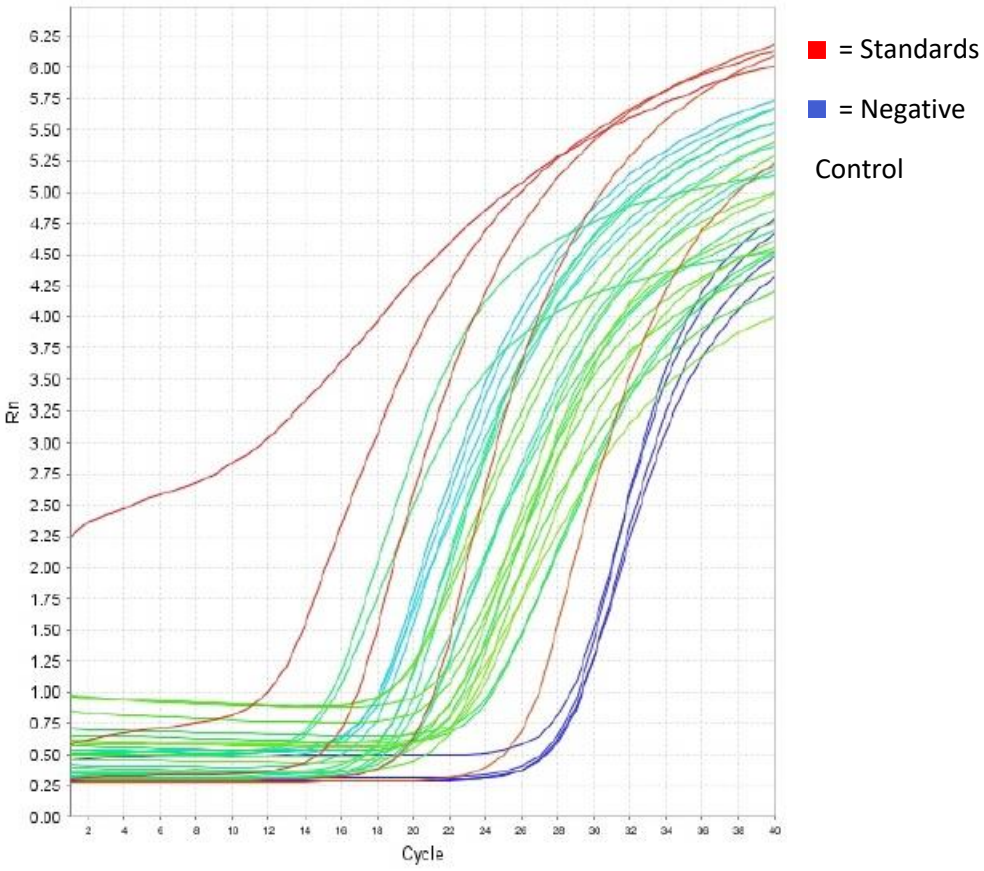


Fig.4.13 Rn vs Cycle amplification plot of all samples. The threshold for the reaction was determined by the 7500 Software to be 0.131459

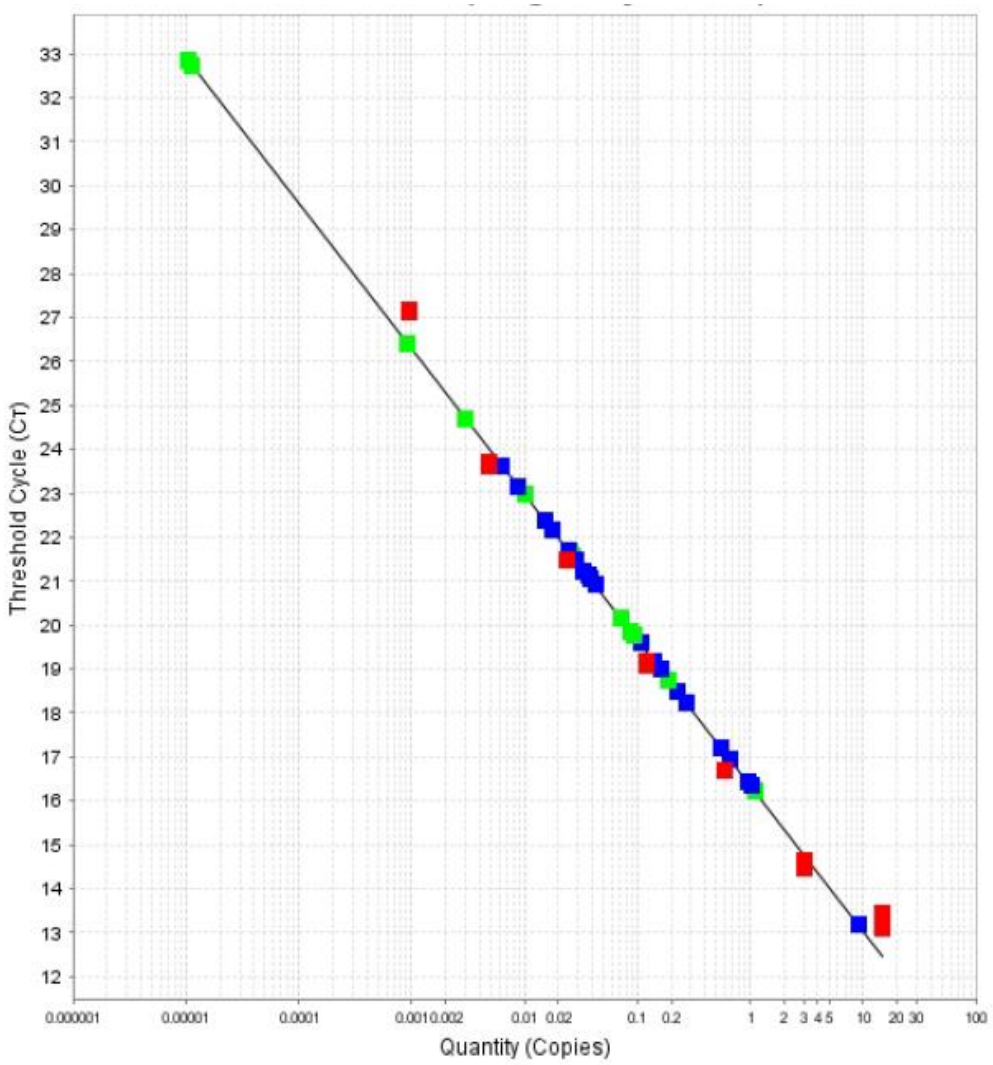


Fig.4.14 Standard curve plotted in accordance to the quantity of standards to estimate the quantity of DNA in unknown samples. ■ = standard ■ = Unknown ■ = Unknown (Flagged). Slope = -3.31 ; Y-Intercept = 16.37 ; R^2 = 0.99

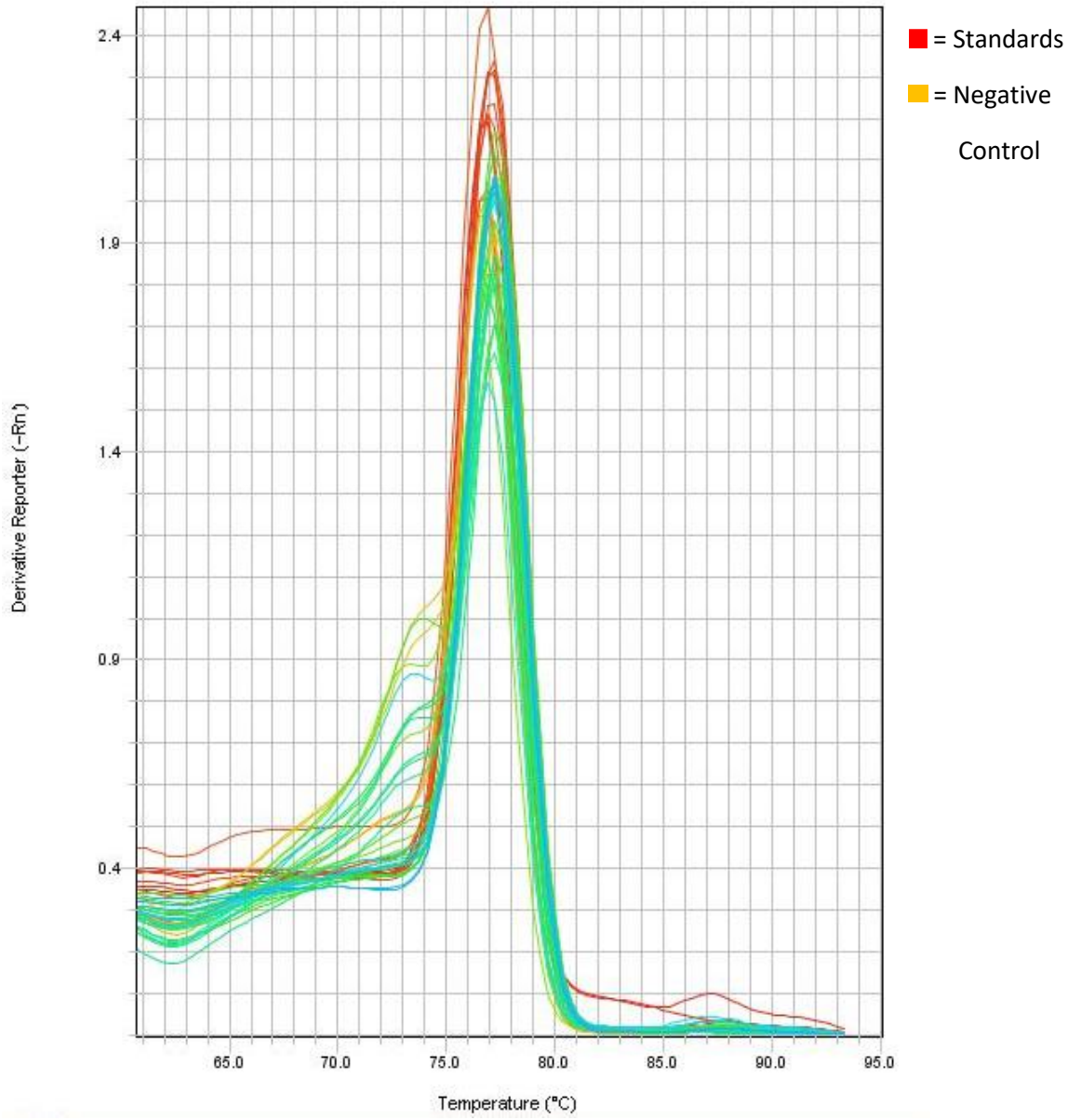


Fig.4.15 Melt curve analysis confirmed the amplification of only the target region as only one peak per sample was observed. Tm (melting temperature) of most of the samples is between 76˚C - 78 ˚C.

Table 4.2. Results table showing quantification of the unknown samples. Taking into account these results, samples **3** (1 larval crop fixed only with EtOH) , **4** (3 larval crops fixed with hot water) ,**5** (1 larval crop fixed with hot water) , **6** (3 larval crops fixed only with EtOH), **7** (5 larval crops fixed only with EtOH) , **10** (5 larval crops fixed first by freezing them placing in EtOH, **12** (3 larval crops fixed by freezing them) , **14**(1 larval crop fixed first with hot water then placing it in EtOH) and **15** (3 larval crops fixed first with hot water then placing it in EtOH) were selected for STR analysis as all of them have a quantity $\geq$ 0.05ng/µl.

SAMPLE	TARGET	TASK	QUANTITY (ng/µl)	C	Tm
S1	Cyt B Bos	Standard	15	13.4551	76.7128
S2	Cyt B Bos	Standard	3	14.62	77.0626
S3	Cyt B Bos	Standard	0.6	16.6793	77.0626
S4	Cyt B Bos	Standard	0.12	19.1622	77.0626
S5	Cyt B Bos	Standard	0.024	21.4997	76.7128
S6	Cyt B Bos	Standard	0.0048	23.7186	76.8877
Water	Cyt B Bos	Negative	-	27.1851	77.2374
1	Cyt B Bos	Unknown	0.0253	21.6536	77.2374
2	Cyt B Bos	Unknown	0.0367	21.1206	77.2374
3	Cyt B Bos	Unknown	0.1431	19.1652	77.2374
4	Cyt B Bos	Unknown	0.0937	19.7738	77.2374
5	Cyt B Bos	Unknown	1.0218	16.4173	77.2374
6	Cyt B Bos	Unknown	0.6664	17.2216	77.0626
7	Cyt B Bos	Unknown	1.1226	20.1734	77.0626
8	Cyt B Bos	Unknown	0.0089	23.1604	77.2374
9	Cyt B Bos	Unknown	0.0268	21.5737	77.2374
10	Cyt B Bos	Unknown	0.1901	19.8584	77.4123
11	Cyt B Bos	Unknown	0.0367	21.0553	77.2374
12	Cyt B Bos	Unknown	0.0426	20.9067	77.2374
13	Cyt B Bos	Unknown	0.01	22.9823	76.8877
14	Cyt B Bos	Unknown	0.1071	19.5886	77.2374
15	Cyt B Bos	Unknown	0.2769	18.4893	77.2374

4.6 Genetic Profiling

Of all the above samples that underwent capillary electrophoresis, a full profile (12 loci) was obtained from samples 3, 6 and 7 (showing 100% efficiency for the method wherein EtOH was used to fix larvae) and a partial profile from samples 4 and 14 (showing 25% efficiency each, for methods in which hot water only and hot water along with EtOH was used to fix larvae). This also suggests an overall success rate of 56% of the analysis.

The genotyping results of all the samples have been summarised in Table 4.3 and Fig. 4.16 displays the electropherogram of the reference standard Bovine meat in comparison to one of the samples. Electropherograms of the complete STR profiles along with the positive control and negative have been attached in the Appendix E.

Table 4.3. Summary of genotyping results of the positive samples in comparison to the genotyping of DNA extracted from Bovine meat that was fed to the larvae while breeding (standard). Samples 3, 6 and 7 show exact matches which proves the DNA extracted from the gut content was similar to the one fed to the larvae.

Sample	**Standard**	**3**	**6**	**7**
Locus	**Allele Calling at 50 RFU**			
TGLA227	79, 97	79, 97	79, 97	79, 97
BM2113	135, 139	135, 139	135, 139	135, 139
TGLA53	158, 166	158, 166	158, 166	158, 166
ETH10	219, 221	221	219, 221	221
SPS115	252, 258	252, 258	252, 258	252, 258
TGLA126	123	123	123	123
TGLA122	151	151	151	151
INRA23	200, 216	200, 216	200, 216	200, 216
BM1818	270	270	270	270
ETH3	117, 127	117, 127	117, 127	117, 127
ETH225	140, 150	140, 150	140, 150	140, 150
BM1824	178, 180	178, 180	178, 180	178, 180

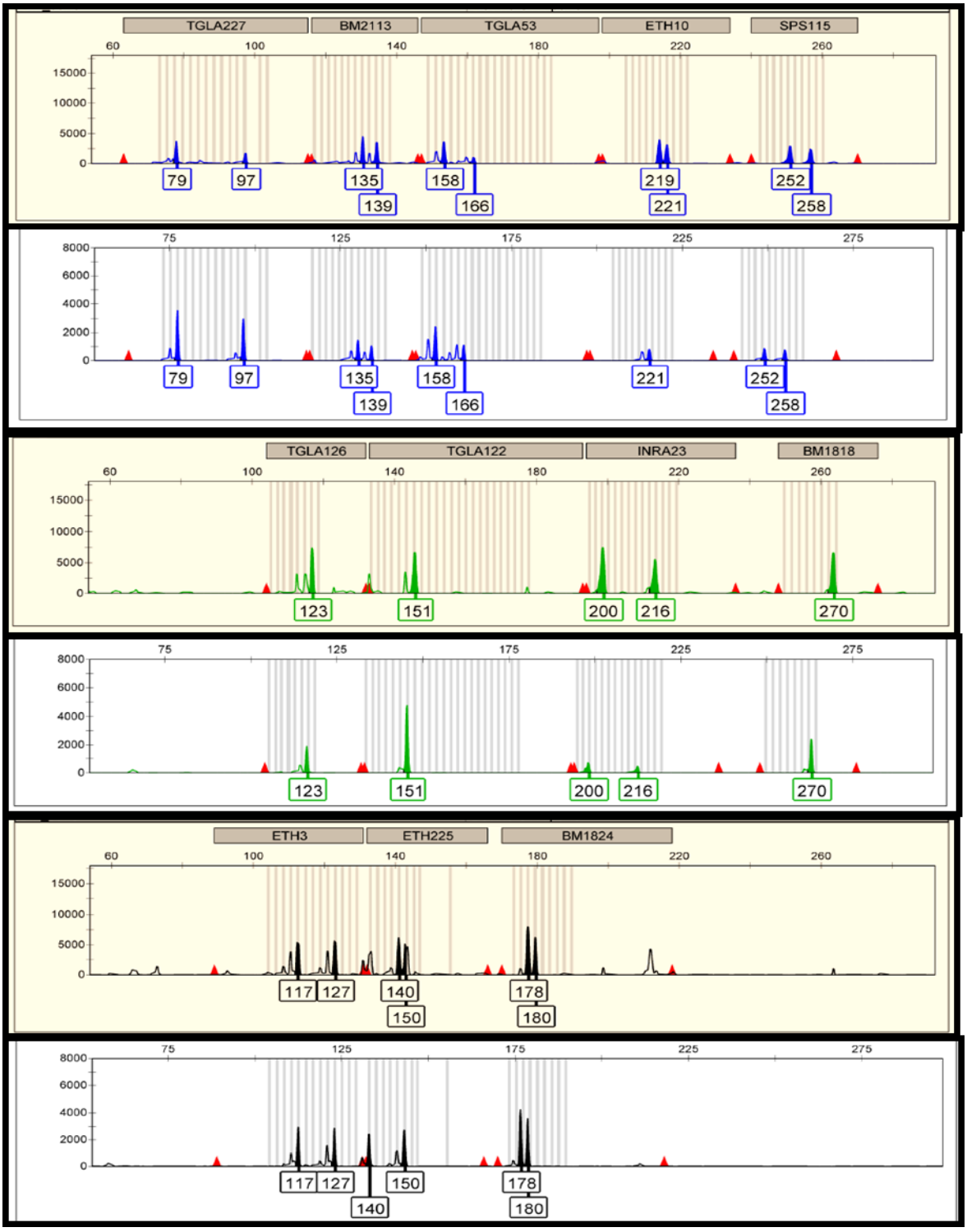


Fig. 4.16 Electropherogram depicting a comparison of complete STR profile (12 loci), of Bovine meat which was fed to the *M. scalaris* flies while breeding and was used as standard for comparing the STR profiles of the samples (yellow background) and DNA sample obtained from the gut content of 1 larva (white background).

5. Discussion:

This study shows that even though the larvae of *Megaselia scalaris* are very small in size as compared to blow-flies or flesh-flies (Fig.1.19), successful molecular analysis of larval gut contents is possible in order to characterize trace amount of DNA of the larval food source. This is predominantly useful in circumstances where a body found is too decomposed to perform conventional methods of DNA identification of the deceased. It can also be used in instances where a body has been moved from the primary crime scene and only maggots are found in that location (Vanin, 2016; Wells & Stevens, 2008). *Megaselia scalaris* is an important fly of forensic interest and is among the first colonisers in indoor crime scenes as demonstrated by Bugelli et al. (2015) and Reibe and Madea (2010) and is also the dominant species in cases involving buried corpses as shown by Pastula and Merritt (2013). Although previous studies have been conducted on molecular analysis of maggot gut content (de Lourdes Chavez-Briones et al., 2013; Di Luise et al., 2008; Li et al., 2011; Linville et al., 2004; Njau et al., 2016; Wells et al., 2001), none of them have been focused on this particularly important species.

This study began with first testing if it was possible to extract non-insect DNA from the gut content of 3rd instar larva of *Megaselia scalaris*. Larvae, fed on bovine meat, were selected for dissection after they attained their fully fed stage and arrived at the wandering phase of the life cycle. As proposed by Amendt et al. (2007), they were fixed by pouring hot water (≥80˚C) for 30s and were dissected as described by Tuccia et al. (2016). DNA extraction was carried out using the QIAamp® DNA Mini Extraction Kit and PCR amplification was divided into two steps. Firstly, insect universal primer COI-I (LCO and HCO) was used to amplify the *M. scalaris* DNA. Successful amplification was obtained from the DNA extracted from larval tissues, which did not have any trace of non-insect DNA, and thus this DNA sample was used as a negative control in the consecutive PCR process to distinguish mammalian DNA in the insect gut content. Primer Cyt B Long (MTCB-F,R) was used for this process. Gel electrophoresis, after the PCR, revealed that the primer was unsuccessful in amplifying mammalian DNA and this was true for all DNA samples obtained from 1, 3, 5 and 10 crops. Thus to increase specificity, a nested PCR was performed with the primer Cyt B short (mcb398, mcb869) which resulted in positive amplification of the mammalian DNA. No amplification was obtained in the *M. scalaris* DNA sample which was used as a negative control.

This therefore confirmed that it was possible to extract DNA from the gut content of *M. scalaris* larvae, regardless of their small size.

The next step involved selecting a DNA extraction kit which gave the maximum yield of DNA. The QIAamp® DNA Investigator Kit, PrepFiler® Forensic DNA Extraction Kit and QIAamp® DNA Mini Extraction Kit were selected as these are the most preferred extraction kits in majority of forensic laboratories. Larvae were again fixed by pouring hot water (≥80˚C) for 30s and were dissected as described by Tuccia et al. (2016). Out of the three DNA extraction kits used, the QIAamp® DNA Investigator Kit was found to provide the maximum amount of DNA and the reagents used in this process also did not cause any inhibitions in the subsequent analysis processes. This was concluded after observing the results of the ANOVA test for the amount of DNA extracted per specimen using several methods (Fig.4.2). Gel electrophoresis of the PCR products (amplification of mitochondrial target region of 116bp by Cyt B Bos primer) obtained after extraction using each kit also showed the brightest amplification bands in samples extracted using QIAamp® DNA Investigator Kit (Fig.4.6). Effectiveness of the QIAamp® DNA Investigator Kit was also demonstrated in the 2009 Victorian bushfire identification effort, where DNA was successfully extracted from bone, tissue, buccal swabs and blood stains, for disaster victim identification (Hartman et al., 2011). It has also been used for successfully extracting DNA from toe nails of decomposed human bodies as shown by Schlenker et al. (2016). There have been studies conducted, such as by Sturk-Andreaggi et al. (2011) and Barbaro et al. (2009), which report that the PrepFiler® Forensic DNA Extraction Kit provides better results than silica-based methods of DNA extraction, in terms of DNA yield and inhibitor removal. In contradiction to these, in this study, although PrepFiler® Forensic DNA Extraction Kit gave good quantification results, it however failed to provide positive results in qPCR and was thus not used. It showed the presence of multiple Tm peaks in the melt curve analysis, thus effectively suggesting amplification of multiple regions simultaneously, therefore indicating the presence of a fragmented DNA sample. QIAamp® DNA Mini Extraction Kit did not provide high amount of DNA, as is observed after ANOVA tests and gel electrophoresis of PCR products, and was thus not employed for further DNA extractions (Fig. 4.2, Fig. 4.6). Figure 4.12 summarizes the parameters that were taken into account to assess overall performance of all the three kits.

So as to confirm if the DNA extracted was of *Bos taurus,* the PCR products were purified and sent for Sanger sequencing to Eurofins Genomics. The sequences received back were run in BlastN and upon comparison with the sequences in the GeneBank database, it was found that most of the samples of DNA belonged to *Bos taurus* (Table 4.4).

Following the selection of best suited extraction kit and confirming that it was possible to extract DNA from larval gut content of *M. scalaris*, 5 methods of larval fixation were adopted and DNA from the gut contents, using QIAamp® DNA Investigator Kit, was extracted. Method 1 consisted of pouring hot water ($\geq$ 80˚C) for 30s on the larvae, method 2 involved only freezing the larvae (-20˚C), method 3 involved freezing (-20˚C) the larvae first and then placing them in EtOH, method 4 comprised of placing the larvae directly in EtOH (100%) and method 5 consisted of pouring hot water ($\geq$ 80˚C) for 30s on the larvae and then placing them in EtOH (100%). This was done in order to assess the method which resulted in easier dissection, more weight of the crops and better overall molecular analysis results. Weight of 10 larval crops, obtained post dissection, from all the above methods was recorded and ANOVA tests followed by Post-Hoc revealed crops obtained after fixing larvae with only EtOH, had the maximum weight among all (Fig. 4.1 a & b). This method also proved to be helpful in carrying out dissection of larvae along with the method comprising of freezing the larvae (-20˚C) first and then placing them in EtOH, as minimum liquefaction of organs was observed. This is because EtOH has a dehydrating effect on the maggot. This also results in increasing fragility of the alimentary canal of larva as reported by Linville et al. (2004) hence care was taken while dissecting. Storage in EtOH, provides the best preservation technique for molecular analysis of larva, it is however not recommended if morphological analysis is to be carried out, as the EtOH leads to morphological disruptions like shrinking, hardening and distortion (Carter, 2003). ANOVA test results followed by Post-Hoc analysis (Tukey HSD) of the method of fixation on the crop weight showed that temperature (Hot water or frozen) had an effect on the overall dry weight of the crops ($p<0.05$), while presence or absence of EtOH did not ($p=0.165$). Also, the interaction between both the parameters showed a statistically significant difference on the results ($p<0.05$).

DNA was then extracted from 1, 3 and 5 crops respectively, using each fixation method with the help of QIAamp® DNA Investigator Kit. The quantification results obtained suggested that

maximum quantity of DNA was obtained from the gut content of larvae fixed with only EtOH, frozen and then placed in EtOH and only frozen. This was comparable to the amount of DNA obtained from the PrepFiler® Forensic DNA Extraction Kit, from larvae fixed by only pouring hot water for 30s, as observed in Fig.4.3. ANOVA test results confirmed that that there was statistically significant difference in the quantification results, between all methods adopted for fixing the larvae and extracting DNA ($p<0.05$) (Table. 4.3). This was followed by a Post-Hoc analysis (Tukey HSD) which suggested that the presence or absence of EtOH as an individual variable does not affect the DNA quantifications ($p= 0.133$, i.e., $p>0.05$), while temperature (hot water or frozen) ($p= 0.000$, i.e., $p<0.05$) and number of crops ($p= 0.01$, i.e., $p<0.05$) does. The interaction among the variables i.e., temperature, EtOH and number of crops, also does not affect the results ($p>0.05$) (Table 4.4).

The next step, after assessing the quantifications of DNA extracted from all methods, involved PCR amplification of the target region of 116bp (Cyt B Bos) and 131bp (16S rRNA Bos) specific to *Bos taurus*. These primers were selected as the mammalian specific primer Cyt B Long (MTCB-F,R) did not provide results before the nested PCR and hence obtaining results required more effort and was time consuming. Bovine specific primers on the other hand are more reliable in terms of result and also have further application in qPCR quantification as demonstrated by Drummond et al. (2013) and Zhang et al. (2007). Gel visualisation after electrophoresis showed positive amplification of the target region in all methods, however Cyt B Bos showed better results and brighter amplification bands as compared to 16S rRNA (Fig.4.6-4.9). All samples showing positive results were purified and sent for Sanger sequencing to Eurofins Genomics to confirm the source of DNA obtained, as discussed earlier. Table 4.4 shows the results obtained after BlastN, which suggests most of the samples belonged to *Bos taurus*. This established the fact that DNA was extracted from the larval gut content and the source was the larva's last meal (Bovine meat).

Following this qPCR was performed to assess the accurate amount of *Bos taurus* DNA present in the samples and if it was adequate to perform an STR analysis. As mentioned in section 4.2. the Invitrogen™Qubit® 3.0 does not give species specific quantifications, hence it was not pragmatic to proceed for STR analysis on the basis of the quantifications obtained from it. For qPCR, a bovine

specific prime (Cyt B Bos) was used, hence the quantification of DNA obtained by this method is more reliable. Table 4.5 describes the results attained after successful qPCR run and the quantifications of *Bos taurus* DNA present in each sample. As the minimum quantity of DNA required for a successful STR analysis is 0.05ng/µl, samples 3 (1 larval crop fixed only with EtOH) ,4 (3 larval crops fixed with hot water) ,5 (1 larval crop fixed with hot water) ,6 (3 larval crops fixed only with EtOH),7 (5 larval crops fixed only with EtOH) ,10 (5 larval crops fixed first by freezing them placing in EtOH,12 (3 larval crops fixed by freezing them) ,14(1 larval crop fixed first with hot water then placing it in EtOH) and 15 (3 larval crops fixed first with hot water then placing it in EtOH, were selected.

The Bovine Genotypes Panel 1.2 was used to successfully perform an STR analysis on the samples, with full STR profiles obtained from samples 3, 6 and 7 (12loci) and a partial profile from samples 4 and 14. The allele calling has been described in Table 4.6. The profiles of samples obtained were compared to one obtained from the DNA extracted from Bovine meat, which was fed to the flies while rearing. Both the profiles showed exact matches and thus established the fact that DNA was successfully extracted from *M. scalaris* larva gut content in accordance to studies by de Lourdes Chavez-Briones et al. (2013), Li et al. (2011), Linville et al. (2004), Wells et al. (2001) and several others, irrespective of the small size of *M.scalaris.* It was also established that fixing the larvae with only EtOH was the best method to obtain maximum weight of the crops, which resulted in more *Bos taurus* DNA as observed after quantifications with Invitrogen™Qubit® 3.0 (Life technologies, USA) and qPCR, and thus resulted in complete STR profiles for 5, 3 and 1 crops.

Amendt et al. (2007) suggest immersing larvae for 30s in hot water and then placing them in EtOH for preservation or replacing the hot water with placing the larvae in freezer, as ideal methods for larval collection. This study suggests that placing *M.scalaris* larvae directly in EtOH is more practical and achievable for crime scene officers, for when they find larvae they could immediately collect them and preserve them in EtOH and this could make the process quicker and also suitable for positive results in molecular analysis.

A future research can be employed on determining the time-period till which DNA can successfully by extracted from larvae preserved and fixed only in EtOH. It can also be researched

if methods of pouring hot water for 30s and then placing the larvae in EtOH can provide complete STR profiles as they have provided partial STR profiles in this study.

6. Conclusion

Although previous studies have been conducted on extraction and STR analysis of genomic DNA from larval gut content, all of them were focused on insect species having larvae big in size (> 1cm) and concerned with early colonisers in outdoor crime scenes (Calliphoridae and Sarcophagidae). The result of this study proves that successful DNA extraction and STR analysis is possible from gut contents of *M. scalaris* larva with a successful result with a minimum of 1 crop. This result is also important because *M. scalaris* is one of the early colonisers in indoor crime scenes and also the dominant species in cases involving buried corpses. Apart from this *M. scalaris* is also found colonising corpses during later stages of decomposition in outdoor crime scenes. From a procedural point of view this study also ascertained that placing larvae directly in EtOH not only proves to be a good preservation technique but also provides ease in dissection and also the best results in molecular analysis when compared to other preservation techniques. This method can also be useful and achievable for crime scene officers for quicker and efficient collection of samples. Although *Bos taurus* was used as a model in this study, this research can be exercised in forensic cases wherein the corpse is too decomposed to perform conventional methods of human identification or when a number of larvae are present at the primary scene of crime and it is suspected that a corpse has been moved.

This research also opens up new horizons in the field of entomology, by showing that successful molecular analysis of larval gut contents is possible irrespective of the size of larvae and new studies can be conducted on other species of forensic interest which may be smaller in size when compared to Calliphoridae or Sarcophagidae. Further research with respect to *M. scalaris* can also be conducted on the time-period till which successful molecular analysis from the gut is possible with EtOH as method of fixation or other methods of fixation can be explored to perform effective molecular analysis of larval gut content.

Appendix A:

1. QIAamp® DNA Mini Extraction Kit (Qiagen, 2016)

- The larval crops, post dissection, were placed in 1.5 ml microcentrifuge tubes containing 180µl of tissue lysis buffer (Buffer ATL) as described in section 4.2.
- A sterilised plastic pestle was used to carefully crush and homogenise the tissues.
- 20µl of Proteinase K (Promega©, Madison, Wisconsin, USA) was added to the tubes which were then vortexed for 15s to mix all the contents.
- The samples were then subjected to overnight incubation at 56°C in order to increase the yield of DNA. For this Thermo shake lite™ was used. The temperature was adjusted to 56°C and the rocking platform speed was set as 300rpm.
- After overnight incubation the samples were briefly centrifuged in order to remove any droplets that may be present on the lid.
- 4µl RNase A (100 mg/ml) (Promega©, Madison, Wisconsin, USA) was added to obtain a RNA-free DNA sample. The samples were then vortexed for 15s and incubated at room temperature (20°C-25°C) for 2 minutes.
- 200µl of Buffer AL was added to the samples after briefly centrifuging them.
- All the contents were then mixed by vortexing the tubes for 15s. The samples were allowed to incubate at 70°C for 10 minutes using the Thermo shake lite™.
- Following incubation, 200µl of ethanol (98%) was added to the samples and was mixed by vortexing.
- All the lysate was then carefully transferred to QIAamp Mini spin columns and samples were centrifuged at 8000 rpm for 1 minute.
- The filtrate, along with the collection tube, was discarded and the QIAamp Mini spin columns containing DNA sample were placed in new clean 2ml collection tubes.

- To these 500µl of wash buffer AW1 added, and the samples were again centrifuged at 8000 rpm for 1 minute.
- Similar to the above steps, the filtrate along with the collection tube was discarded and the QIAamp Mini spin columns were placed in clean 2ml collection tubes.
- 500µl of wash buffer AW2 was added to each QIAamp Mini spin column and they were centrifuged at 14,000 rpm (full speed) for 3 minutes.
- Again, the collection tube containing the filtrate was discarded and the QIAamp Mini spin columns were placed in clean 1.5 ml microcentrifuge tubes.
- 200µl of Buffer ATE was added to the samples in order to elute DNA.
- The samples were then incubated for 1-3 min at room temperature and then centrifuged at 8000 rpm for 1 minute.
- The QIAamp Mini spin columns were discarded and the eluted DNA sample was then stored at -20°C for further use in analysis.

2. QIAamp® DNA Investigator Kit (Qiagen, 2012)

- As soon as the larval crops were dissected, they were placed in 1.5 ml microcentrifuge tubes containing 180µl of tissue lysis buffer (Buffer ATL) as described in section 4.2.
- 20 µl of Proteinase K (Qiagen®) was added to each tube and was mixed by vortexing them for 15 seconds.
- The microcentrifuge tubes were then kept for overnight incubation at 56°C in the Thermo shake lite™ set at a rocking platform speed of 300rpm.
- The next day 200µl of Buffer AL was added to each sample tube and along with this Carrier RNA was added.
- These were mixed by pulse-vortexing for 15 seconds.

- After adding 200µl of ethanol (98%) to the samples and vortexing them, the samples were set aside for incubation at room temperature for 5 minutes.
- The 1.5ml tubes were briefly centrifuged to remove any droplets that may be present inside of the lid.
- The entire lysate was then carefully transferred to the QIAamp MinElute columns which were placed in 2ml collection tubes.
- These were centrifuged at 8000 rpm for 1 minute.
- The collection tube having the flow-through was discarded and the QIAamp MinElute columns were placed in clean 2ml collection tubes.
- To this 500µl of wash buffer AW1 was added and the columns were seubjected to centrifugation at 8000 rpm for 1 minute.
- Collection tube containing the flow-through was discarded and the QIAamp MinElute columns were again placed in clean 2ml collection tubes.
- 700µl if wash buffer AW2 was carefully added to each QIAamp MinElute column and they were centrifuged at 8000 rpm for 1 minute.
- After this the QIAamp MinElute columns were placed in new collection tubes and the collection tubes containing the flow-through were discarded.
- Carefully 700µl of ethanol (98%) was put in the QIAamp MinElute columns and they were centrifuged at 8000 rpm for 1 minute.
- The QIAamp MinElute columns were then placed in clean collection tubes and the ones having the flow-through were discarded.
- Each of the QIAamp MinElute column was then centrifuged at full speed (14,000 rpm) for 3 minutes in order to completely dry the membrane and remove any ethanol residues that may be present.

- New sterilised 1.5ml microcentrifuge tubes were taken and the QIAamp MinElute columns were placed in them after discarding the collection tubes containing the flow-through.
- Carefully the lid of each QIAamp MinElute column was opened and they were set aside to incubate at room temperature for 10 minutes.
- After this, very carefully, Buffer ATE was added exactly at the centre of the membrane for complete elution of DNA.
- The lid of the QIAamp MinElute columns was closed and they were incubated at room temperature for 5 minutes.
- They were then centrifuged at full speed (14,000 rpm) for 1 minute.
- The eluted DNA was then stored at -20°C for future analysis.

3. PrepFiler® Forensic DNA Extraction Kit (Applied Biosystems™, 2008)

- A 1.5ml microcentrifuge tube was taken and to it 250 µL PrepFiler™ Lysis Buffer and 3µL of 1 M DTT was added.
- The larval crops after dissection were placed in these tubes and were vortexed for 5 seconds.
- Each of the micro-centrifuge tubes were then allowed to incubate at 70°C for 30 minutes on the Thermo shake lite™ set at a rocking platform speed of 900rpm.
- Following this the sample tubes were taken and set aside at room temperature for 5 minutes in order to have evenness with the surrounding temperature.
- 15µl of magnetic particles were added to each sample tube and these were centrifuged briefly.
- After this 180µl of isopropanol (100%) was added to it and vortexed for 5 seconds.
- The sample lysate tubes were placed in the Thermo shake lite™ set at room temperature and 1000 rpm rocking platform speed for 10 minutes in order to mix all the contents.

- The tubes were then vortexed to resuspend the magnetic particles and were then placed on a magnetic stand for 2 minutes so that the magnetic particles can form a pellet at the back of the tube.
- Very carefully a micropipette was used to discard all the visible liquid medium.
- 300µL of PrepFiler™ wash buffer was added to the tubes and they were then vortexed for 5 seconds.
- The above two steps were repeated 3 times until there was no visible magnetic particle pellet present at the side of the tube.
- The magnetic particles-bound DNA was allowed to air dry at room temperature for 10 minutes.
- 50 µL of PrepFiler™ Elution Buffer was added to each sample tube and they were then placed in the Thermo shake lite™ set at 70°C and 900 rpm for 5 minutes.
- Following incubation, the tubes were vortexed briefly and were placed in the magnetic stand for 2 minutes.
- After a magnetic pellet was formed at the side of the tube, all the liquid phase was pipetted and transferred to a fresh 1.5ml microcentrifuge tube.
- The liquid phase in these new tubes contains the extracted genomic DNA.
- These were then stored at -20°C for future analysis.

Appendix B:

Table.1 Post-Hoc analysis (Tukey HSD) of dry weight showing the significant statistical difference on weight of each method of fixation with others. (1= Hot water (≥90˚C) , 2= Frozen Only, 3= Frozen + EtOH, 4= Hot water + EtOH and 5 = EtOH only)

Method	(J) Method	Mean Difference (I-J)	Std. Error	Sig. (p- value)
1.00	2.00	.00053333*	.00004714	.000
	3.00	.00003333	.00004714	.950
	4.00	.00040000*	.00004714	.000
	5.00	-.00040000*	.00004714	.000
2.00	1.00	-.00053333*	.00004714	.000
	3.00	-.00050000*	.00004714	.000
	4.00	-.00013333	.00004714	.102
	5.00	-.00093333*	.00004714	.000
3.00	1.00	-.00003333	.00004714	.950
	2.00	.00050000*	.00004714	.000
	4.00	.00036667*	.00004714	.000
	5.00	-.00043333*	.00004714	.000
4.00	1.00	-.00040000*	.00004714	.000
	2.00	.00013333	.00004714	.102
	3.00	-.00036667*	.00004714	.000
	5.00	-.00080000*	.00004714	.000
5.00	1.00	.00040000*	.00004714	.000
	2.00	.00093333*	.00004714	.000
	3.00	.00043333*	.00004714	.000
	4.00	.00080000*	.00004714	.000

* The mean difference is significant at the 0.05 level.

Table.2 Post-Hoc analysis (Tukey HSD) showing significant statistical difference between the Kits used for extraction on DNA per specimen. (Kit 1 = PrepFiler® Forensic DNA Extraction Kit, Kit 2 = QIAamp® DNA Investigator Kit, Kit 3 = QIAamp® DNA Mini Extraction Kit)

(I) Kit	Mean Difference (I-J)		Std. Error	Sig. (p-value)
1.00	2.00	1.12905*	0.19650	0.000
	3.00	3.09332*	0.24402	0.000
2.00	1.00	-1.12905*	0.19650	0.000
	3.00	1.96427*	0.17006	0.000
3.00	1.00	-3.09332*	0.24402	0.000
	2.00	-1.96427*	0.17006	0.000

* The mean difference is significant at the 0.05 level.

Table.3 Post-Hoc analysis (Tukey HSD) showing significant statistical difference between the Kits used for extraction on DNA per specimen. (method 1 – PrepFiler Kit; method 2 – Hot water for 30s (Investigator Kit); method 3 – Only Frozen (Investigator Kit); method 4 – Mini Kit; method 5- Frozen + Ethanol (Investigator Kit); method 6 – Only ethanol (Investigator Kit); method 7 - Hot Water (≥90˚C) + Ethanol (Investigator Kit)

(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Sig. (p-value)
1.00	2.00	1.69293*	0.15093	0.000
	3.00	.78109*	0.15588	0.000
	4.00	3.09332*	0.16162	0.000
	5.00	.76264*	0.15588	0.000
	6.00	0.23027	0.15588	0.758
	7.00	2.06553*	0.15588	0.000
2.00	1.00	-1.69293*	0.15093	0.000
	3.00	-.91184*	0.12925	0.000
	4.00	1.40038*	0.13611	0.000
	5.00	-.93030*	0.12925	0.000
	6.00	-1.46266*	0.12925	0.000
	7.00	0.37259	0.12925	0.065
3.00	1.00	-.78109*	0.15588	0.000
	2.00	.91184*	0.12925	0.000
	4.00	2.31223*	0.14158	0.000
	5.00	-0.01845	0.13499	1.000
	6.00	-.55081*	0.13499	0.001
	7.00	1.28444*	0.13499	0.000
4.00	1.00	-3.09332*	0.16162	0.000
	2.00	-1.40038*	0.13611	0.000
	3.00	-2.31223*	0.14158	0.000
	5.00	-2.33068*	0.14158	0.000
	6.00	-2.86304*	0.14158	0.000
	7.00	-1.02779*	0.14158	0.000
5.00	1.00	-.76264*	0.15588	0.000
	2.00	.93030*	0.12925	0.000
	3.00	0.01845	0.13499	1.000
	4.00	2.33068*	0.14158	0.000
	6.00	-.53236*	0.13499	0.002
	7.00	1.30289*	0.13499	0.000
6.00	1.00	-0.23027	0.15588	0.758
	2.00	1.46266*	0.12925	0.000
	3.00	.55081*	0.13499	0.001
	4.00	2.86304*	0.14158	0.000
	5.00	.53236*	0.13499	0.002
	7.00	1.83525*	0.13499	0.000
7.00	1.00	-2.06553*	0.15588	0.000
	2.00	-0.37259	0.12925	0.065

3.00	-1.28444*	0.13499	0.000
4.00	1.02779*	0.14158	0.000
5.00	-1.30289*	0.13499	0.000
6.00	-1.83525*	0.13499	0.000

* The mean difference is significant at the 0.05 level.

Appendix C:

Table 1. Quantification results of samples extracted with QIAamp® DNA Mini Extraction Kit (Qiagen, Netherlands)

SAMPLE	AVERAGE OF TOTAL AMOUNT OF DNA	SD OF TOTAL AMOUNT OF DNA	AVERAGE OF AMOUNT OF DNA PER SAMPLE	SD OF AMOUNT OF DNA PER SAMPLE
1.1	Too low			0
1.2	0.21	0.01	0.21	0.04
1.3	0.19	0.04	0.19	0.01
1.4	0.15	0.01	0.15	0.01
1.5	0.21	0.01	0.21	0.01
1.6	0.18	0.01	0.18	0.01
1.7	0.19	0.01	0.19	0.01
1.8	Too low			0.00
1.9	0.22	0.01	0.22	0.01
5.1	0.22	0.04	0.04	0.01
5.2	1.46	0.01	0.29	0.00
5.3	0.44	0.06	0.09	0.01
5.4	0.32	0.02	0.06	0.00
5.5	0.71	0.03	0.14	0.01
5.6	0.40	0.01	0.08	0.00
5.7	0.47	0.01	0.09	0.00
5.8	1.09	0.13	0.22	0.03
5.9	0.55	0.05	0.11	0.01
5.10	0.72	0.02	0.14	0.00

5.11	0.48	0.01	0.10	0.00
5.12	0.47	0.04	0.09	0.01
10.1	0.15	0.01	0.02	0.00
10.2	0.68	0.01	0.07	0.00
10.3	1.13	0.09	0.11	0.01
10.4	1.07	0.00	0.11	0.00
10.5	1.57	0.01	0.16	0.00
10.6	0.66	0.01	0.07	0.00

Table.2 Quantification results of samples extracted with QIAamp® DNA Investigator Kit (Qiagen, Netherlands)

Method of Fixation - Hot Water (≥90˚C)				
SAMPLE	**AVERAGE OF TOTAL AMOUNT OF DNA**	**SD OF TOTAL AMOUNT OF DNA**	**AVERAGE OF AMOUNT OF DNA PER SAMPLE**	**SD OF AMOUNT OF DNA PER SAMPLE**
1.1	2.24	0.39	2.24	0.39
1.2	3.03	0.03	3.03	0.03
1.3	2.41	0.02	2.41	0.02
1.4	0.36	0.01	0.36	0.01
1.5	0.58	0.00	0.58	0.00
1.6	1.06	0.08	1.06	0.08
1.7	3.00	0.05	3.00	0.05
1.8	1.49	0.06	1.49	0.06
1.9	1.74	0.06	1.74	0.06
1.10	0.76	0.01	0.76	0.01
3.1	4.77	0.03	1.59	0.01
3.2	5.62	0.09	1.87	0.03

3.3	8.23	0.32	2.74	0.11
3.4	3.59	0.06	1.20	0.02
3.5	8.46	0.34	2.82	0.11
3.6	5.36	0.25	1.79	0.08
3.7	4.60	0.50	1.53	0.17
3.8	3.52	0.03	1.17	0.01
3.9	1.83	0.02	0.61	0.01
3.10	3.20	0.05	1.07	0.02
5.1	12.51	0.49	2.50	0.10
5.2	10.46	0.48	2.09	0.10
5.3	12.20	0.00	2.44	0.00
5.4	8.66	0.54	1.73	0.11
5.5	10.42	0.51	2.08	0.10
5.6	11.40	0.40	2.28	0.08
5.7	10.48	0.18	2.10	0.04
5.8	14.20	0.35	2.84	0.07
5.9	10.05	0.09	2.01	0.02
5.10	12.63	0.03	2.53	0.01
10.1	16.63	0.22	1.66	0.02
10.2	21.57	1.28	2.16	0.13
10.3	18.49	0.19	1.85	0.02
10.4	26.33	0.11	2.63	0.01
10.5	18.87	0.66	1.89	0.07
10.6	20.65	0.51	2.06	0.05

Method of Fixation - Only Frozen

SAMPLE	AVERAGE OF TOTAL AMOUNT OF DNA	SD OF TOTAL AMOUNT OF DNA	AVERAGE OF AMOUNT OF DNA PER SAMPLE	SD OF AMOUNT OF DNA PER SAMPLE
1.1	3.91	0.11	3.91	0.11
1.2	1.71	0.01	1.71	0.01
1.3	3.01	0.05	3.01	0.05
1.4	2.86	0.02	2.86	0.02
1.5	2.78	0.01	2.78	0.01
1.6	2.82	0.04	2.82	0.04
1.7	3.64	0.33	3.64	0.33
1.8	1.58	0.11	1.58	0.11
1.9	2.61	0.34	2.61	0.34
1.10	4.02	0.02	4.02	0.02
3.1	5.27	0.23	1.76	0.08
3.2	6.81	0.08	2.27	0.03
3.3	7.27	0.30	2.42	0.10
3.4	6.59	0.29	2.20	0.10
3.5	5.58	0.28	1.86	0.09
3.6	7.22	0.06	2.41	0.02
3.7	7.25	0.27	2.42	0.09
3.8	6.07	0.07	2.02	0.02
3.9	7.25	0.13	2.42	0.04
3.10	6.51	0.21	2.17	0.07
5.1	15.01	0.01	3.00	0.00
5.2	16.49	0.56	3.30	0.11
5.3	16.80	0.26	3.36	0.05
5.4	15.56	0.25	3.11	0.05
5.5	16.74	0.02	3.35	0.00
5.6	17.18	0.53	3.44	0.11

5.7	17.28	0.07	3.46	0.01
5.8	16.81	0.06	3.36	0.01
5.9	17.37	0.19	3.47	0.04
5.10	16.21	0.01	3.24	0.00

Method of Fixation - Frozen + Ethanol				
SAMPLE	**AVERAGE OF TOTAL AMOUNT OF DNA**	**SD OF TOTAL AMOUNT OF DNA**	**AVERAGE OF AMOUNT OF DNA PER SAMPLE**	**SD OF AMOUNT OF DNA PER SAMPLE**
1.1	2.34	0.02	2.34	0.02
1.2	2.53	0.02	2.53	0.02
1.3	2.79	0.10	2.79	0.10
1.4	3.15	0.10	3.15	0.10
1.5	2.89	0.05	2.89	0.05
1.6	2.18	0.07	2.18	0.07
1.7	3.20	0.05	3.20	0.05
1.8	3.65	0.01	3.65	0.01
1.9	2.60	0.07	2.60	0.07
1.10	3.79	0.05	3.79	0.05
3.1	7.56	0.02	2.52	0.01
3.2	8.21	0.01	2.74	0.00
3.3	8.10	0.13	2.70	0.04
3.4	8.47	0.14	2.82	0.05
3.5	7.80	0.15	2.60	0.05
3.6	8.51	0.01	2.84	0.00
3.7	8.43	0.02	2.81	0.01
3.8	8.99	0.15	3.00	0.05
3.9	8.54	0.21	2.85	0.07
3.10	7.11	0.07	2.37	0.02

5.1	14.84	0.01	2.97	0.00
5.2	11.25	0.01	2.25	0.00
5.3	11.28	0.35	2.26	0.07
5.4	14.19	0.21	2.84	0.04
5.5	15.52	0.10	3.10	0.02
5.6	15.18	0.01	3.04	0.00
5.7	15.47	0.04	3.09	0.01
5.8	14.37	0.01	2.87	0.00
5.9	14.82	0.68	2.96	0.14
5.10	13.93	0.53	2.79	0.11
Method of Fixation - Only Ethanol				
SAMPLE	**AVERAGE OF TOTAL AMOUNT OF DNA**	**SD OF TOTAL AMOUNT OF DNA**	**AVERAGE OF AMOUNT OF DNA PER SAMPLE**	**SD OF AMOUNT OF DNA PER SAMPLE**
1.1	3.28	0.23	3.28	0.18
1.2	4.91	0.04	4.91	0.03
1.3	3.22	0.01	3.22	0.00
1.4	3.52	0.01	3.52	0.00
1.5	3.75	0.03	3.75	0.02
1.6	2.82	0.07	2.82	0.06
1.7	3.32	0.06	3.32	0.04
1.8	4.55	0.01	4.55	0.00
1.9	4.17	0.06	4.17	0.04
1.10	4.36	0.06	4.36	0.06
3.1	8.73	0.24	2.91	0.07
3.2	9.45	0.03	3.15	0.01
3.3	9.21	0.04	3.07	0.01
3.4	9.35	0.01	3.12	0.00

3.5	8.82	0.27	2.94	0.07
3.6	9.70	0.13	3.23	0.03
3.7	7.36	0.11	2.45	0.03
3.8	8.23	0.02	2.74	0.01
3.9	8.88	0.12	2.96	0.03
3.10	9.62	0.18	3.21	0.05
5.1	17.53	0.05	3.51	0.01
5.2	15.33	0.01	3.07	0.00
5.3	16.33	0.13	3.27	0.02
5.4	15.62	0.09	3.12	0.01
5.5	16.86	0.23	3.37	0.04
5.6	16.23	0.16	3.25	0.03
5.7	17.80	0.08	3.56	0.01
5.8	15.47	0.16	3.09	0.03
5.9	16.74	0.18	3.35	0.03
5.10	16.08	0.06	3.22	0.01

Method of Fixation -Hot Water (≥90˚C) + Ethanol				
SAMPLE	**AVERAGE OF TOTAL AMOUNT OF DNA**	**SD OF TOTAL AMOUNT OF DNA**	**AVERAGE OF AMOUNT OF DNA PER SAMPLE**	**SD OF AMOUNT OF DNA PER SAMPLE**
1.1	1.43	0.19	1.43	0.19
1.2	1.50	0.13	1.50	0.13
1.3	2.07	0.06	2.07	0.06
1.4	1.55	0.04	1.55	0.04
1.5	2.37	0.10	2.37	0.10
1.6	0.98	0.19	0.98	0.19
1.7	1.59	0.05	1.59	0.05
1.8	1.23	0.02	1.23	0.02

1.9	2.26	0.08	2.26	0.08
1.10	1.75	0.04	1.75	0.04
3.1	3.29	0.08	1.10	0.03
3.2	3.46	0.02	1.15	0.01
3.3	3.65	0.01	1.22	0.00
3.4	3.00	0.01	1.00	0.00
3.5	3.55	0.08	1.18	0.03
3.6	3.03	0.20	1.01	0.07
3.7	2.44	0.16	0.81	0.05
3.8	3.38	0.13	1.13	0.04
3.9	3.78	0.14	1.26	0.05
3.10	3.21	0.17	1.07	0.06
5.1	8.55	0.09	1.71	0.02
5.2	8.38	0.03	1.68	0.01
5.3	9.61	0.03	1.92	0.01
5.4	8.91	0.14	1.78	0.03
5.5	8.41	0.06	1.68	0.01
5.6	8.62	0.06	1.72	0.01
5.7	9.18	0.03	1.84	0.01
5.8	8.35	0.14	1.67	0.03
5.9	9.47	0.16	1.89	0.03
5.10	9.46	0.06	1.89	0.01

Table. 3 Quantification results of samples extracted with PrepFiler® Forensic DNA extraction Kit (Applied Biosystems, USA)

SAMPLE	AVERAGE OF TOTAL AMOUNT OF DNA	SD OF TOTAL AMOUNT OF DNA	AVERAGE OF AMOUNT OF DNA PER SAMPLE	SD OF AMOUNT OF DNA PER SAMPLE
1.1	4.13	0.03	4.13	0.03
1.2	4.40	0.23	4.40	0.23
1.3	4.25	0.05	4.25	0.05
1.4	3.65	0.09	3.65	0.09
1.5	3.22	0.03	3.22	0.03
1.6	4.62	0.09	4.62	0.09
3.1	8.35	0.04	2.78	0.01
3.2	9.37	0.12	3.12	0.04
3.3	9.64	0.25	3.21	0.08
3.4	8.39	0.11	2.80	0.04
3.5	9.30	0.48	3.10	0.16
3.6	9.30	0.12	3.10	0.04
5.1	18.12	0.01	3.62	0.00
5.2	18.64	0.02	3.73	0.00
5.3	18.28	0.20	3.66	0.04
5.4	17.97	0.67	3.59	0.13
5.5	18.53	0.04	3.71	0.01
5.6	18.73	0.19	3.75	0.04

Appendix D:

Sequences received, of PCR products purified, from Eurofins Genomics, Germany. These were compared to sequences available in the GeneBank database to establish identity of the sample.

1 - TTTATAGGATACGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACC AACCT

2 - ATGAAATATCGGAGTAATCCTTCTGCTCACAGTAATAGCCACAGCATTTATAGGATACGT CCTACCAT

3 - CCCCCTCACATCAAACCCGAGTGATACTTCTTATTTGCCTACGCAATCTTACGATCATCC AAA

4 - AATACTACTAGTACTATTCTCACCCGACCTCCTCGGAGACCCAGACAACTACACCCCAGC
CAATCCACTCAACACACCCCCTC

5 - CCATTCCACCCCTACTATACCATTAAGGACATCTTAGGGGCCCTCTTACTAATTCTAGCT
CTAATACTACTAGTACTATTCGCACCCGACCTCCTCGGAGACCCAGATAACTACACCCCA
GCCAATCCACTCAACACACCCCCTCACATCAAACCCGAGTGATACTTCTTATTTGCATAC
GCAATCTTACGATCAATCCCAAAA

6 - TACTACTAGTACTATTCTCACCCGACCTCCTCGGAGACCCAGACAACTACACCCCAGCCA
ACCCACTCAACACCCCCCCTCACATCAAACCCGAATGATACTTCTTATTTGCCTACGCAA
TCTTACGATCAATCCCACA

7 - CTCGGAGACCCAGACAACTACACCCCAGCCAATCCACTCAACACTCCCCCTCACATCAAA
CCCGAATGATACTTCTTATTTGCCTACGCTATCTTACGATCATCCCCA

8 - TACAAGAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTAT
AGAGAAAGTACCTGGGA

8 - GATACGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCT
TATCAGCAATCCCGCA

9 - TACCAGAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTAT
AGAGAAAGTACCCACTAG

9 - GACAGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCTT
ATCAGCAATCCAGCA

10 - CAAACGAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTAT
AGAGAAAGTACCCAAGC

10 - TTCGTCCTACCATGAGGACAAATATCATTCTGAAGGAGCAACAGTCATCACCAACCTCTT
ATCAGCAATCCCGCTC

11 - AGAATAAAACAAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGC
GCTATAGAGAAAGTACCAAACG

11 - GTAAGGAAGCAACAGTCATCACCAACCTTCTTATCAGCAATCACGT

12 - GTCACCAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTAT
AGAGAAAGTACCCACTG

12 - CGGATACGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCT
CTTATCAGCAATCCAGCA

13 - TACCAGAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTAT
AGAGAAAGTACCCACGTA

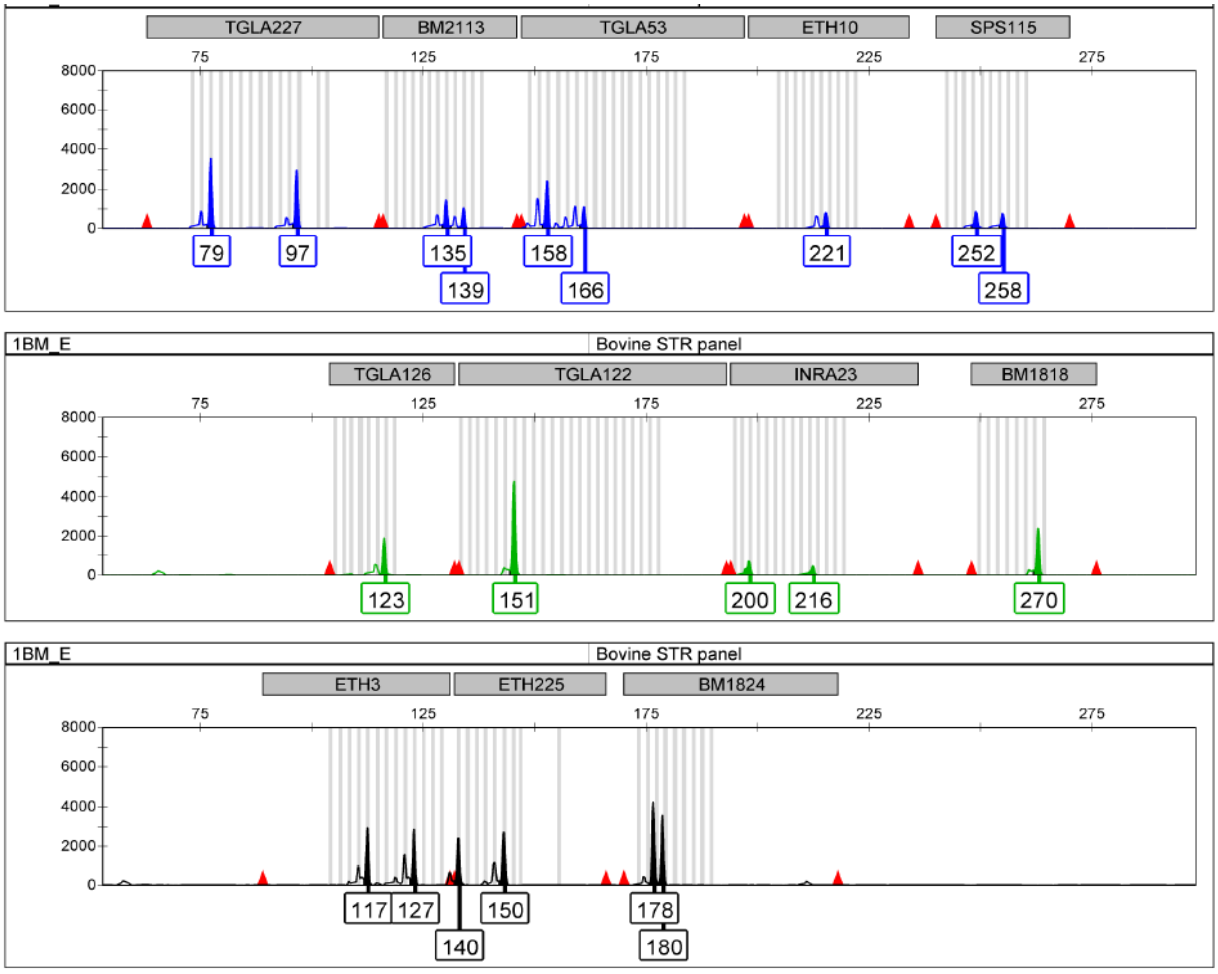
13 - TTAAGTCCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCTT
ATCAGCAATCATACC

14 - ACCAGAACATTTAATCCCAATTTAAAGTATAGGAGATAGAAATCTAAGTACGGCGCTATA
GAGAAAGTACCCACTAA

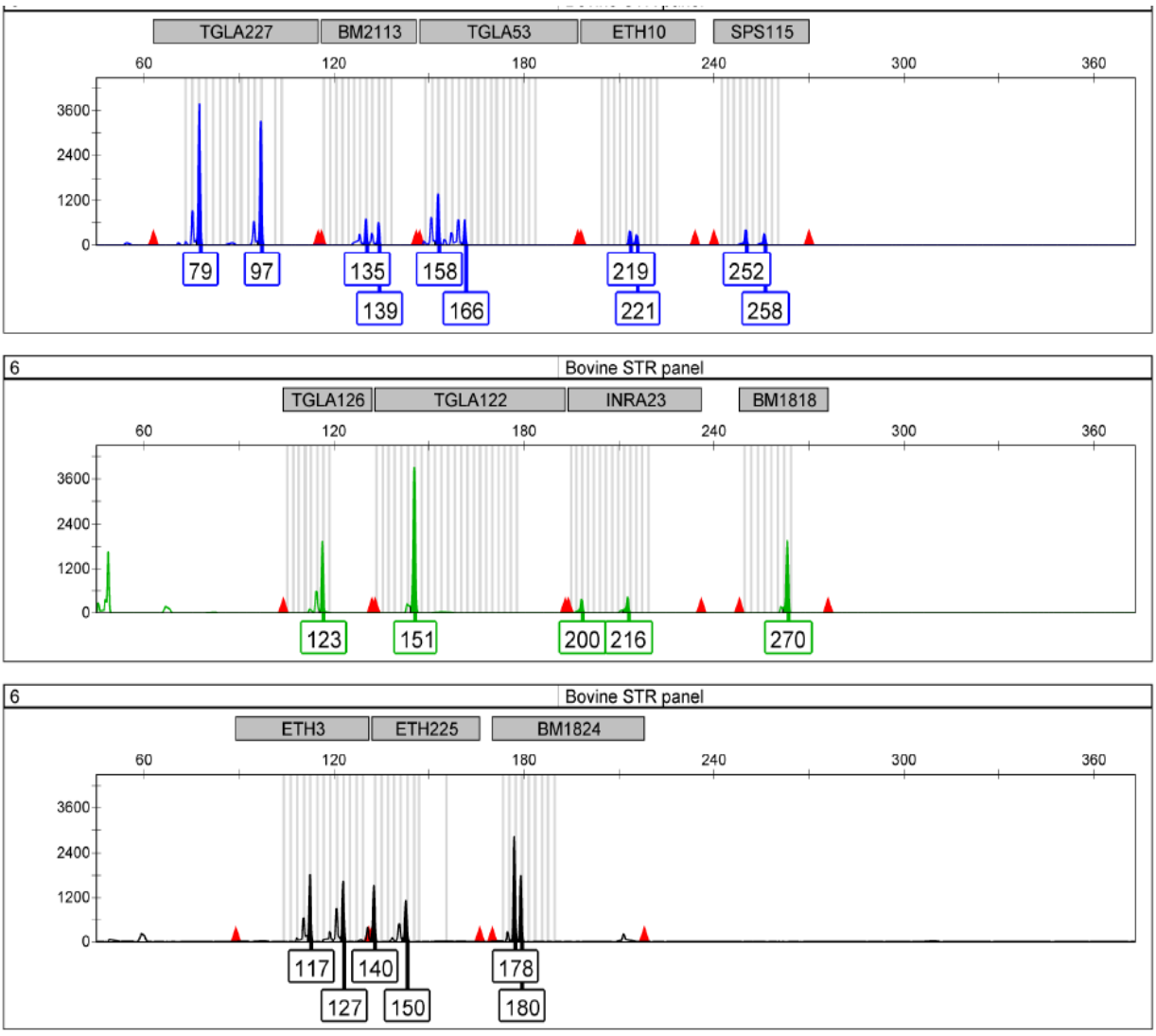
14 - GCTACCTACCATGAGGACAAATATCATTCTGAGGAGCAACAGTCATCACCAACCTCTTAT
CAGCAATCATGCAA

Appendix E:

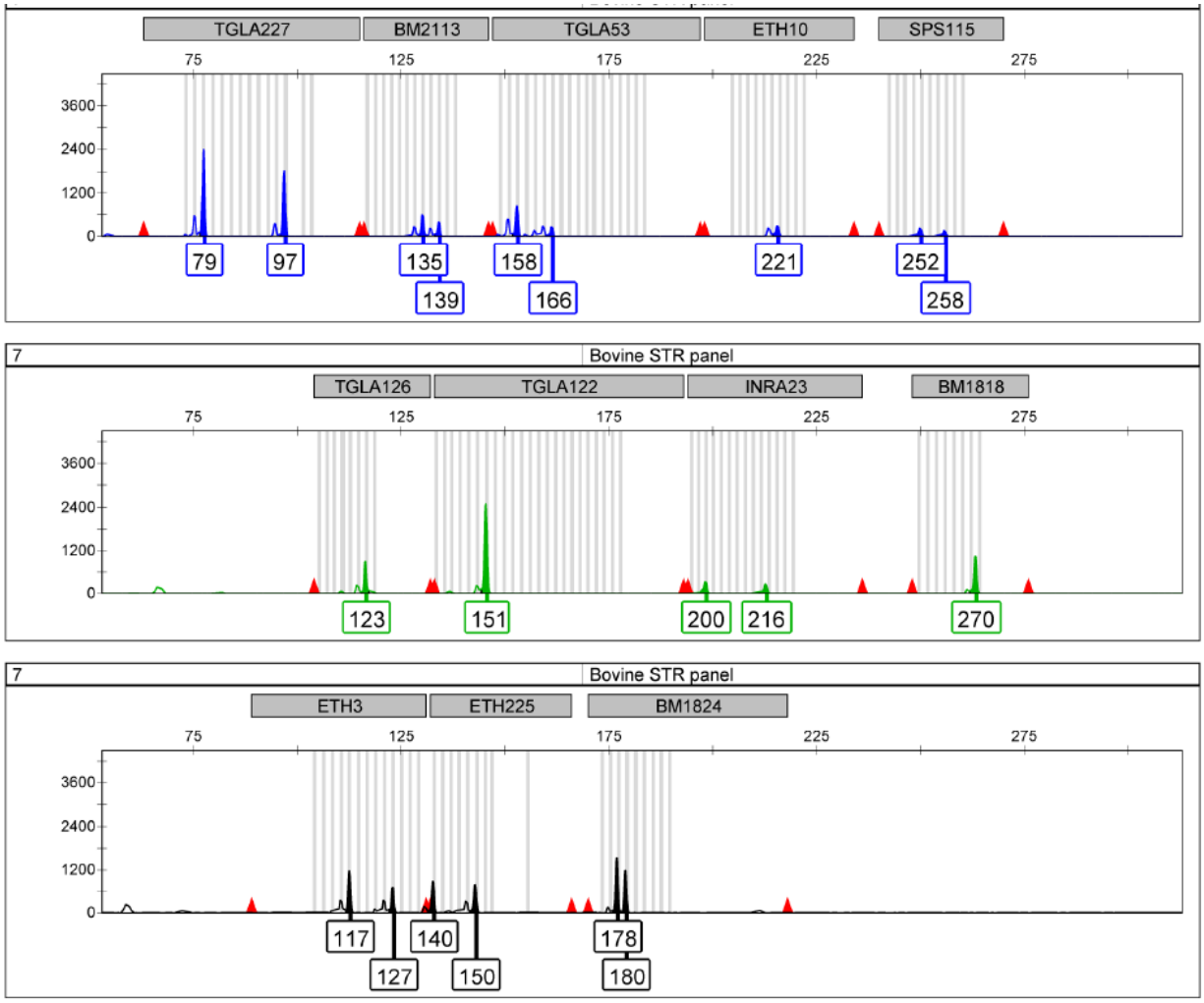
1. Electropherograms showing complete STR profile (12 loci) for DNA extracted from gut content of one *M. scalaris* larva



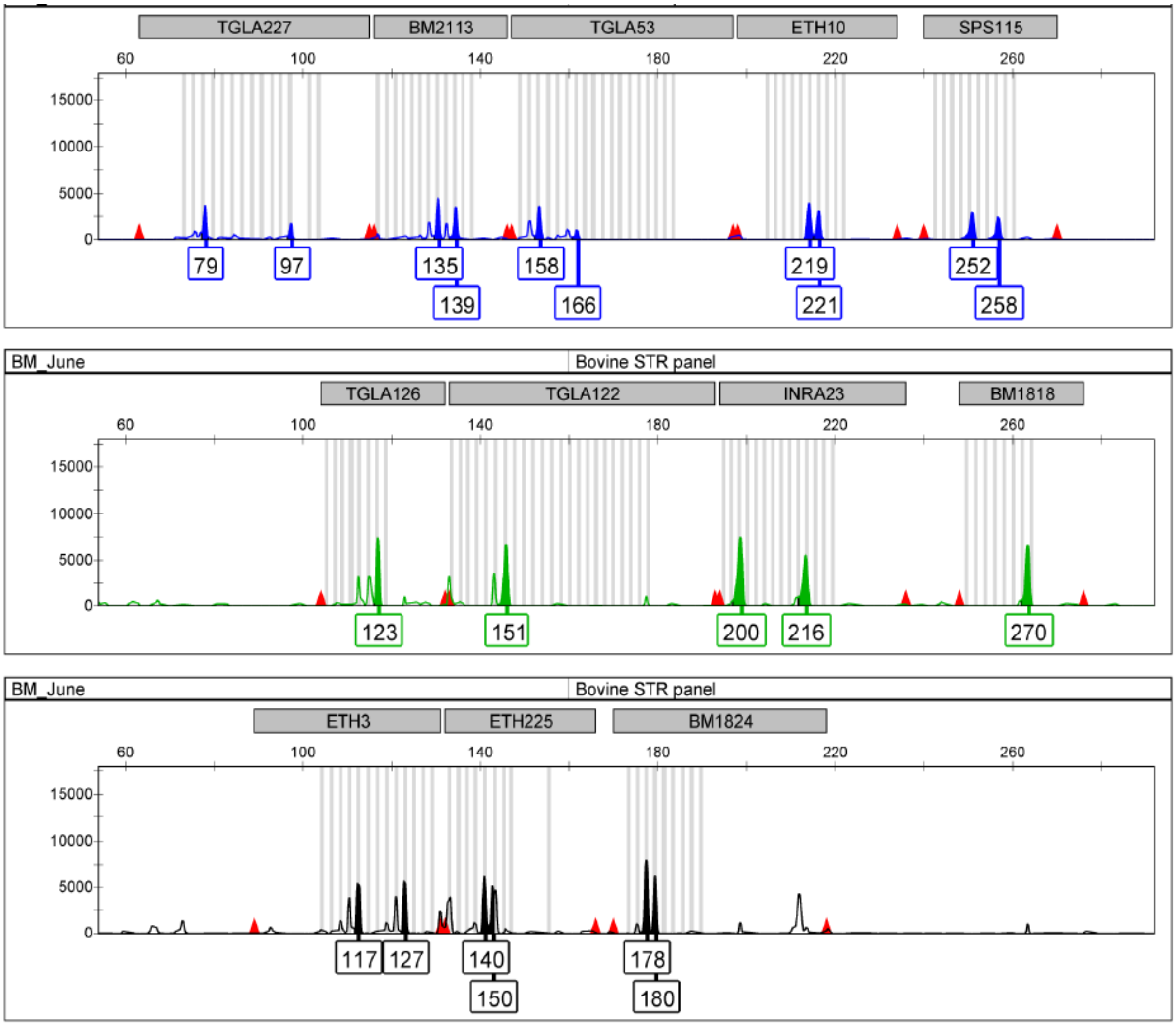
2. Electropherograms showing complete STR profile (12 loci) for DNA extracted from gut content of 3 *M. scalaris* larvae



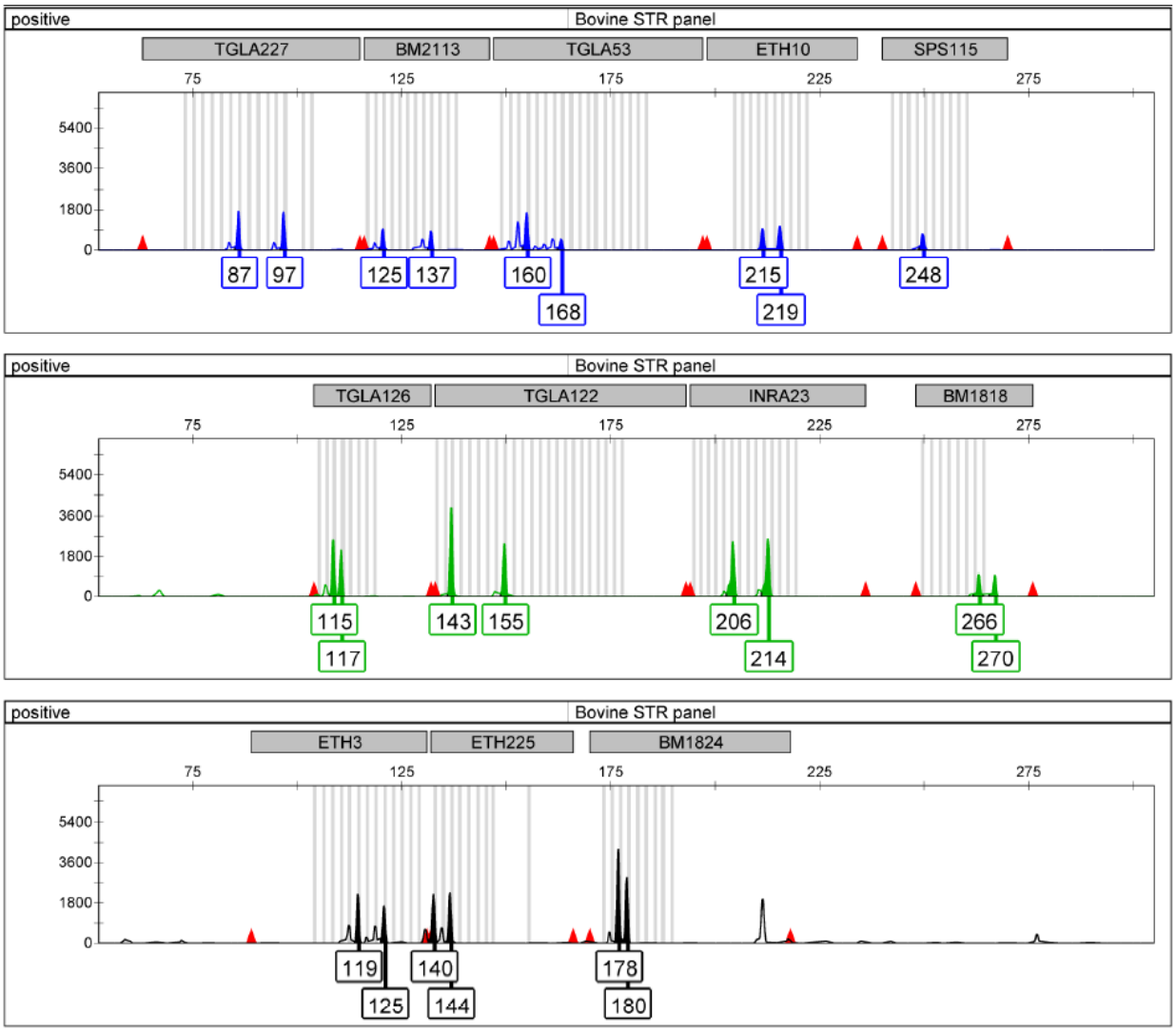
3. Electropherograms showing complete STR profile (12 loci) for DNA extracted from gut content of 5 *M. scalaris* larvae



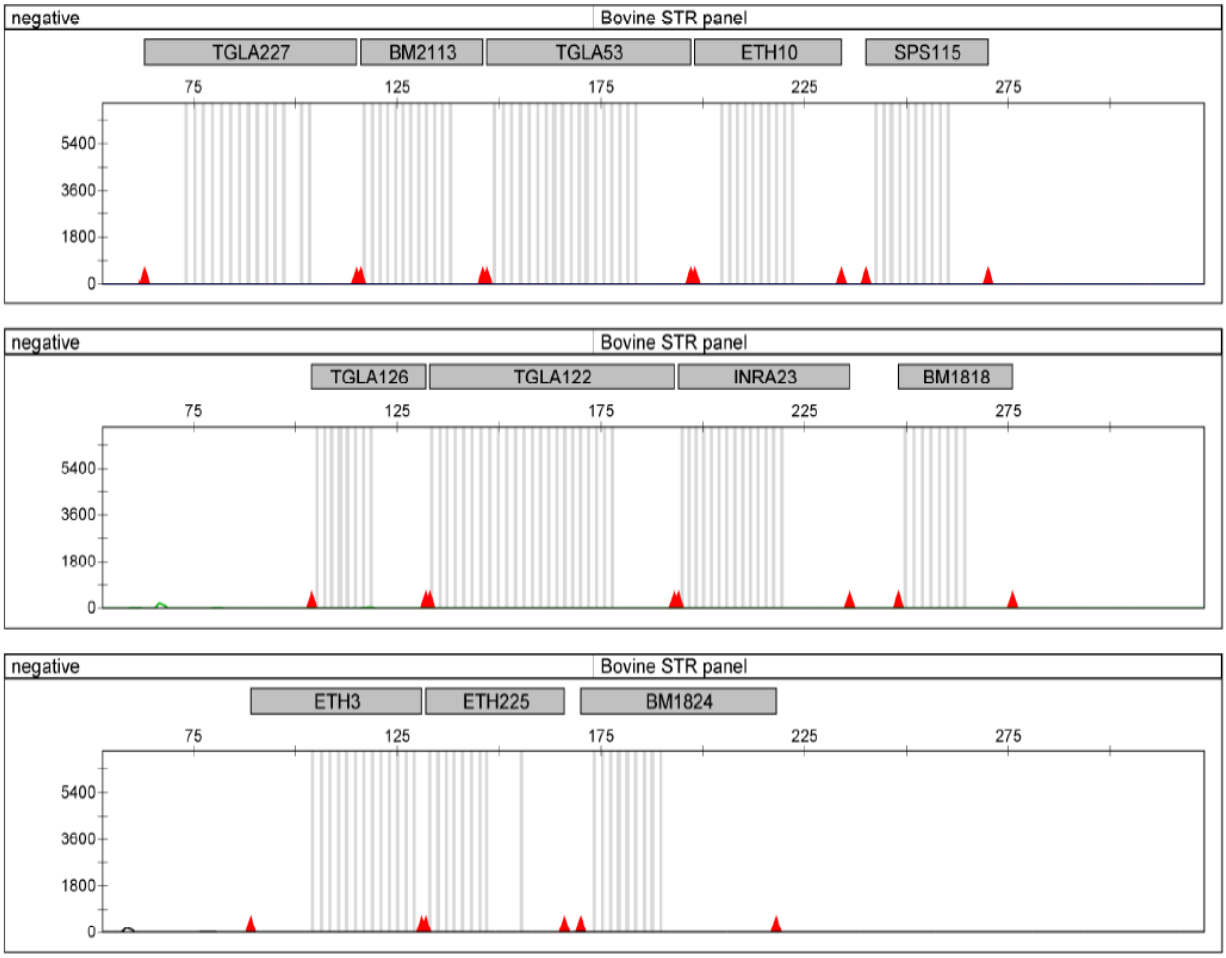
4. Electropherograms showing complete STR profile (12 loci) for DNA extracted from Bovine meat, which was fed to *M. scalaris* larvae while breeding. It was used as a reference to compare profiles obtained from DNA extracted from the larval gut content. Samples showing an exact match prove that the DNA extracted was of the same Bovine meat which was fed to the larvae.



5. Electropherograms showing complete STR profile (12 loci) for the positive control which was provided by the Bovine Genotypes Panel 1.2 Kit, to test proper functioning of the reaction.



6. Electropherograms showing CE results for the negative control for which Ultrapure™ PCR grade water (Invitrogen, USA) was used. Absence of any peaks or disturbances confirms that no contamination occurred during the entire process.



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