Efficacy of different DNA polymerase enzymes in PCR amplification for forensic analysis of bovine DNA

By

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DEDICATION

I would like to dedicate this work to my mom and my son Teddy for being my inspiration and being there for me at all times. To Mr Netshilema for the support and encouragement he always gives me. To my husband Zweli for his support and for being there for me in good and bad times. And lastly to God for His protection and life He gave me.

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I declare that this thesis is my own work and effort and that it has never been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged

Signature: ……

Date: ……..

ABSTRACT

DNA profiling of exhibits that originate from forensic stock theft cases is routinely used as a tool to link suspects to the crime or scene. DNA derived from aged or degraded samples is often highly fragmented which compromises the efficiency for obtaining a complete genotypic profile using PCR. Conventional polymerases such as *Taq*, lack certain repair mechanisms for use on degraded DNA templates. New generation polymerases are known to have high fidelity characteristics. The aim of this study was to determine the efficiency of Restorase®, a novel DNA polymerase blend that is known to repair damaged DNA and the FastStart High Fidelity PCR System enzymes, on degraded forensic bovine samples using PCR-based methodology. Bovine meat samples were subjected to different degrees of degradation in the sun and in the shade during summer and winter seasons. DNA was extracted, subjected to PCR amplification using 16 bovine microsatellites and genotypes were generated for analyses. Rapid degradation of samples was observed during winter while during summer samples tend to dry out. Restorase® exhibited high enzyme activity on degraded samples as compared with FastStart and *Taq* DNA polymerase. Some of the markers that failed to be successfully amplified by *Taq* polymerase, such as ETH10 and SPS115 were recovered using Restorase®. Markers such as BM1818, BM2113, ETH3, INRA23 and TGLA227 remained active throughout the experiment using all the enzymes, and therefore can form a basis of the bovine marker panel. Restorase® was found to be an alternative enzyme for use in bovine forensic analysis.

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List of Abbreviations

ABI	Applied Biosystems
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed PCR
ARC	Agricultural Research Council
bp	Base pair
°C	Degree Celsius
ChiSq	Chisquare
DAF	DNA Amplification Fingerprinting
DIG-dUTP	Digoxigenin-Deoxyuridine Triphosphate
DNA	Deoxyribonucleic Acid
dCTP	Deoxycytidine Triphosphate
dNTP	Deoxyribonucleotide
dUMP	Deoxyuridine Monophosphate
dUTP	Deoxyuridine Triphosphate
g	Gram
H2G2	DNA Fingerprinting Guide
H_20	Water
HCl	Hydrocloric Acid
HGMIS	Human Genome Management Information System
IHGSC	International Human Genome Sequencing Consortium
ISAG	International Society of Animal Genetics
ISSR	Inter Simple Sequence Repeat
IVF	*In vitro* Fertilization
kb/kbp	Kilo-base pair
kD	kiloDalton
kg	Kilogram
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium Chloride
mM	Millimolar

ng	Nanogram
NTP	Nucleoside Triphosphate
OH	Hydroxyl
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	Picomole
QTL	Quantitative Trait Loci
RADP	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Unit
RNA	Ribonucleic Acid
SA	South Africa
SAPS	South African Police Services
SAS	Statistical Analysis Software
SNP	Single Nucleotide Polymorphism
STR	Short Tandem Repeats
TdT	Terminal Deoxynucleotidyl Transferase
Tris-HCl	Tris Hydrochloride
U	Unit
µL	Microlitre
VNTR	Variable Number Tandem Repeats

Chapter 1

1.1. Introduction

The growth of the world population exerts enormous pressure on agriculture for increased food production on ever decreasing agricultural land and natural resources (Pimentel, 2004). The aim of livestock production is to produce more animal protein more efficiently. In Southern Africa and South Africa (SA) livestock production is not only an important sector in commercial food production, but a major source of protein for households and income for small farmers in deep rural areas. Livestock are also kept as a source of investment, insurance against disaster and also for cultural purposes (Kunene and Fossey, 2006).

Stock theft in the livestock industry has been one part of South Africa's main challenges and dates back as early as the 17th century, when livestock farmers crossed the Kei River and cattle theft was already a problem (Walker and Wyndham, 1941). Today it still remains one of our country's most common and persistent crimes (Johnson, 2010). It especially affects the sustainability of rural farming practices, restricting their production capacity and eventually threatening food security (www.nafufarmers.co.za). The impact on resource poor farmers is devastating due to the fact that in many circumstances their livelihoods consist of few animals. Recently, stock theft has also become more violent and an organized crime.

Studies have shown that stock theft syndicates transport animals from one village to another and eventually to local butcheries and market outlets in South Africa and across the borders. Stolen animals can be transported easily within the neighboring countries of Lesotho, Swaziland and other nearby countries and readily exchanged for cash, dagga (drugs made from *Cannabis sativa*) and guns and therefore it renders stock theft a profitable venture. Unfortunately it is a difficult matter for police, prosecutors and magistrates, who are unable to arrest and convict thieves making the problem of stock theft worse. Moreover there is a perception that some police, chiefs, officials and business men are involved in these criminal networks (Dzimba and Matooane, 2005; Kynoch and Ulicki, 2001).

Although there is some recovery of animals, this seems to be of little consolation to the owners. In South Africa, from 2003 to 2009, more than 190 000 stock theft cases were opened and approximately 700 000 animals (cattle, goats and sheep) to the value of more than R1.2 billion were reported stolen. The calculated net loss in the economy was estimated to be approximately R2.5 billion (South African Police Services (SAPS) stock theft statistics, 2008/2009). Stock theft prevents the profitability of livestock stock production and furthermore it is interfering with the government's land reform process as well as empowering of the emerging farmers (News 24, 2007; Agri SA media release, March 2010).

The three major challenges in stock theft is the problem of identification, tracing of legal owners and recovery of stolen animals. Conventional methods such as brand marking and ear tagging have proved not to be reliable or individual-specific because they can be tampered with, and therefore the conviction rate in stock theft is low. Few animals are branded and ear tagged especially in rural areas. Often stud and high producing animals such as dairy cows are at high risk.

Deoxyribonucleic acid (DNA) technology provides a new approach for the effective identification of animals (Loftus, 2005). It can be used as a tool to resolve the problem of identification and also offers a reliable form of identification (Vazquez *et al*., 2004). DNA technology is the chemical manipulation of the genotypes and resulting phenotypes of organisms, such that living organisms are modified (Campbell and Reece, 2002). Its role in studying genetics, biochemistry, ecology and evolutionary biology of organisms is well known (Campbell and Reece, 2002). The application of DNA technology has now become more popular and widely accepted, and it is used routinely for animal identification and parentage analyses (Vazquez *et al*., 2004; Kashi *et al*., 1990) as well as individual traceability systems (Evans and Van Eenennaam, 2005).

Forensic DNA profiling is now being successfully applied and has become an established forensic tool for individual identification (Curran, 1997; Loftus, 2005). Although polymerases are used for Polymerase Chain Reaction (PCR)-based analysis, difficulties are encountered when analyzing degraded DNA samples such as skin, meat, blood and faecal samples from forensic investigation (Yang and Speller, 2006).

DNA degradation is a challenge in forensic analysis, and it can easily lead to false results and/or no results (Yang and Speller, 2006). DNA templates may be damaged by exposure to acid, alkylating agents, heat, light, phenol/chloroform extraction, and reactive oxygen species or simply by time (Curan, 1997). Over time, unless frozen, dried or preserved, DNA in a cell will degrade and even PCR-based DNA testing methods will no longer be successful. The inefficient amplification of a DNA sequence can lead to an increase in PCR cycle number for optimization purposes, which in turn increases the probability of the mis-incorporation of nucleotides. Poor specificity can furthermore result in numerous artifacts which then interfere with the interpretation of experimental results (Wang *et al*., 2004; Foran, 2006).

For many years *Taq* DNA polymerase has served as the most appropriate enzyme in the PCR amplification of DNA. However, a major limitation of *Taq* is its inability to amplify damaged DNA, thereby restricting its usefulness in forensic application (McDonald *et al*., 2006). Restorase® is a special enzyme designed to facilitate repair and provide reliable amplification of damaged DNA. It allows for the amplification of highly degraded DNA samples which are unable to be amplified by other polymerases such as *Taq* polymerase. In addition, it has the ability to increase both yield and specificity and it's a blend for use on a daily basis (Kobilinsky *et al*., 2004).

1.2. Aim of the study

The Animal Genetics laboratory of the Agricultural Research Council (ARC) located in Irene, Centurion, South Africa is a genetics laboratory performing research on different livestock and wildlife species as well as offering commercial genetic tests for farmers as well as for livestock forensics. Forensic stock theft cases are analyzed on a routine basis and a major challenge is the problem of degradation of samples collected from different crime scenes.

In South African courts, the expression of the DNA evidence in terms of likelihood ratios is compulsory for any DNA analysis. In order to calculate likelihood ratios for animals accurately and give expert evidence in court, allele frequencies for a specific microsatellite loci set must be known for each specific livestock breed. Allele frequency results are added to a database, and a number of analyses from animals in each species or breed within species must be compiled into a database as a prerequisite for the accurate calculation of likelihood ratios for forensic purposes (Harris *et al*, 2006; ARC database). The database often contains information that may be useful during forensic investigations, and produce valuable output for application in court cases.

The aim of this study was to determine the efficiency of Restorase®, a novel DNA polymerase blend that is known to repair damaged DNA and the FastStart High Fidelity PCR System enzymes, on degraded forensic bovine samples using PCR-based methodology. These enzymes were compared to *Taq* polymerase, which was used as a reference enzyme.

Chapter 2

2. Literature Review

The advancement made in molecular genetics over the past years has led to the development of DNA technology that has had an important contribution to forensic studies (Baldi and Hatfield, 2002). As early as 1944 Watson and Crick described the double helical structure of DNA and Maxam, Gilbert and Sanger laid the foundations for understanding the functions of DNA with sequencing methodology (Roberts *et al*., 2001). These discoveries resulted in genome mapping and sequencing of a large number of species including farm animals such as cattle, sheep, pigs and chicken (Gama Sosa, *et al*., 2010; Womack, 2005; Burt *et al*., 1995; Bishop *et al*., 1994).

It is likely that the massive global investment in genomic research will have significant benefits for livestock production. An increase in DNA data generated will also influence the development of bio-informatics, for studying fundamental and complex physiological mechanisms (Nyren, 2006). The aim of this chapter is firstly to provide a brief overview on DNA markers and their application and secondly to review detail on the role of PCR and polymerase enzymes used in DNA forensics.

2.1. DNA technology and DNA markers

Deoxyribonucleic acid

DNA is a unit of inheritance for generational transfer of heritable traits. It governs the inheritance of eye colour, hair, size, bone density and many other human and animal characteristics (Olivier, 2001). An individual's DNA is formed by combination of DNA from the parents with half coming from the mother and half from the father, and for this reason DNA can be used as evidence to determine the paternity of an individual (Olivier, 2001; Luftig and Richey, 2001). DNA is not visible with the naked eye and is difficult to tamper with and it can thus be described as a fingerprint which can be used to identify an animal, dead or alive (Luftig and Richey, 2001).

DNA is present in almost every cell of the body with the exception of the red blood cells (Goodwin *et al*., 2007). DNA can be recovered from cellular materials such as

blood, semen, hair, saliva, feaces and other biomaterials (Goodwin *et al*., 2007). Most of the DNA in the chromosomes has no known function and it is commonly referred to as "junk" DNA. This type of DNA is of special interest for forensic application (Curran, 1997). DNA can be used in all cases where blood is involved and in cases where the identity of an unidentified body has to be established. This is accomplished by profiling DNA sequence information, which characterizes the small sections of DNA that vary from individual to individual (Olivier, 2001).

DNA markers

DNA sequence information offers the potential to identify a large number of species and sub-species specific markers. A marker is any sequence or locus which can be uniquely assigned to a position within a larger sequence, chromosome or genome. Markers are used as features to define a map of the region, so that structural analysis can be performed, or function investigated (Liu and Cordes, 2004). A genetic marker can be defined as any stable and inherited variation that can be measured or detected by a suitable method through DNA technology, and can be used to detect the presence of a specific genotype or phenotype (Tautz, 1989; Mitra, 1994). Selection of markers for different applications is influenced by the degree of polymorphism, skill or expertise available, possibility of automation, radioisotopes used, reproducibility of the technique and the cost involved and most importantly by the question that needs to be addressed (Tvedebrink, 2009).

Molecular markers reveal variations at the DNA level and possess unique genetic properties. Methodological developments have also made them more useful for genetic analysis. Moreover, molecular markers are numerous and distributed throughout the genome (Mitra *et al*., 1999). They remain unaffected by environmental factors, and generally do not have pleiotropic effects on quantitative trait loci (QTL) (Geldermann, 1975). These markers include Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLP's), Amplified Fragment Length Polymorphisms (AFLP's), Short Tandem Repeats (STRs) or microsatellites and Single Nucleotide Polymorphisms (SNPs). SNP markers are currently used in DNA fingerprinting for identification analysis, paternity, animal breeding, and association studies with phenotypic performance (Teneva and Petrovic, 2010; Budowle and Van Daal, 2008; Jobling and Gill, 2004).

RAPD, AFLP and RFLP markers

RAPD markers are DNA fragments used in PCR amplification of random segments of genomic DNA, with a single primer of random nucleotide sequence (Smith *et al.*, 1994). RAPD has been one of the first markers to be developed in DNA technology. It's a marker technique that requires no cloning or sequencing, it is PCR-based and it is possible to detect several loci simultaneously. The technique is easy, inexpensive and fast and because a single RAPD primer may detect more than one locus, it is useful for phylogenetic studies. The short primer (single 10-mer oligonucleotide) may amplify random pieces of DNA that produces a series of products of different sizes (Bardakci, 2001; Williams *et al.*, 1990). This however can be easily affected by annealing conditions and results may not be highly reproducible (Smith *et al.*, 1994).

RAPDs have several advantages. For example, multiple amplifiable fragments from different loci are usually present for each set of primers in each genome. Fragments can be separated by size on a standard agarose gel and visualized by ethidium bromide, eliminating the need for radiolabeled probes. Since the primers consist of random sequences and do not discriminate between coding and noncoding regions, it is easy to sample the genome more randomly compared to conventional methods such as biotyping and staining methods (Lynch and Milligan, 1994; Michelli *et al.*, 1994).

Despite these advantages, in the case of DNA fingerprinting where multiple markers appear on the same gel, there can be uncertainty in assigning markers to specific loci in the absence of preliminary pedigree analysis. An additional concern is the possibility that the products of different loci will have similar molecular weights and therefore will be indistinguishable on a gel. RAPD is also a dominant marker and heterozygotes cannot be distinguished from marker/null heterozygote which reduces the accuracy of estimation compared to codominant markers (Lynch and Milligan, 1994; Michelli *et al.*, 1994).

AFLPs are fragments that have been amplified using primers from digested genomic DNA (Chial, 2008; Matthes *et al.*, 1998). This technique requires no cloning or sequencing and is also PCR-based. It is based on much the same principle as RAPD but the primer consists of a longer fragment, approximately 15 base pairs (bp) fixed portion and a short 2-4bp random portion. The long fixed portion gives the primer

stability while the short random portion enables the amplification of many loci which may allow one to amplify over 100 loci with a single AFLP primer. Since the primer can detect so many loci it is very useful for fingerprinting (Smith *et al.*, 1994).

RFLP is the most widely used hybridization-based molecular marker. It was first used in 1975 to identify DNA sequence polymorphism for genetic mapping of a temperature-sensitive mutation of adeno-virus serotypes (Semagn *et al.*, 2006). The basic principle of RFLP is separation of the desired repetitive sequences by cleaving them out from the genome using an appropriate endonuclease enzyme, followed by electrophoresis of the digested DNA and subsequent detection by DNA probes (Kashyap *et al.*, 2004).

RFLP is the marker of interest in forensic DNA analysis and typing, and was the first technique that was adapted for individual identification (Kashyap *et al.*, 2004; Curran, 1997). In this technique a restriction enzyme is used to cut DNA into small pieces. The size of each fragment is distinct based on the location within the specific sequences of the DNA. Thus, a restriction profile for an individual's DNA can be generated (Luftig and Richey, 2001).

Short Tandem Repeats/Microsatellite markers

Microsatellites are simple tandem repeat markers that are highly polymorphic and used for genetic analyses (Ellegren *et al.*, 1997; Tautz, 1989). These markers are stretches of DNA consisting of tandem repeated short units of 1-6 base pairs in length and are ubiquitous in prokaryotes and eukaryotes, present even in the smallest bacterial genomes (Chistiakov *et al.*, 2006; Selkoe and Toenen, 2006). In eukaryotic genomes the existence of simple sequence repeats has demonstrated a large number and a wide occurrence of these sequences from yeast through to vertebrates.

Microsatellites are selectively neutral and codominant and are easily amplified with PCR. Since these markers are codominant, it is possible to distinguish between the homozygous and the heterozygous individuals (Wright and Bentzen, 1994; Chistiakov *et al.*, 2006). Microsatellites arise predominantly through slippage synthesis during DNA replication and are mostly found in the non-coding part of the genome (Curran, 1997). These features provide the foundation for their successful application in a wide

range of fundamental and applied fields of biology and medicine, including forensics, molecular epidemiology, parasitology, population and conservation genetics, genetic mapping and genetic classification of complex traits (Szibor *et al*., 2003; Chistiakov *et al*., 2006; Greenhouse *et al*., 2006; Anderson, 2001). Microsatellites are considered selectively neutral markers and always represent functionally significant polymorphisms. These markers contribute to DNA structure, chromatin organization, regulation of DNA recombination, transcription and translation, gene expression and cell cycle dynamics (Rakoczy-Trojanowska and Bolibok, 2004).

The most characteristic feature of microsatellites is their high mutation rates, resulting in high levels of polymorphism with a large number of alleles being present in most populations. Mutation rates of 10^{-4} to 10^{-6} per generation have been reported, which is up to 10 000 times faster than that of coding genes (Curran, 1997). Other advantages of microsatellite markers include the availability of their map positions in the genome on several species, the growing amount of data, the availability of primer sequence information enabling their use as genetic markers, and the large comparative data available from laboratories around the world (Rakoczy-Trojanowska and Bolibok, 2004; Ellegren, 1997).

Although many microsatellites map to non-coding regions, these markers can be used as molecular markers linked to traits of interest for marker assisted breeding (Beuzen *et al*., 2000; Ellegren, 1997). Microsatellites are also widely spread throughout the genome and have cross species conservation of primer sequences, are easy to amplify with the PCR, are easily automated, and multiplexing is also possible (Beuzen *et al*., 2000).

Microsatellites were originally used in genetic mapping and have been widely used for linkage analysis in disease associated genes. These markers remain the markers of choice for the construction of linkage maps and only require a small amount of DNA for each analysis. Linkage maps are known as recombination maps and define the order and distance of loci along a chromosome on the basis of inheritance in families or mapping populations (Chistiakov *et al*., 2006). Microsatellites have proven useful in the analysis of paternity and kinship and in sample identity at both the individual and population level (Roushdy *et al*., 2008).

The use of STRs for the identification of animals has developed alongside similar STR applications for humans. Population studies, kinship analysis, paternity testing, and unique identification have been applied to humans and many other animals. The field of forensic science has adapted this information extensively in the prosecution of suspected criminals with great success (Cassidy and Gonzales, 2005).

DNA evidence can be an important factor in a case when it is required to establish an association between a crime scene and a suspect or in crimes involving a specific animal. The DNA testing of animals may be routine, but its use in court as evidence is less common. As the presentation of animal evidence in court increases, appropriate standards and guidelines must be applied to ensure the admissibility of the DNA testing results (Cassidy and Gonzales, 2005).

Single Nucleotide polymorphism

A SNP is a site in the genome where individuals differ in their DNA sequence by a single base (Collins *et al.*, 2003). For such a base to be considered as a SNP, the least frequent allele should have a frequency of 1 percent (%) or greater. SNPs are usually bi-allelic although any of the four possible nucleotide bases can be present. Therefore, the probability of two independent base changes occurring at the single position is very low. Another reason that differentiates individual SNPs is a bias in mutations, leading to the prevalence of two SNP types (Vignal *et al.*, 2002).

Early studies in humans showed that it is possible to identify thousands of SNPs and to perform highly multiplexed genotyping by means of DNA microarrays. The SNP Consortium, built an initial map of 1.4 million SNPs and this has grown to more than 10 million SNPs which are estimated to constitute 80% of all SNPs with frequencies of >10% (Altshuler *et al.*, 2008). SNPs are suitable for use in identification and kinship analysis in cattle populations. Advances in high throughput DNA sequencing, computer software and bioinformatics have facilitated the identification of a SNP marker from amplified amplicons. SNPs therefore are the fundamental unit of genetic variation and are attractive markers because they are abundant in cattle (Veneroni *et al.*, 2010; Heaton *et al.*, 2002).

Standardized sets of SNPs could be used to produce digital DNA signatures for animal tagging. After performing blind genotyping and allowing for a non-null error rate in the analysis, a minimal set of eight microsatellites could be kept, to ensure reliable traceability of bovine meat. Using this as a reference, a comparison with SNPs was done by drawing random bi-allelic markers assuming statistical independence with equal and uniformly distributed allelic frequencies (Heaton *et al*., 2002; Grosse *et al*., 1999). The presence of rare alleles leads to a dramatic fall in power, with the maximum power being reached with 50-60% allelic frequencies. A set of at least 30 uniformly distributed bi-allelic markers was necessary to obtain perfect individual traceability (Vignal, 2002).

SNPs also have some limitations. The number of SNPs required is four times the number of STR; this means that 60 SNPs are necessary to have similar discriminatory power than new STR multiplexes used in forensic science (Sobrino *et al*., 2005). So far paternity testing is simple and cost efficient using STRs. In addition knowledge on STRs has increased during the last 10 years and the information on mutations or polymorphisms in flanking regions for STRs are more available. This can be difficult for SNPs since a proper validation in population groups is required for an increased number of markers. Whether SNPs will replace STR's as a primary method of choice in forensics is not yet clear (Sobrino *et al*., 2005; Butler *et al*., 2007; Beecher-Monas, 2008).

2.2. Application of DNA markers

DNA Fingerprinting

DNA fingerprinting is the molecular genetic method that enables identification of individuals using hair, blood, semen or other biological samples. This method identifies individuals based on their unique DNA patterns. It was first described in 1984 by British scientist Alec Jeffreys that focused on sequences of DNA called minisatellites, which contain repeating patterns with no known function (Jobling and Gill, 2004).

The first direct application of DNA technology in beef cattle genetic improvement has been providing highly accurate forms of identity testing. By evaluating a panel of 10-

15 highly variable genetic markers, a unique genetic fingerprint of an individual can be obtained. This relatively simple technology is now routinely used for parentage determination and pedigree verification. As the genotyping process becomes faster and cheaper, DNA fingerprinting has become a more routine and important tool in beef breeding programmes (Collins *et al.*, 2003).

Different DNA fingerprinting methods exist using either RFLP or PCR-RFLP, targeting different areas of DNA with known variants such as SNPs, STRs and other various repeating polymorphic regions (Cohen, 1990). The odds of identifying an individual correctly depend on the number of repeating sequences. In forensics, the use of probes, which target regions of DNA that are specific to animals/humans, eliminate any possibility of contamination of DNA by bacteria, plants, insects or other sources (Tamaki and Jeffreys, 2005). Using only a single probe, the match probability can be estimated and two probes together can give a very low probability value, such that the only individuals sharing identical DNA fingerprints are monozygotic twins (Jobling and Gill, 2004).

In animals, DNA fingerprinting helps in effective animal identification which benefits agriculture with regard to traceability by facilitating efficient responses to disease outbreaks. DNA fingerprinting is used to resolve disputes in cases where samples were collected at the point of origin before a disease outbreak occurred. In the absence of pre-existing samples, DNA fingerprinting may identify the origin of animal disease if samples from a parent are available (Heaton *et al.*, 2002).

The efficiency of DNA fingerprinting lies in the accuracy, which is the ability of the test to detect an incorrect DNA profile. The overall accuracy evaluation is determined by the number of genetic markers examined, the degree of variation that exists for each genetic marker, and also if the genetic markers used show a large degree of variation (Burns, 2007).

Forensic DNA technology

Archimedes was the world's first forensic scientist when he proved that his king's crown was not pure gold by measuring its density. However in the late nineteenth century, Sir Arthur Conan Doyle first anticipated the use of science in solving crimes. At about the same time, Sir Francis Galiton's studies revealed that fingerprints are unique and do not change with age. In early 1858, a British official in India used imprints of inked fingers and hands as signatures on documents for people who could not write (http://science.jrank.org).

The use of DNA technology in forensic science is recent, but already has been well established and is a widely accepted branch of science (Vazquez *et al*., 2004). DNA markers have been shown useful in forensics since 1984 (Tamaki and Jeffrey's, 2005). The first application of DNA technology in forensics was in an immigration case. A boy from Ghana wanted to immigrate to Britain, claiming that his mother was already a resident. Conventional blood tests were not conclusive to confirm that the two could be related. DNA analysis however showed that the relationship could be claimed (Olivier, 2001).

Forensic DNA typing was also used in 1986 in England in the case of Collin Pitchfork, who was convicted of the sexual assault and murder of two teenage girls. In an attempt to identify a suspect, nearly 400 men of the local area between the ages of 13 and 34 were requested to give DNA samples for testing. Pitchfork was arrested before the forensic-DNA profile testing. The method that was used in this case was RFLP, which remains a widely used method of DNA identity testing. The DNA typing procedures proved that Pitchfork did indeed commit both crimes, and he was sentenced to two life sentences for murder (Friedman, 1999; Curran, 1997).

In another case a murder was solved when the suspect's DNA taken from saliva matched with the DNA swabbed from a bite mark on the victim. A masked rapist was convicted of forced oral copulation when his victim's DNA matched DNA swabbed from the suspect's penis six hours after the offence. Cases have been solved by DNA analysis of saliva on cigarette butts, postage stamps and from ski masks (when sampled from the area around the mouth opening) (Olivier, 2001).

Forensic science reflects a multidisciplinary scientific approach to examine crime scenes and evidence used in legal proceedings (Roffey and Harmon, 1993). It aims to assist judges and juries to solve legal issues, not only in criminal law but also in civil cases (Jobling and Gill, 2004). A basic principle in forensic science investigations is that a criminal always brings to and leaves something at the crime scene that can be used as proof of evidence for detectives and prosecutors. This includes fingerprints, footprints, tooth marks, blood, semen, hair, fibers, broken glass, a knife or a gun, a bullet or less tangible things such as the nature of wounds or bruises left on a victim's body, which might indicate the nature of weapon used or the method of assault (Norrgard, 2008; Jobling and Gill, 2004).

It was indicated that a forensic DNA profile does not represent the complete genetic make-up of an individual; rather it is a selection of DNA fragments which can be used as identification markers. The key to the usefulness of the DNA typing procedure is the fact that the use of an appropriate number and combination of probes demonstrates that each individual has a unique pattern, with the exception of identical twins (Loftus, 2005). One of the most important and central issues associated with forensics is the individuality of a DNA fingerprint. Unique identification with DNA typing is therefore possible provided that enough sites of variation are examined. However the DNA typing systems used today examine only few sites of variations and have only limited resolutions for measuring the variability of each site. Using 3-5 loci, a match between two DNA patterns can be considered strong evidence that the two samples came from the same source (Cummings, 2008). It is however important that the source of DNA is of good quality for reliable amplification.

The use of DNA testing in animal identification has been developed along with human identification. Population studies, kinship analysis, paternity testing and unique identification have been applied to humans, and have been adapted in animal identification (Cassidy and Gonzales, 2005). Animal evidence can be an important element when it is used to establish an association between the crime scene and a suspect, or a crime involving specific animals (Cassidy and Gonzales, 2005; Jobling and Gill, 2004). Many species-specific genotyping panels have been developed for most animal species. The recently sequenced bovine genome demonstrates a continued genetic exchange between cattle populations (van de Goor *et al.*, 2009).

Since the domestication of cattle several years ago, more than 50 distinct breeds have been identified. Since then forensic cases involving cattle such as identity forgery and cattle theft are relatively common (van de Goor *et al.*, 2009).

Parentage Verification

For centuries it has been difficult to prove and resolve paternities. During the Roman Empire children had no rights and they couldn't expect maintenance from their natural fathers. There was still a lack of clarity about the true mechanism of fathering even after the discovery of the anatomical structures until the 19th century (Albrecht and Schultheiss, 2004). Paternity identification is a process of excluding potential fathers/sires on the basis of their genotype and it is therefore important that DNA from all possible sires can be included in a paternity test (Evans and Van Eenennaam, 2005). The mode of inheritance of markers provide a powerful means for determining parent-offspring links and also serves as a useful tool for animal identification, particularly for verification of the semen used for artificial insemination (Mitra *et al.*, 1999). The classes of genetic markers that have proved most suitable are those that provide highly variable qualitative character states with known transmission properties (Avise, 1994). Genetic markers yield a much higher exclusion probability (> 90%) than testing with blood groups (70-90%) or other biochemical markers (40-60%) (Mitra *et al.*, 1999).

Several panels of markers are available for determination of parentage in farm animal species (Van Eenennaam *et al.*, 2007). In buffalo, studies showed that DNA fingerprinting with oligoprobes (OAT18 and ONS1) has been successfully used to determine the parentage of an *in vitro* fertilized (IVF) buffalo calf. With the introduction of PCR-based microsatellite assays, a large number of microsatellite panels have been reported that are useful for parentage testing in different livestock species (Mitra *et al.*, 1999; Gomez-Raya *et al.*, 2007). Recently SNPs have become an important type of marker for commercial diagnostic and parentage genotyping applications as automated genotyping systems have been developed that yield accurate genotypes (Rohrer *et al.*, 2007; Matukumalli *et al.*, 2009).

2.3. Polymerase Chain Reaction

The polymerase chain reaction developed by Kary Mullis in 1984, is a technique used to enzymatically amplify a specific DNA locus *in vitro* (Roffey and Harmon, 1993). It entails the exponential amplification of DNA fragments using oligonucleotide primers (Kashyap *et al.*, 2004). PCR allows amplification of a small amount of DNA from biological samples. Its ability to amplify such quantities of DNA enables even highly degraded samples to be analyzed (Luftig and Richey, 2001). The PCR process is similar to the mechanism by which DNA duplicates itself in a cell (Curran, 1997).

Early PCR-based genotyping systems targeted a small number of SNPs in the HLA-DQA1 gene. The system used was not effective due to a low discriminating power and mixtures were difficult to interpret and this led to the development of short tandem repeats (STRs). The use of STRs substituted the early PCR tests worldwide because of their high discriminating power, sensitivity and the ability to resolve simple mixtures, and the time needed to carry out an analysis was reduced (Jobling and Gill, 2004). Since then PCR has rapidly gained acceptance and has been adapted to a variety of applications in research, medicine, industry, agriculture and justice (Roffey and Harmon, 1993).

In PCR amplification, a specific region of DNA is selected based on known sequences of small pieces of DNA, generally 20 to 30 bases in length, called primers, which flank the selected region of interest. Primers are added to the DNA sample along with the DNA polymerase, an enzyme that polymerizes DNA monomers and free monomers. DNA polymerase facilitates the attachment of the complementary nucleotides to rebuild each strand, resulting in two double stranded molecules (Curran, 1997; Luftig and Richey, 2001).

The PCR amplification process consists of three steps: The double-stranded DNA molecule is denatured into single strands by incubation at high temperature [94 degree Celsius (°C)], the temperature is subsequently lowered to allow the primers to specifically bind to their complementary sequences adjacent to the target site at 55-65°C (primer specific temperature) and the temperature is again raised to 72°C which is the optimum temperature for polymerase enzyme activity, to allow the synthesis of

the DNA region flanked by the primers. After the completion of the synthesis, the temperature is again raised to denature the DNA strands so that the next cycle can begin. The process is repeated for about 30-35 cycles and the completion of each cycle doubles the number of each target DNA molecule, causing the target DNA to amplify exponentially (Roffey and Harmon, 1993; Luftig and Richey, 2001; Semagn *et al*., 2006).

After the amplification process the amplified DNA is separated to allow follow-up analysis on agarose gel electrophoresis, reverse dot blot, Polyacrylamide Gel Electrophoresis (PAGE) or automated electrophoresis. These are a few commonly employed methods for separating the amplified allele segments based either on their size or sequence. Accuracy, precision and rapidness are the important factors in PCR-based technology (Kashyap *et al*., 2004). PCR is useful in the genetic analysis and the diagnosis of hereditary diseases, the identification of genetic fingerprints (used in forensics and paternity testing), the detection and diagnosis of infectious diseases, and the creation of transgenic organisms (Curran, 1997).

In forensic science, the use of PCR has revolutionalised the process of criminal identification (Kashyap *et al*., 2004). Forensic science relies heavily on PCR technology to amplify specific sequences of DNA that will establish a connection between a specific suspect and a crime scene. The primary concern in forensics, using PCR on extracted DNA is to verify the validity of the DNA and the information it contains. Additional concern is whether tissue samples of different ages and conditions of preservation will give positive results and whether the information obtained is conclusive (Golenberg *et al.*, 1996).

DNA degradation

Biological samples collected from crime scenes may have been exposed to harsh environmental conditions such as heat, direct sunlight and water (Misner *et al*., 2009). Environmental exposure damages DNA by breaking the molecules into smaller pieces and this occurs as a result of endogenous endonuclease activity and spontaneous depurination. Damaged DNA blocks the extension step in PCR and the ability to recover large fragments via PCR reduces levels of DNA damage (Deagle *et al*., 2006).

Inhibitors of the PCR, such as some textile dyes, can also interfere with the ability to recover a full DNA profile from biological evidence (Misner *et al*., 2009). New DNA tests are being developed to recover information from smaller regions of DNA, which are more likely to be intact following DNA damage. These new DNA tests include miniSTRs (using PCR primers close to the STR repeat region) and SNPs. Whole genome amplification and DNA repair methods are also being evaluated to determine the possibility of enriching PCR amplifiable material from limited or damaged DNA templates (Misner *et al*., 2009).

Degraded DNA can also result in incomplete genetic profiles, resulting in allele dropout. It also blocks the progression of the DNA polymerase and increases miscoding, which can have an impact on forensic DNA analysis (Sikorsky *et al*., 2007). One approach to recover information from degraded DNA samples is to reduce the size of the PCR products, by moving primers in as close as possible to the STR repeat region (Butler *et al*., 2007).

2.4. The role of DNA polymerases in PCR

DNA polymerases are enzymes that help in DNA replication by catalyzing the polymerization of deoxyribonucleotide triphosphate (dNTP) using the template DNA strand (Li *et al*., 1998; Berg *et al*., 2002). The polymerase first binds to a primer/template, then a dNTP is incorporated loosely to the complex (Figure 2.4.1).

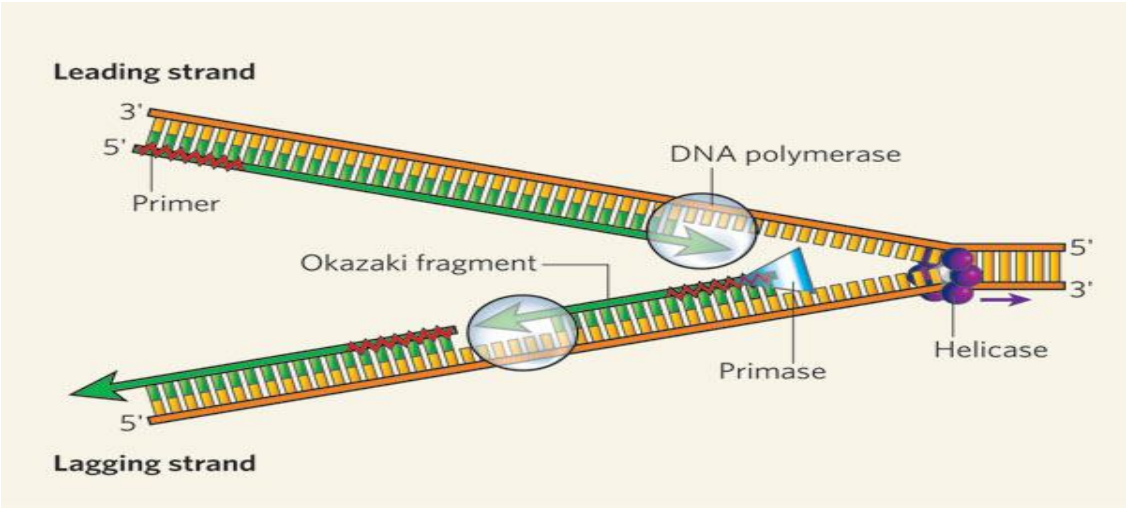

Figure 2.4.1. The role of DNA polymerase in DNA replication (taken from Bell, 2006).

Binding of the correct nucleotides leads to a conformational change, which converts the loose ternary complex into a tight activated complex capable of undergoing chemical bond formation. After the chemistry occurs, the pyrophosphate product is released and the DNA product is either translocated or dissociated (Berg *et al*., 2002).

DNA polymerase can only add free nucleotides to the 3´end of a newly forming strand, onto a pre-existing 3´-hydroxyl (OH) group. For this reason, during PCR DNA polymerase needs a primer (Figure 2.4.1) at which it can add the first nucleotide. This results in elongation of the new strand in a 5´-3´end direction (Berg *et al*., 2002; Mar Alba, 2001). Metal ions (e.g. Mg^{2+}) can participate as co-factors in the DNA polymerase reaction. One metal ion coordinates the 3'-hydroxyl group of the primer, whereas the phosphate group of the nucleoside triphosphate bridges between the two metal ions. The hydroxyl group of the primer attacks the phosphate group to form an O-P bond (Lodish, *et al*., 2000).

DNA must be replicated with high fidelity and each base added to the growing chain should be the complement of the base in the corresponding position in the template strand. The binding of the nucleoside triphosphate (NTP) containing the proper base is followed by the formation of a base pair, which is stabilized by specific hydrogen bonds. The hydrogen bonds linking two complementary bases make a significant contribution to the fidelity of the DNA replication (Berg *et al*., 2002).

Some DNA polymerases enhance fidelity of DNA replication by the use of proofreading mechanisms. This relies on the probability that the end of the growing strand with an incorrectly incorporated nucleotide will leave the polymerase site and move to the exonuclease site, where the added nucleotide is removed by hydrolysis. The mismatched bases are more likely to leave the polymerase site and proofread the sequence of the DNA being synthesized (Lodish *et al*., 2000; Berg *et al*., 2002).

Based on the sequence homology, DNA polymerase can be further divided into seven different families; A, B, C, D, X, Y and RT. These polymerases are involved in excision repair and processing of Okazaki fragments generated during lagging strand synthesis (Mar Alba, 2001). This is also shown in Figure 2.4.2.

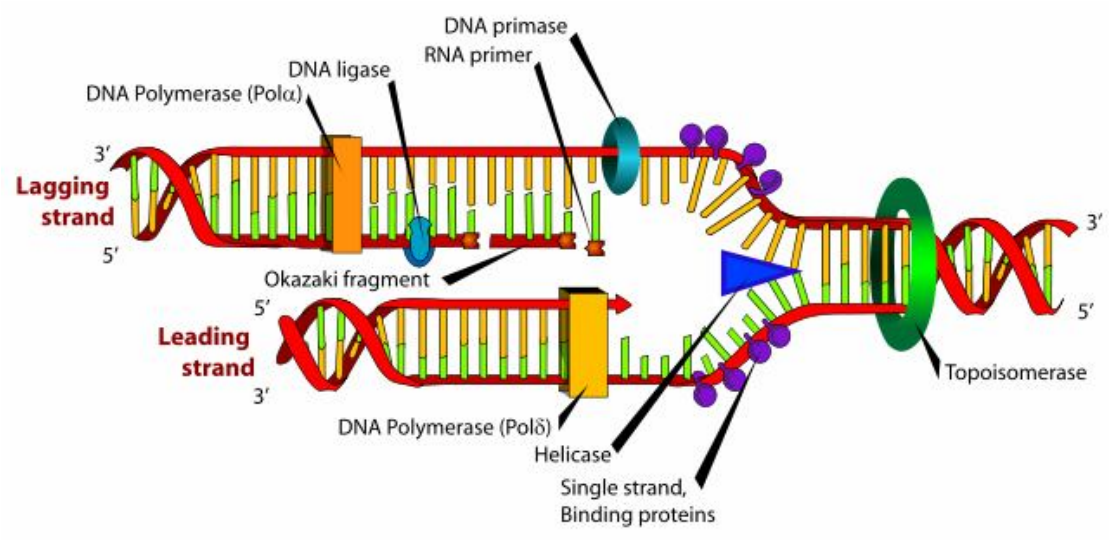

Figure 2.4.2. The role of DNA polymerase families on a DNA template during replication (taken from Campbell and Farrell, 2007).

Family B polymerases are involved in both leading and lagging strand synthesis and they include α (alpha), δ (delta) and ε (epsilon) and also include DNA polymerase encoded by some bacteria and bacteriophages. Family C is the primary bacterial chromosomal replication enzymes, e.g. DNA polymerase III. Family D is still not very well characterized and all known examples are found in Euryarchaeota and are thought to be polymerases that could be used in replication (Mar Alba, 2001).

Family X polymerase contains the well known eukaryotic polymerase pol β (beta), pol σ (sigma), pol λ (lambda) , pol μ (mu) and terminal deoxynucleotidyl transferase (TdT) (Figure 2.4.3). Family RT is the reverse transcriptase family containing examples from both retroviruses and eukaryotic polymerases. Family Y polymerases differ from others in having a low fidelity on undamaged templates and their ability to replicate damaged DNA. These polymerases can bypass the damage in an error-free fashion (McDonald *et al*., 2006).

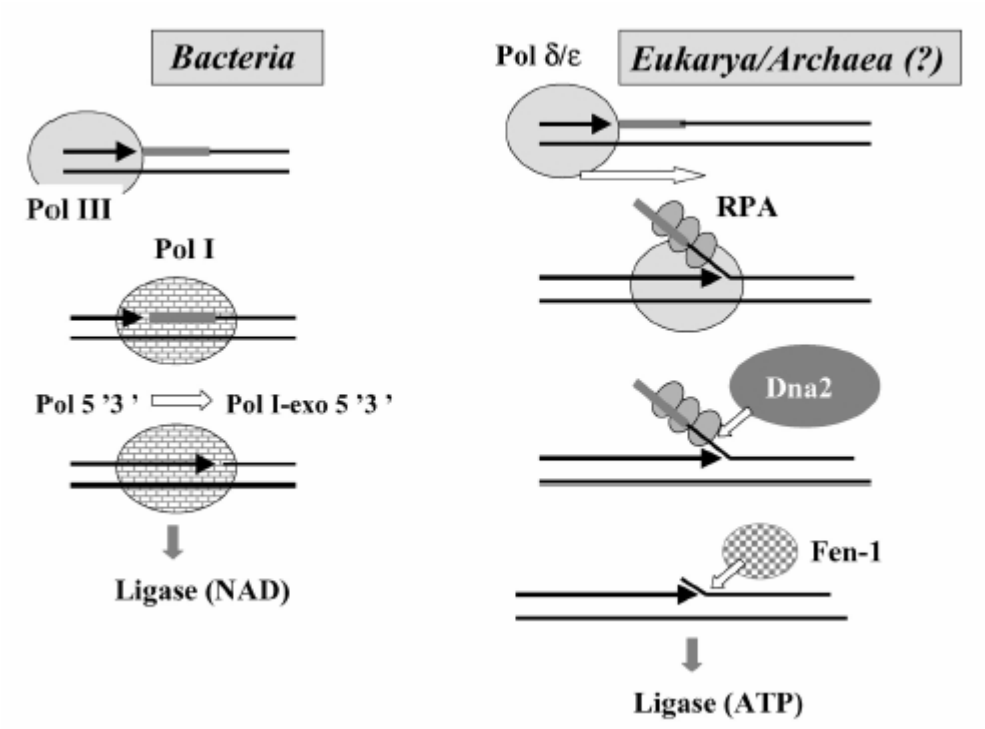

Figure 2.4.3. Family B, C and X polymerase (taken from Forterre *et al*., 2004).

Taq polymerase

Taq polymerase is a highly thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (Hajibabaei *et al*., 2005). It consists of a single polypeptide chain with a molecular weight of 95 kiloDaltons (kD) (Mburu and Hanotte, 2005). The enzyme catalyzes template-dependent polymerization of nucleotides into duplex DNA in the 5´-3´ direction. *Taq* DNA polymerase exhibits no detectable 3´-5´ exonuclease activity and it exhibits deoxynucleotidyl transferase activity, which results in addition of extra adenines at the 3´-end of PCR products (http://www.fermentas.com/catalog).

The discovery of *Taq* polymerase has been important for the development of PCR-based techniques. The original function of this enzyme was to facilitate *in vivo* replication of DNA in the thermophilic bacteria. Since then *Taq* polymerase has been known to operate at high temperatures, as required for the *in vitro* replication of DNA (Semagn *et al*., 2006). It is stable at high temperatures whereas other DNA polymerases become denatured. More complex PCR multiplex's that include one or more repair enzymes offer an improved alternative for the amplification of degraded DNA (Hajibabaei *et al*., 2005).

Restorase® DNA polymerase

The enzyme, Restorase®, represents one of the recently introduced commercial enzymes that couples Accu*Taq*, a high accuracy polymerase with a repair enzyme (Hajibabaei *et al*., 2005). DNA derived from aged or degraded samples is often highly fragmented due to autolysis, bacterial degradation and spontaneous depurination (Golenberg *et al*., 1996; Alaeddini, 2009). This fragmentation severely reduces the efficiency of PCR and the activity of DNA polymerase. It is expected that PCR of ancient or degraded DNA should only amplify small fragments because the template of DNA itself comprises only small fragments, and the amount of amplified product should be small compared with similar reactions with undegraded DNA (Mitchell *et al*., 2005; Golenberg *et al*., 1996).

DNA can be damaged by improper storage, aging, or exposure to acid, heat or light (Golenberg *et al*., 1996). Restorase® DNA polymerase was developed to enable the amplification of damaged DNA templates. It also increases amplification of undamaged templates, making it a powerful enzyme blend that is versatile (Skage and Schander, 2007).

Restorase® DNA polymerase combines Sigma' Long and Accurate enzyme technology with a DNA repair enzyme. This results in a blend that facilitates repair and amplification of damaged DNA. Restorase® is often used to amplify templates that have failed amplifications using standard PCR enzymes. It modifies the damaged sites allowing subsequent template copying. The level of template damage dictates optimal Restorase® treatment of the DNA. DNA repair begins the moment the enzyme is added to the multiplex and ends when the reaction is heated to >50°C. Restorase® can have the ability to amplify damaged DNA, but some templates are irreversibly damaged (www.sigmaadrich.com).

FastStart High Fidelity PCR System

The FastStart High Fidelity PCR System is a novel blend of chemically modified *Taq* DNA polymerase and a thermostable protein which allows for proofreading of the synthesized DNA. The thermostable protein mediates proofreading activity, but does not have polymerase activity itself. FastStart High Fidelity PCR System contains a *Taq* polymerase enzyme which can amplify fragments up to 5 kilo-base pairs (kbp)

from all types of DNA with high yield, fidelity and accuracy. This unique blend also incorporates deoxyuridine triphosphate (dUTP) and when combined with Uracil-DNA Glycosylase, can be used to protect PCR reactions against cross contamination. It is also highly suitable for the labeling of PCR products with modified nucleotides such as digoxygenin (DIG)-dUTP, biotin-dUTP, and fluorescein-dUTP (www.roche-applied-science.com).

The FastStart High Fidelity PCR System produces better yields with fewer cycles as compared to other systems. This is due to the mechanism of the proofreading protein and how the FastStart *Taq* DNA polymerase is processed. The role of the proofreading activity is to reduce the number of prematurely terminated amplification products formed which in turn increases the yield of full-length product. It also increases the sensitivity by allowing detection of 1 nanogram (ng) of complex templates (www.roche-applied-science.com).

2.5. Conclusion

Forensic DNA profiling has become an accepted and vital technology in forensic science. It can answer important questions of identity and lineage from the decades and even centuries through the use of the genetic code (Curran, 1997). However, the type of genetic material used plays an important role in this process. This study will aim to address some of the challenges regarding forensic DNA profiling on aged and or degraded DNA which can have an impact on identification of the animal or individual.

Chapter 3

3. Materials and Methods

Bovine meat samples were collected from ARC abattoir and exposed to different environmental conditions, in winter and in summer, in the sun and also in the shade. DNA was extracted from the bovine samples and PCR was performed using three polymerase enzymes, *Taq* polymerase, Restorase® and FastStart High Fidelilty PCR System. This was done in order to compare the efficiency of these polymerases even on degraded samples that were exposed for 15 days in the environment. Samples exposure to the environment was done in order to obtain a complete degradation of the samples so as to mimic those samples that are collected in the crime scene.

3.1. Sample preparation, degradation and collection

Two kilograms (kg) of meat from the silver side portion of Bonsmara beef cattle was obtained from the abattoir in Animal Nutrition Section, ARC-Irene, Pretoria. Thirty two samples each weighing 2.0 grams (g), having the same size and shape were cut and put into sealed containers (Figure 3.1.1).

In order to initiate degradation, 15 samples were placed in the field at ARC-Irene, Animal Genetics Section and exposed to sun, while another 15 samples were exposed to shady conditions for 15 days. Two fresh samples were put in -20 degrees (ºC) freezer and were used as controls (day 0). Samples were collected daily for 15 consecutive days (Figure 3.1.1) and were stored in the freezer immediately after sampling.

Figure 3.1.1. Sample degradation in sunny and shady conditions behind the ARC Genetics building.

In order to determine the effect of the season on the samples, samples were exposed at the locality described above during winter (May 2008) and during summer (November 2008). Temperature, humidity, wind speed and rainfall were monitored (data provided by the South African Weather Services (SAWS), Irene, Pretoria) on a daily basis in order to determine the environmental effects on the samples. Characteristics such as morphology and texture of the sample were also observed in the process of degradation. On completion of the degradation process, the samples were removed and were stored at -20°C. Aliquots (0.1g) from each sample, starting at day 0 till day 15, were taken and prepared for DNA extraction.

3.2. DNA extraction and selection of markers

DNA extraction and purification was performed according to the manufacturer's standard protocol using a High Pure PCR Template DNA extraction kit (Roche). DNA was quantified using a nanodrop (Thermo Scientific).

A total of 16 bovine microsatellite markers were selected for PCR amplification (Table 3.3.1). These microsatellite markers were recommended by the International Society of Animal Genetics (ISAG) for use in bovine mapping and were selected and optimized in ARC for daily use. These markers were selected because of their known high polymorphic state, high heterozygosity, large number of alleles and are conserved (van de Goor *et al*., 2009; Navani *et al*, 2002). These markers have no known null alleles and are suitable for multiplex PCR. These markers cover different chromosomes numbers with base pairs ranging between 35 and 350bp.

Table 3.3.1. Summary of the 16 cattle microsatellite markers and their sequences used to type bovine forensic samples (van de Goor *et al.*, 2009)

Msat ID	Chrom no.	Size Range (bp)	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
TGLA227	18	176-198	CGAATTCCAAATCTGTTAATTTGCT	ACAGACAGAAACTCAATGAAAGCA
BM2113	2	111-129	GCTGCCTTCTACCAA ATA CCC	CTTCCTGAGAGAAGCAACACC
TGLA53	16	207-211	GCTTTCAGAAATAGTTTGCATTCA	ATCTTCACATGATATTACAGCAGA
ETH10	5	138-142	GTTCAGGACTGGCCCTGCTAACA	CCTCCAGCCCACTTTCTCTTCTC
SPS115	15	204-216	AAAGTGACACAACAGCTTCTCCAG	AACGAGTGTCCTAGTTTGGCTGTG
TGLA126	20	225-252	CTAATTTAGAATGAGAGAGGCTTCT	TTGGTCTCTATTCTCTGAATATTCC
TGLA122	21	142-150	CCCTCCTCCAGGTAAATCAGC	AATCACATGGCAAATAAGTACATA
INRA23	10	71-77	GAGTAGAGCTACAAGATAAACTTC	TAACTACAGGGTGTTAGATGAACTC
BM1818	23	104-132	AGCTGGGAATATAACCAAAGG	AGTGCTTTCAAGGTCCATGC
ETH3	19	133-193	GAACCTGCCTCTCCTGCATTGG	ACTCTGCCTGTGGCCAAGTAGG
ETH225	9	89-131	GATCACCTTGCCACTATTTCCT	ACATGACAGCCAGCTGCTACT
BM1824	1	248-276	GAGCAAGGTGTTTTTCCAATC	CATTCTCCAACTGCTTCCTTG
CSRM60	10	79-115	AAGATGTGATCCAAGAGAGAGGCA	AGGACCAGATCGTGAAAGGCATAG
HAUT27	26	120-168	TTTTATGTTCATTTTTTGACTGG	AACTGCTGAAATCTCCATCTTA
CSSM66	14	171-209	ACACAAATCCTTTCTGCCAGCTGA	AATTTAATGCACTGAGGAGCTTGG
ILST006	7	277-309	TGTCTGTATTTCTGCTGTGG	ACACGGAAGCGATCTAAACG

3.3. PCR Amplification

The 16 markers were optimized for a multiplex as shown in Table 3.3.2 for the three enzymes tested in this study. The amount varied depending on the enzyme used during optimization.

PCR protocol

Three protocols were applied using the three different polymerase enzymes. The concentration of all the markers used in each plex was 10 picomoles (pmol). With *Taq* polymerase, a standard protocol was followed with optimized primers. The protocol was used on a regular basis in the lab.

Table 3.3.2. Bovine Multiplex for *Taq*, Restorase® and FastStart DNA polymerase

Msat ID	Colour	Plex X5 (µl)	Plex X7 (*Taq* & Fas) (µl)	Plex X7 (Res) (µl)
TGLA227	Fam	0.85	1.1	2
BM2113	Fam	0.6	0.7	0.6
TGLA53	Fam	1.6	1.15	3
ETH10	Fam	0.7	0.9	0.7
SPS115	Fam	1.5	2.5	1.5
TGLA126	Vic	1.6	4.4	3
TGLA122	Vic	0.4	0.6	1
INRA23	Vic	1.35	1.35	2
BM1818	Vic	1.26	0.63	1.26
ETH3	Ned	0.5	1.8	0.5
ETH225	Ned	0.76	0.5	1.5
BM1824	Ned	0.75	0.76	0.75
CSRM60	Pet	0.63	1	1.5
HAUT27	Pet	0.93	1.3	0.93
CSSM66	Pet	1.26	1.6	1.26
ILST006	Pet	1.95	5.15	3
Total		**16.64**	**25.44**	**24.5**
Water		**184.01**	**174.56**	**175.5**

[Refer to text for plex concentrations]

For a reaction of 7.5µl with 0.5µl of template DNA, the following mixture was added to one tube as a reaction mix, 1.0µl PCR Primer solution, 0.4µl *Taq* [5 units (U)/µl SuperTherm Gold (Southern Cross)] 0.75µl dNTP (2.5mM), 1.5µl 10X Reaction buffer (15 millimolar (mM) $MgCl_2$, 100mM Tris (Hydroxymethyl-aminomethane Hydrochloride (Tris-HCl, pH 8.3 at 25ºC, at 25 ºC), 3.67µl H_2O and 0.18µl Tween 20 (1%). Template DNA (0.5µl) was added to each of the reaction tubes and the reaction tubes were placed into the thermal cycler (Applied Biosystems, Model 9700) and conditions consisted of : 11 minutes 95 ºC Hot-Start polymerase activation step, 32 cycles of : denaturation in 45 seconds 94 ºC, 45 seconds 61 ºC annealing step, 60 seconds 72 ºC extension step. This was followed by a final extension at 72 ºC for one hour after which the reaction was cooled to 4 ºC prior to sample preparation for fragment analyses on a DNA sequencer.

The same protocol was adapted using Restorase® and FastStart and differences were shown in Table 3.3.3 with the conditions for the multiplex and differences between the protocols printed in bold.

Table 3.3.3. Summary of the PCR amplification protocol using *Taq*, Restorase® and FastStart DNA polymerase

	Enzymes		
Multiplex	***Taq* Polymerase**	Restorase®	**Faststart DNA polymerase**
Primer mix (µl)	1	**0.75**	1
10x Buffer, with $MgCl_2$ (µl)	1.5	**0.75**	1.5
dNTPs (2.5mM) (µl)	0.75	**0.75**	0.75
Taq (µl)	0.4	**0.38**	0.4
Water (µl)	3.67	**4.87**	3.67
1%Tween 20	0.18	-	-
DNA (µl)	0.5	0.5	0.5
Final volume (µl)	**7.5**	**7.5**	**7.5**

The PCR programmes used on different enzymes are shown in Table 3.3.4. PCR program used on *Taq* polymerase and FastStart was the same with the additional cycles on FastSstart.

Table 3.3.4. PCR programmes used for different enzymes

	Enzymes		
Multiplex	***Taq* Polymerase**	Restorase®	**Faststart**
Preincubation	-	**37 ºC: 10min** **72 ºC: 5min**	-
Initial denaturation	-	**94 ºC: 30s**	-
Activation step	95 ºC: 11min	**70 ºC: 5min**	95 ºC: 11min
Danaturation	94 ºC: 45s	**94 ºC: 30s**	94 ºC: 45s
Annealing	61ºC: 45s	**61 ºC: 30s**	61 ºC: 45s
Extension	72 ºC: 60s	72ºC: 60s	72 ºC: 60s
Final extension	72 ºC: 1h	**72 ºC: 10min**	72 ºC: 1h
Final hold	4 ºC: ∞	4 ºC: ∞	4 ºC: ∞
No. of cycles	**32**	**35**	**35**

3.4. Fragment analyses through capillary gel electrophoresis

Capillary electrophoresis is an analytical and micro preparative tool use for DNA sequencing. It provides faster separation at high resolution and with great separation efficiencies than conventional electrophoresis (Swerdlow and Gesteland, 1990). From the PCR products, 1µl was added to 9µl [Liz 500 (6µl) + Formamide (168µl)] in the MicroAmp Optical 96-Well Reaction plate. Reaction mixtures were loaded as a single injection onto an ABI 3130xl DNA sequencer (Applied Biosystems) following denaturation at 94^{o}C for 4 minutes. PCR products were separated by size and dye colour through capillary gel electrophosesis. This was followed by laser-induced fluorescence with multiwave length detection. A size standard (Liz 500) containing DNA fragments of known size was labeled with a different dye colour and was elecrophoresed with each sample to caliberate fragment sizes. The collected data in a form of electrophorograms were analysed by software that automatically determines allele sizes on a standard curve produced from the size standard.

3.5. Genotypic analysis

GeneMapper software version 4 (Applied Biosystems) was used for genotypic analysis. The software provides an alternative method for obtaining high quality annotations of genomes by transferring reference annotations (Chatterji and Patcher, 2006). GeneMapper Software Version 4 provides quality allele calls for all Applied Biosystems electrophoresis-based genotyping systems. It analyses microsatellite markers that contain mono-, penta-, and hexa-nucleotide repeats as well as the existing di- and tetra-nucleotide repeat functionality (Biosystem Product Catalogue, 2008).

Genotypic analysis was done using the manufacturer guide on microsatellites analysis using GeneMapper software version 4 (Applied Biosystems). The panel of markers was created with different colours for each marker as indicated in Table 3.3.2. A standard (Liz 500) was labeled with red colour. To set up the sample analysis, a new project was created. The analysis parameters and the table were specified in the sample tab with Size Standard: Liz 250, Panel: ShoniBov, Analysis Method: ForensicBovine, Instrument: Frg36pop7dailyrun, Results group: Finzymekitbov. In

order to analyze and examine the data, the samples in the project were analysed. The size quality and the size-calling data were reviewed and the data was examined though sample plots.

3.6. Statistical Analysis

Statistical analysis was done on the Mainframe Computer of the University of Pretoria using SAS V8.2 software running under VM/CMS, using PROC LOGISTIC within SAS as a specific procedure. PROC LOGISTIC is a common technique used to describe how a binary response variable is associated with a set of explanatory variables from independent trials (binomial counts) or in 1/0 observation (success/failure) data sets. The model also provides the odds ratio and the estimated adjusted odds ratio for a given predictor as well as approximate confident intervals (Downer and Richardson, 2009). In this study the PROC LOGISTIC with CLASS varbs from data set was done using a linear regression model. Linear regression attempts to model the relationship between two variables by fitting a linear equation to observed data. It is calculated by a formula Y=a+ßX where X is the explanatory variable and Y is the independent variable. The log odds of success versus failure was determined and was calculated as the exponentiation of the parameter estimate for the independent variable by the number e (Log Ratio = $e^{b} = 2.71^{b}$) (Karp, 2000; Guido, 2006).

Chapter 4

4. Results

Bovine tissue samples were degraded during winter and summer, both in the shade and direct sunlight over a period of 15 days. Results were generated using a standard set of primers for forensic analyses. Three different *Taq* polymerase enzymes were tested and genotypes were analysed and compared for enzyme activity on degraded bovine samples.

4.1. Sample degradation

In order to compare the level of sample degradation under different environmental conditions, bovine beef samples were degraded in both shady and sunny conditions in both the winter and summer season. It was found that sample degradation differed between winter (average maximum temperature of 16.2°C) and summer (average minimum temperature of 19.5°C) (Table 4.1.1). Even in winter, samples in the sun degraded faster as compared to the shade. In the shade the temperature remained cold throughout the day and this resulted in samples being preserved throughout the day, with a lower level of degradation.

Table 4.1.1. Weather Data as obtained from South African Weather Services

(a) Winter collection Data

Date (May 2008)	Average Temperature (°C)	Humidity (%)	Rainfall (mm)	Wind Speed (m/s)
8	16.1	55.6	0	2
9	17.1	50.8	0	1.9
10	17.1	52.2	0	2.1
11	18.4	48	0	3.5
12	15.7	36.8	0	3.9
13	17.6	34.8	0	3.2
14	16.6	42	0	2.3
15	16.1	55.8	0	2.1
16	17.6	52.9	0	2.1
17	18.3	48.2	0	3.5
18	14.9	70	0	2.6
19	15.4	68.2	0	1.6
20	14.2	64.6	0	2.2
21	13.3	57.7	0	1.8
22	14.5	49.3	0	1.8
Average	**16.2**	**52.5**	**0**	**2.4**

(b) Summer collection data

Date (Nov 2008)	Average Temperature (°C)	Humidity (%)	Rainfall (mm)	Wind Speed (m/s)
6	22.8	52.1	0	2.9
7	19.5	76.6	27.2	4
8	17.1	88	11	3.1
9	14.8	95.5	0.6	3.8
10	18.1	87.9	3.8	3.2
11	17.1	97.4	28	1.5
12	20.3	85	4.4	3.2
13	19.9	87.7	23	2.6
14	21.2	85.7	5.8	2.9
15	21.6	67	0	2.6
16	22.3	72.6	0.6	3.2
17	21.9	73.9	4.4	3.7
18	19.8	70.8	10	4.2
19	17.7	85.9	0.4	3.7
20	19.1	77.9	0	2.2
Average	**19.5**	**80.3**	**7.9**	**3.1**

Figure 4.1.1 represents a fresh sample from day 0 and degraded samples in day 15. In the sun during summer, the higher temperature resulted in faster degradation of the samples which was evident from the formation of moisture inside the sample tubes and the change of sample colour. The samples shrank and lost its initial shape and became sticky (Figure 4.1.2). Summer temperatures resulted in even faster degradation in the shade and in the sun samples tended to dry out. In Figure 4.1.2 the samples in the sun and in the shade during the process of degradation are shown. This is also seen in Table 4.1.2 and in Figure 4.1.3 wherein the samples showed a difference in the level of degradation. Samples exposed to the sun were totally dry on day 15 as compared to the samples exposed in the shade that were degraded and had a distinct odour.

Table 4.1.2. Sample degradation in the shade and in the sun during summer

No of samples	**No of days**	**Mass (g)**	**Level of degradation**	**Texture/Colour**
15 (Shade)	15	2.0g	Normal to partially degraded	Red-Brown & Silky
15 (Sun)	15	2.0g	Normal to partially degraded to Dry	Red-Brown & Dry

Figure 4.1.1. Fresh samples of 2.0g on day 0, before exposure to the environment and degraded samples in day 15.

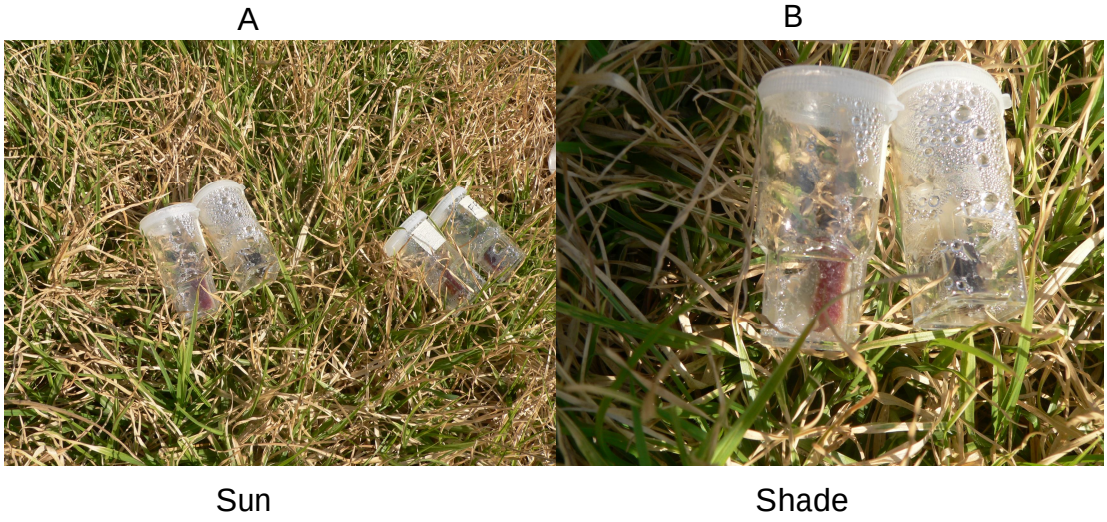

Figure 4.1.2. The degradation of samples during winter from the shade and the sun on day 15 as compared to the fresh samples (Figure 4.1.1).

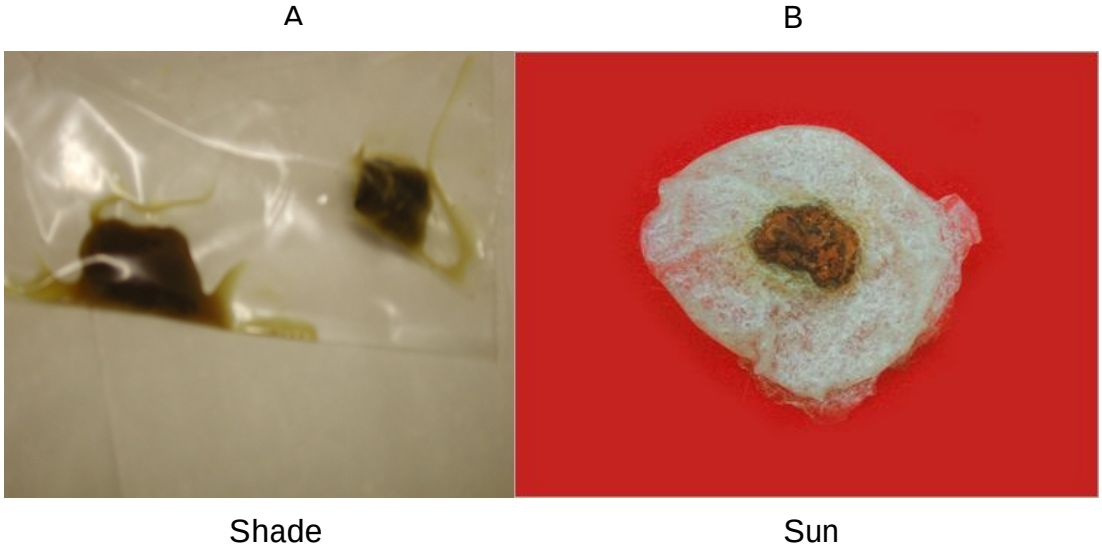

Figure 4.1.3. The degradation of samples exposed to summer conditions on day 15 (Figure 4.1.1).

DNA extraction and quantification

In this study spectrophotometry was used to determine the DNA quantity and quality. Table 4.1.3 indicates the difference in the DNA concentration of the samples degraded during winter and summer. The concentration of DNA quantity varied from day 1 to day 15 and the yield was relatively high compared to the samples degraded in summer.

It was still possible to obtain a reasonable quantity of DNA after day 15 for samples degraded during winter, while the samples degraded in summer had a higher level of degradation with a decrease in concentration of 117ng/µl to only 26.40ng/µl on day 15. Nucleic acid has the maximum absorption at 260nm and the quality of DNA is determined by the ratio 260/280nm with the ratio ranging between 1.8 and 2.0. Quality also deteriorated as indicated by the 260/280 ratio from 1.86 to 1.33.

Table 4.1.3. DNA quantification of samples degraded during winter and summer in the shade

No. of Days	**Sample No.**	**DNA concentration (ng/µl)**		**260/280 ratio**	
		Winter	Summer	Winter	Summer
1	1	137.41	117.05	1.44	1.86
2	2	163.42	108.40	1.56	1.56
3	3	94.95	91.63	1.35	1.35
4	4	45.11	96.99	1.66	1.66
5	5	63.77	88.29	1.63	1.63
6	6	49.80	68.45	1.43	1.43
7	7	57.22	66.98	1.38	1.38
8	8	35.14	59.84	1.53	1.53
9	9	74.86	57.22	1.62	1.62
10	10	68.45	54.88	1.63	1.63
11	11	57.45	50.75	1.50	1.50
12	12	51.10	59.80	1.18	1.18
13	13	48.98	44.87	1.59	1.59
14	14	50.75	35.14	1.26	1.26
15	15	49.50	26.48	1.33	1.33
0	**Cntrl1**	**97.20**	**105.20**	**1.89**	**1.85**

In Figure 4.1.4 and 4.1.5 the concentration of DNA on samples degraded in the shade during summer and winter are shown. There was a decrease in DNA quantity as samples degraded, but the decrease was not constant as compared to the samples that degraded during summer.

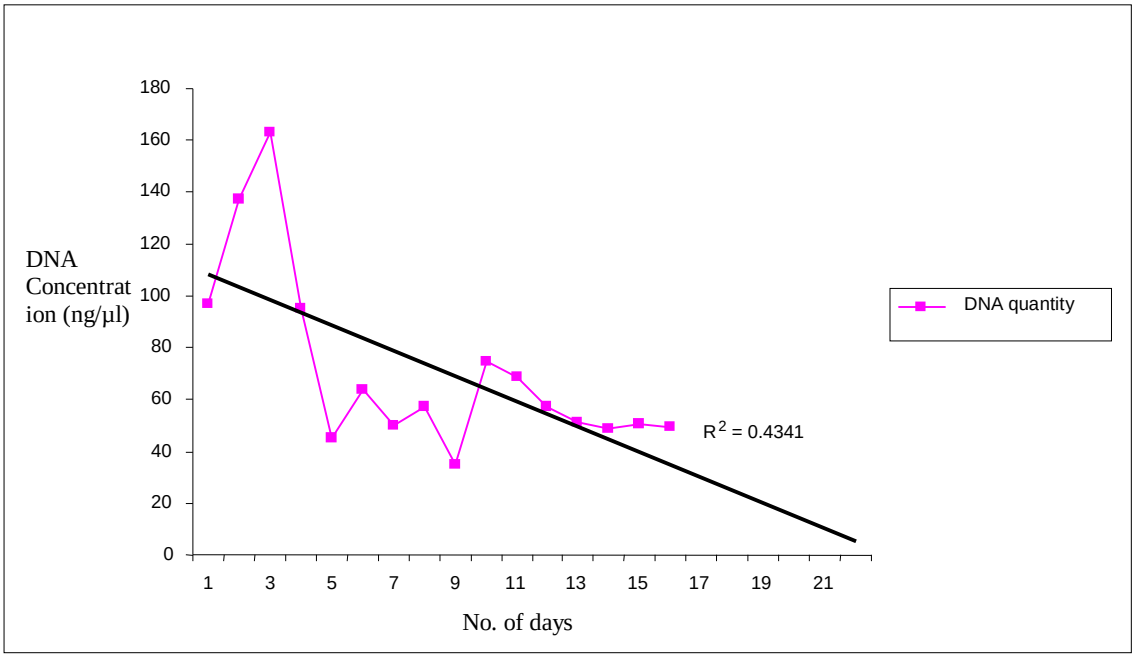

Figure 4.1.4. DNA concentration obtained from samples degraded during winter. DNA concentration decrease was not constant.

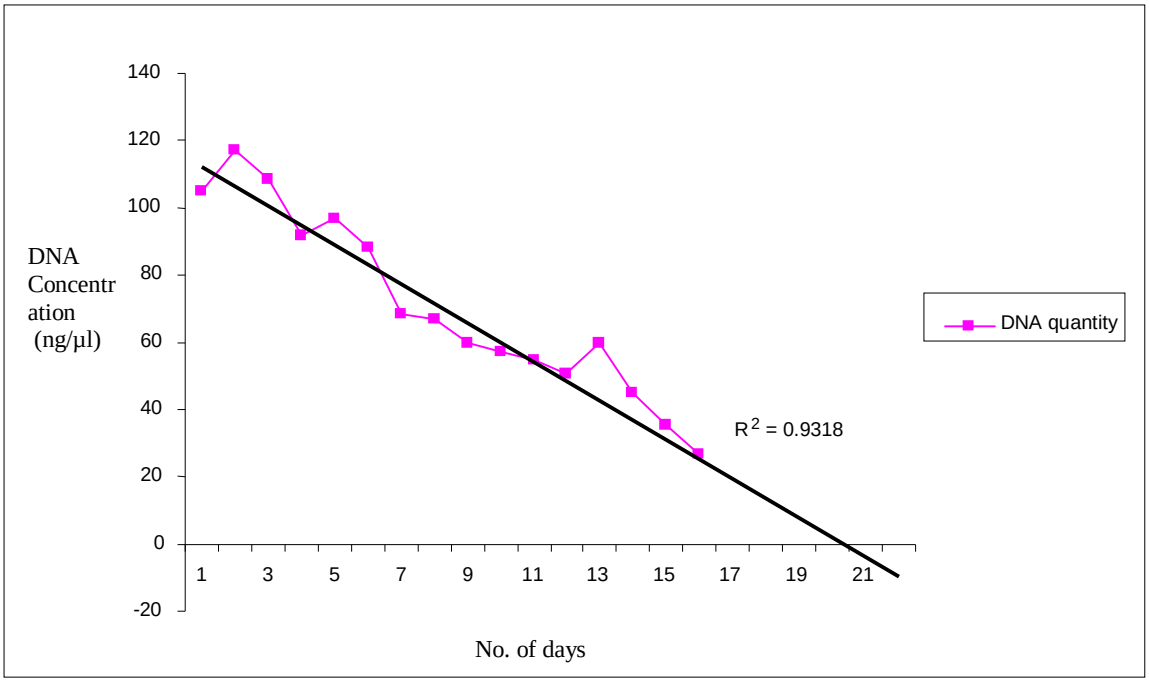

Figure 4.1.5. DNA concentration obtained from samples degraded during summer. The concentration of DNA decreased as the number of days increased.

4.2. PCR Amplification Success

The DNA of the degraded samples was tested and *Taq* polymerase had an amplification success of 100% in all 16 microsatellites markers used. Restorase® amplified only 88% of all markers and FastStart amplified only 10 microsatellites markers with an amplification success of 69% (Table 4.2.1).

Table 4.2.1. A summary of amplification success from all polymerase enzymes for 15 days during summer

Amplification Success				
Enzyme	**No. of markers worked**	**No. of markers failed**	**Markers with poor amplification**	**Amplification Success**
Taq Polymerase	16/16	0	-	100%
Restorase®	14/16	2	9,10	88%
FastStart	10/16	6	2,13,14,15,16	69%

The amplification success was also confirmed by testing a success model (prologistic output) of enzymes in all markers (Table 4.2.2). The activity of FastStart was low with the negative estimate value of -0.4259 as compared to *Taq* polymerase and Restorase®.

Table 4.2.2. Prologistic output of success model on DNA amplification using different enzymes

Enzymes	**Estimate**	**StdErr**	**WaldChiSq**	**Prob ChiSq**
Intercept	*1.7262*	*0.0962*	*332.2039*	*<0.0001*
Taq Polymerase	0.224	0.0813	7.5889	0.0059
Restorase®	0.2019	0.0815	6.1369	0.0132
FastStart	**-0.4259**	0.0787	29.2975	<.0001

There was a significant difference in the enzyme activity as indicated by the probability Chi-Square (ChiSq) value of <0001 on FastStart. There was no significant difference in Restorase® and *Taq* polymerase activity.

4.3. Fragment analysis using *Taq* polymerase

DNA fragments were analysed based on the allele frequencies, peak heights, specificity of peaks and the peaks artifacts. On day 1 to 6 of degradation peaks were clear with no artifacts and there were no non-specific peaks. As the sample started to degrade it lost quality, non-specific peaks and artifacts started to appear and the peak heights were lower. Figure 4.3.1 shows the peaks of the samples that were degraded in winter; there was no major difference in peak heights even on day 10 of sample degradation.

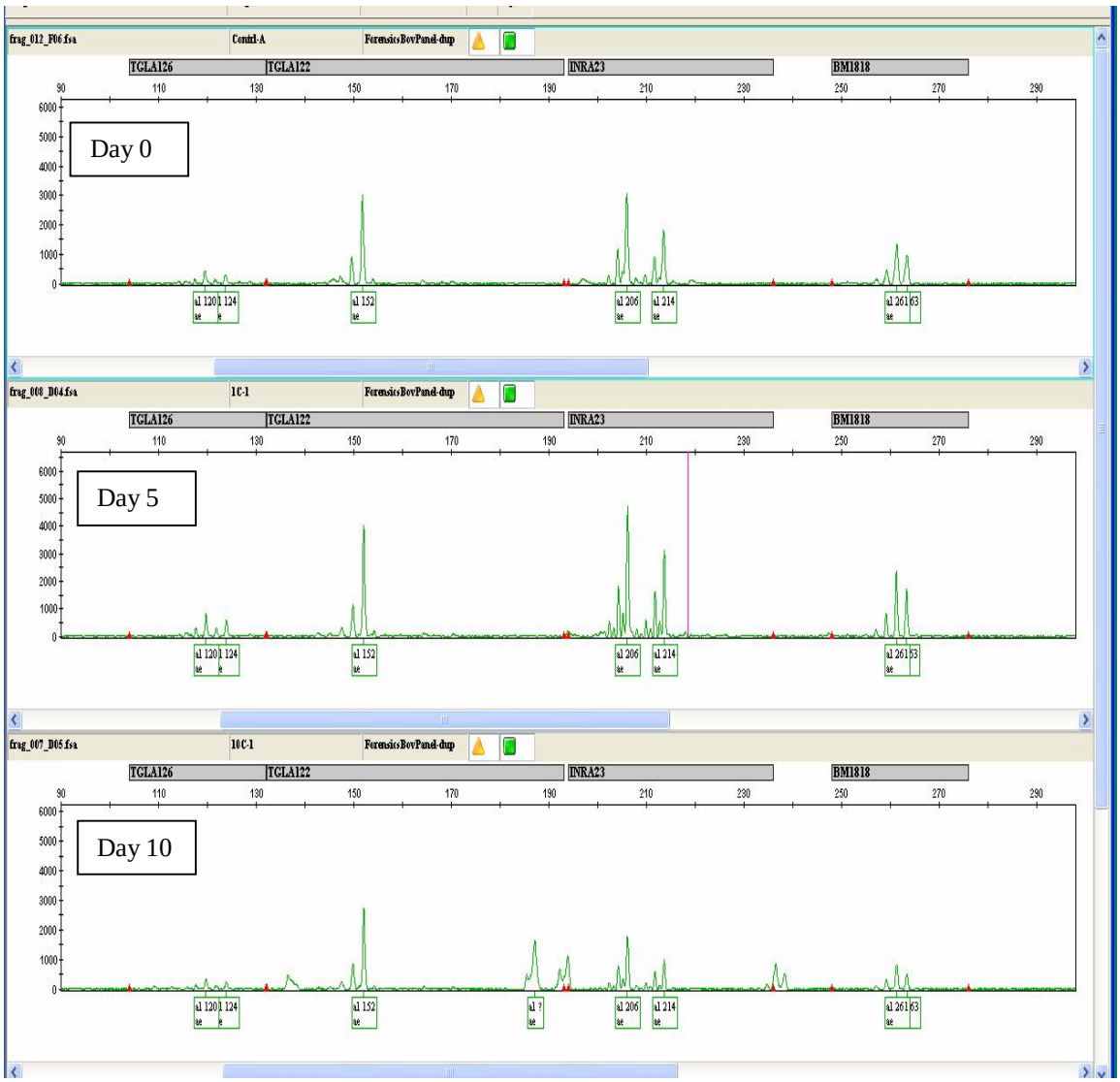

Figure 4.3.1. Chromatogram showing the genotyped profile of samples amplified with *Taq* polymerase. Samples were exposed to winter temperatures and DNA extracted, amplified and genotyped using marker TGLA126, TGLA122, INRA23 and BM1818 (from left to right) on day 0, 5 and 10.

The peaks were clear with no artifacts, few non-specific peaks and there was no allele drop-out from day 0-10. This means that there was limited damage of DNA in samples degraded in winter within the days of the experiment.

In Figure 4.3.2 there was a sudden allele drop-out on day 5 and 10 in samples degraded in summer in the same microsatellites markers (TGLA126, TGLA122, INRA23 and BM1818) as in Figure 4.3.1 on day 0, day 5 and day 10.

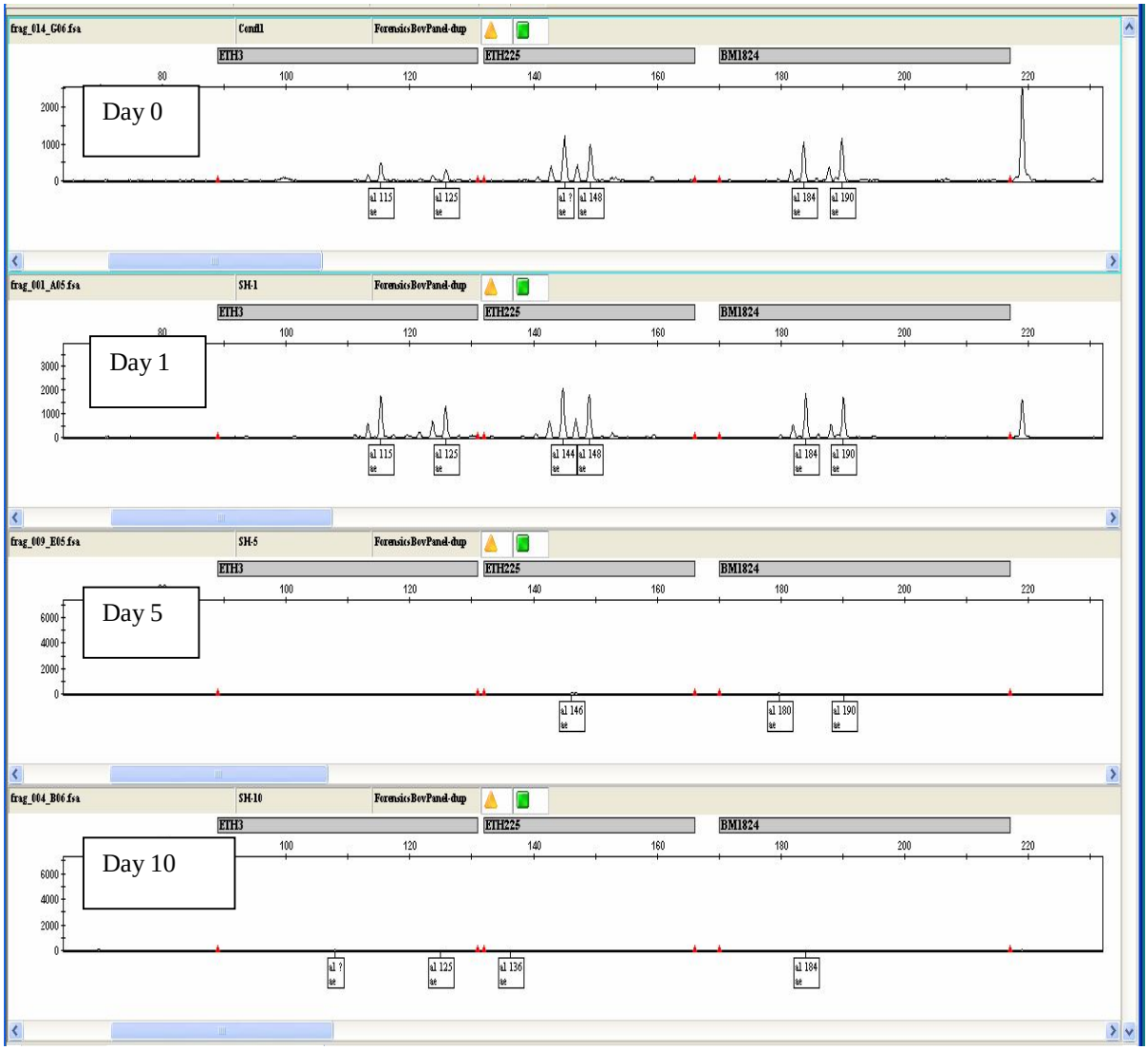

Figure 4.3.2. Peaks from samples exposed to summer temperatures amplified with *Taq* polymerase. Marker TGLA126, TGLA122, INRA23 and BM1818 are shown for day 0, 5 and 10 respectively.

Table 4.3.1 also summarizes the influence of fragments sizes (allele frequencies) of degradation of samples during summer and during winter. A good quality sample resulted in more accurate genotypes. Poor samples tend to yield poor results which were difficult to analyze and gave inconclusive results.

Table 4.3.1. Influence of the level of degradation on the fragment sizes during summer and during winter for marker BM1818

Summer Level of degradation using BM1818					
No. of days	**Sample no.**	**Allele1 (bp)**	**Allele 2 (bp)**	**Height 1 (RFU)**	**Height 2 (RFU)**
1	1	261	263	2481	1695
3	3	261	263	60	45
5	5	0	0	0	0
10	10	0	0	0	0
Winter					
1	1	261	263	2367	1695
3	3	261	263	6138	4319
5	5	261	263	4762	3341
10	10	261	263	811	502

In Table 4.3.2 the difference in peak heights between the samples prior to degradation for markers BM1818 and BM1824 were shown. There was an indication of increase in level of degradation from day seven depending on different markers used.

Table 4.3.2. Influence of the level of degradation in the sun and in the shade during summer using *Taq* polymerase for marker BM1818 and BM1824

	Marker 1 (BM1818) (Homozygous)		**Marker 2 (BM1824) (Heterozygous)**			
	Sun	**Shade**	**Sun**		**Shade**	
	Peak Heights (RFU)		**Peak Heights (RFU)**			
No.of sample/days	Allele 1	Allele 1	Allele 1	Allele2	Allele 1	Allele2
1	1969	3323	1767	1612	2750	2675
2	1873	2970	1621	1479	2326	2141
3	1780	2905	1433	1322	2269	2044
4	1743	2634	1355	1273	2210	2032
5	1195	1768	1329	1165	1499	1398
6	1015	1609	1175	971	1288	1119
7	**982**	**1296**	**1099**	**875**	**1238**	**1078**
8	951	1209	968	850	1117	1045
9	940	1148	869	732	944	945
10	877	1028	817	749	887	930
11	713	605	784	677	857	772
12	244	538	712	680	535	477
13	162	386	583	829	435	440
14	0	235	409	394	253	276
15	0	171	100	56	224	215

An increase in the level of degradation was seen by the drop of peak height over time, followed by allele dropout on day 15 (Figure 4.3.3 and 4.3.4). There was a clear difference in samples that were degraded in the sun and the sample degraded in the shade in both microsatellites markers, BM1818 and BM1824.

In Figure 4.3.3 and 4.3.4 a clear trend can be seen that the peak heights tend to decrease around day 6 in the sun and in the shade, but with a smaller difference in the shade.

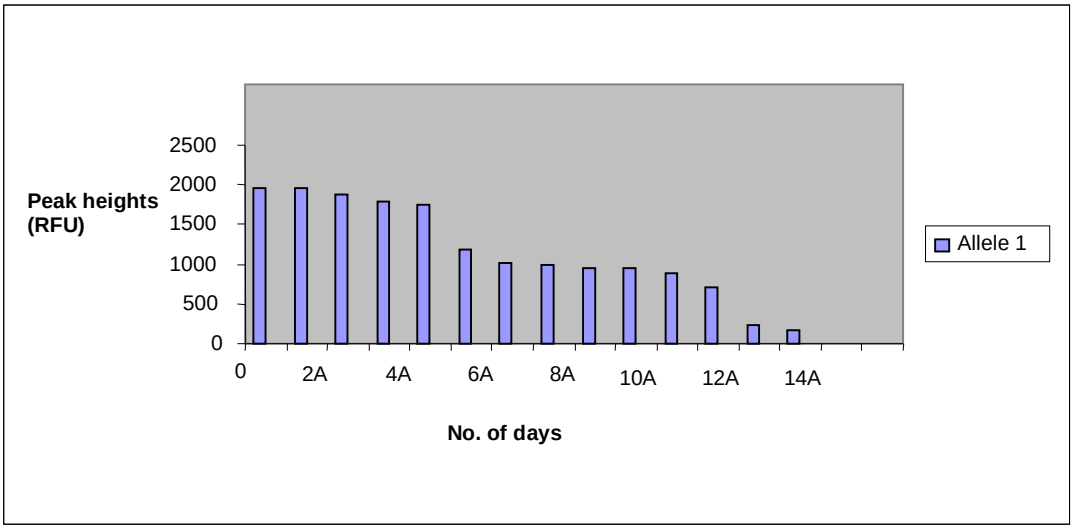

Figure 4.3.3. Peak heights versus number of days in the sun during summer using marker BM1818 with *Taq* polymerase.

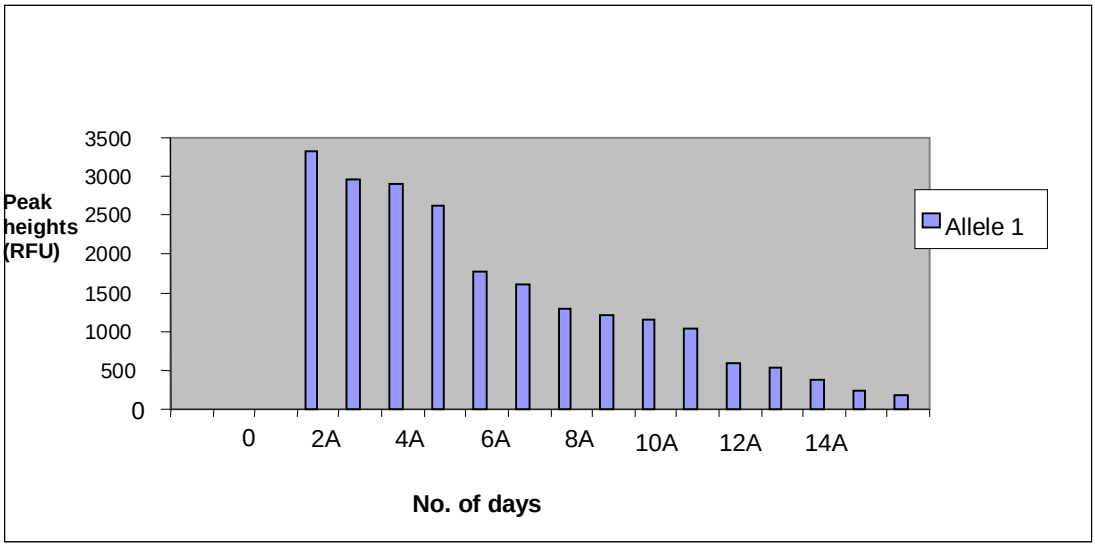

Figure 4.3.4. Peak heights versus number of days in the shade during summer using marker BM1818 with *Taq* polymerase.

4.4. Enzymes activity of Restorase®, *Taq* and FastStart DNA polymerase

Restorase® DNA polymerase and FastStart High Fidelity PCR system are known for use on damaged DNA due to their high fidelity characteristics and were tested on the degraded DNA. Winter samples gave the same results for all three enzymes and were not further analysed. In Table 4.4.1 the peak height ranges of both enzymes between microsatellites markers as compared to the *Taq* polymerase are shown for the summer samples.

There was a difference in the activity of the enzymes which resulted in variability of peak heights between the samples. *Taq* polymerase had the lowest peak range of 30-570 Relative Fluorescence Unit (RFU), Restorase® with 31-111 RFU and FastStart with 33-660 RFU. Restorase® had the lowest peak range of 31-111 RFU compared to *Taq* DNA polymerase and FastStart polymerase. With the FastStart polymerase, the lowest peak range of 33-660 RFU was still higher as compared to the Restorase® enzyme.

Table 4.4.1. Peak height ranges between the polymerase enzymes from all the markers during summer

				Peak Heights range (RFU)		
Marker	**Alleles**	**Allele Size (bp)**		***Taq* polymerase**	**Restorase®**	**FastStart**
1 (BM1818)	Homo	263		137-3323	32-3189	65-2481
2 (BM1824)	Hetero	184	190	100-2750	45-1389	**35-654**
3 (BM2113)	Hetero	131	135	82-1985	91-2592	163-4717
4 (CSRM60)	Hetero	102	112	**36-675**	82-2394	36-3014
5 (CSSM60))	Hetero	187	191	39-1336	39-1377	64-4223
6 (ETH10)	Hetero	215	219	**39-562**	60-2980	282-3084
7 (ETH225)	Hetero	144	154	116-3559	66-1806	76-2014
8 (ETH3)	Homo	115		154-4086	298-8769	116-8681
9 (HAUT27)	Hetero	144	150	43-1989	**34-275**	56-602
10 (ILSTS006)	Hetero	291	297	30-1024	**31-111**	**33-660**
11 (INRA23)	Hetero	196	198	41-3942	72-3770	112-2853
12 (SPS115)	Hetero	244	252	**30-570**	82-5536	329-6825
13 (TGLA122)	Homo	143		454-8842	31-5838	31-3480
14 (TGLA126)	Homo	118		71-1743	**44-166**	40-1321
15 (TGLA227)	Hetero	89	95	110-2977	184-3191	53-1537
16 (TGLA53)	Homo	157		115-8296	41-2279	36-2542

[Bold: Markers that had low RFU]

4.4.1. *Taq* DNA polymerase

In Figure 4.4.1 the peak heights of the samples using marker BM2113, TGLA53, ETH10 and SPS115 during summer in the shade are shown. Peaks were good with no artifacts or non-specific peaks. TGLA53 peaks were higher than BM2113, ETH10 and SPS115 and this shows that amplification efficiency differs between markers for a given sample.

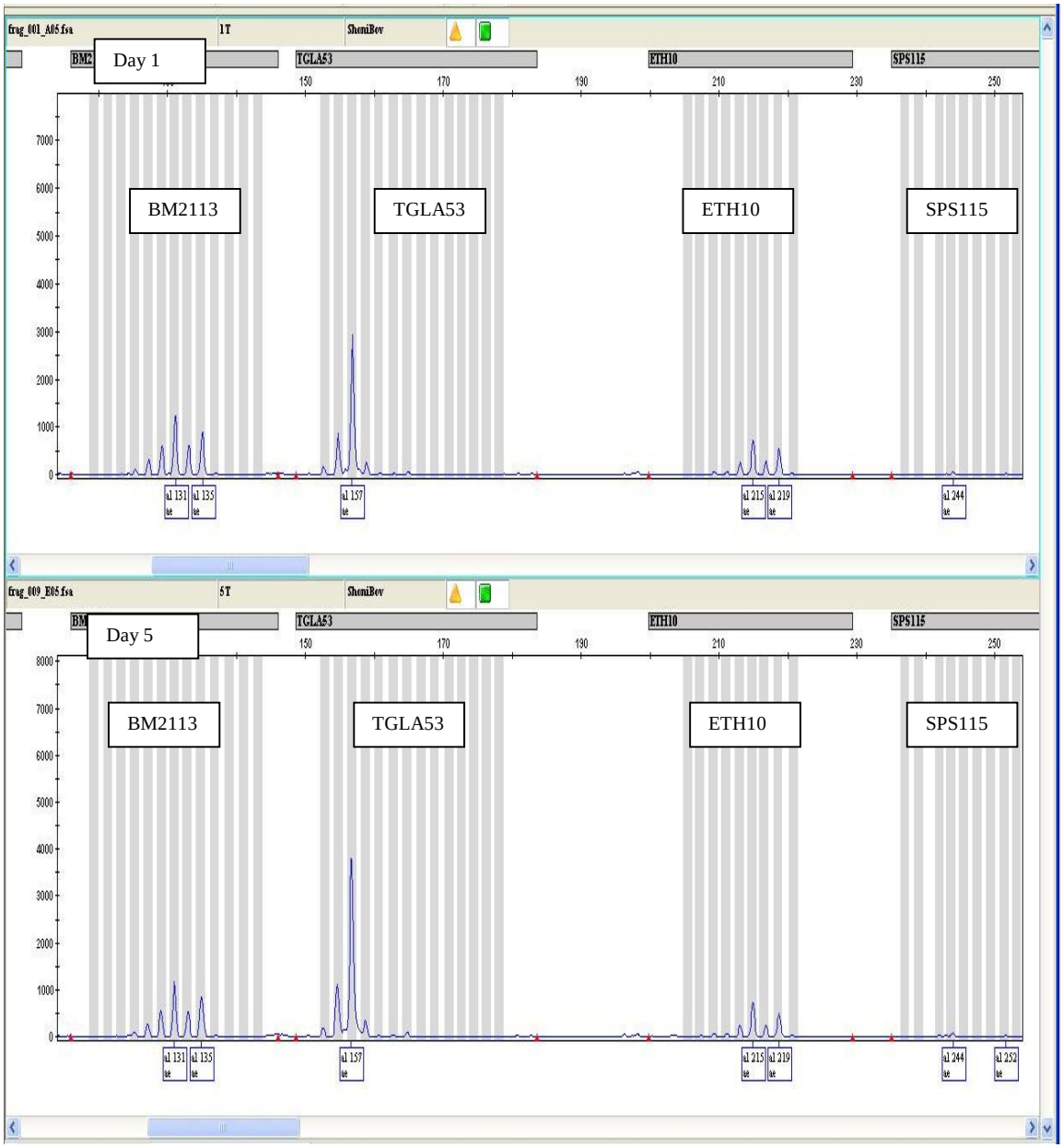

Figure 4.4.1.1. *Taq* DNA polymerase enzyme activity using BM2113, TGLA53, ETH10 and SPS115 markers from day 1 and 5 samples placed in the shade during summer.

In this analysis the success model was performed for combined days, 0- 4, 5-11 and 12-15. Figure 4.4.1.2 and 4.4.1.3 shows the variation in the activity of markers in the samples from day 0-4 in the sun and in the shade using *Taq* polymerase. From both sunny and shady conditions ETH3 showed the highest activity as compared to other markers, but its activity from the samples exposed to the shade was low as compared to the samples exposed to the sun.

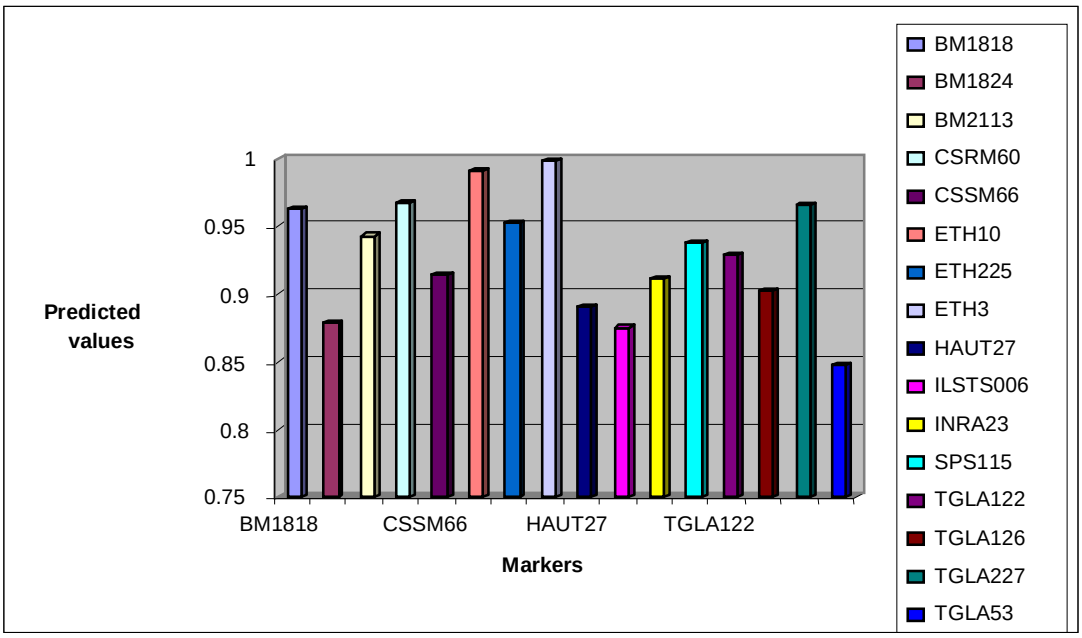

Figure 4.4.1.2. Predicted success rate of markers using *Taq* polymerase from samples exposed to the shade on day 0-4 during summer.

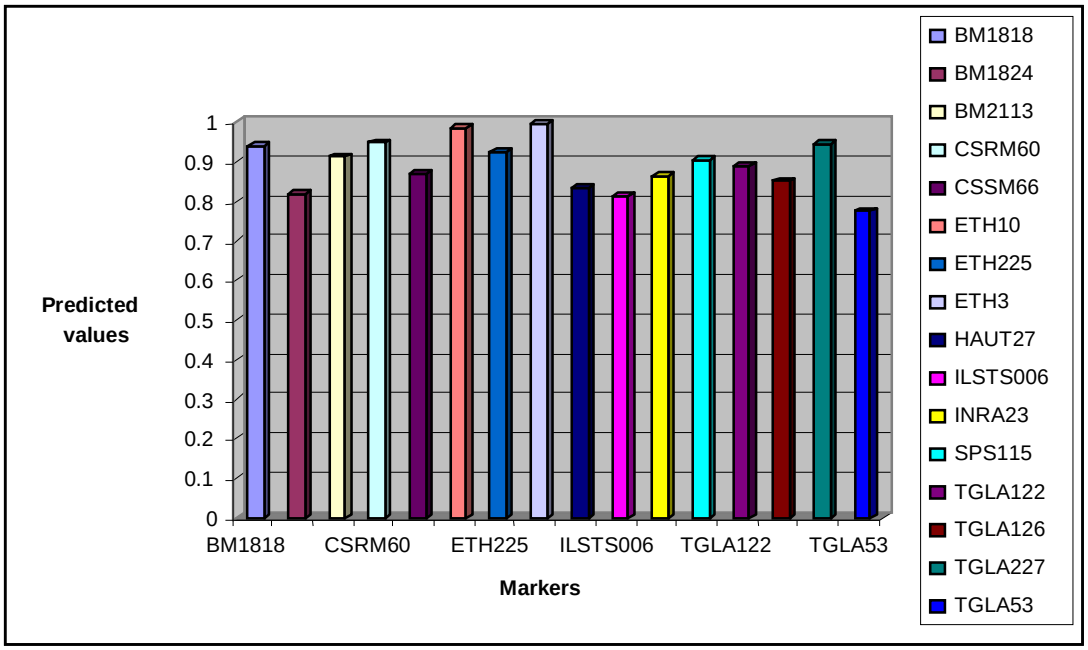

Figure 4.4.1.3. Predicted success rate of markers using *Taq* polymerase from samples degraded in the sun from day 0-4 during summer.

As the number of days the samples were exposed to the environment increased, i.e. day 5-11 (as shown if Figure 4.4.1.4 and 4.4.1.5) using *Taq* polymerase on samples from both sun and shade conditions, the activity of markers also decreased. The more samples were left exposed to the environment, had an impact on the amplification of markers. Markers such as ETH10, ILST006 and SPS115 decreased amplification with sample degradation.

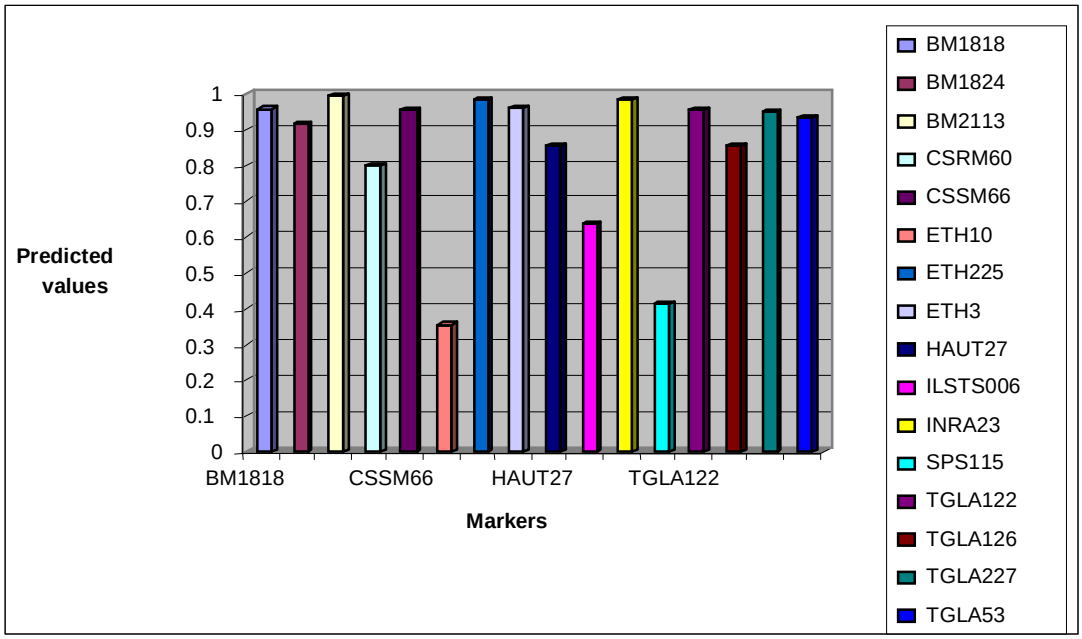

Figure 4.4.1.4. Predicted success rate of markers using *Taq* polymerase from samples exposed to the shade on day 5-11 during summer.

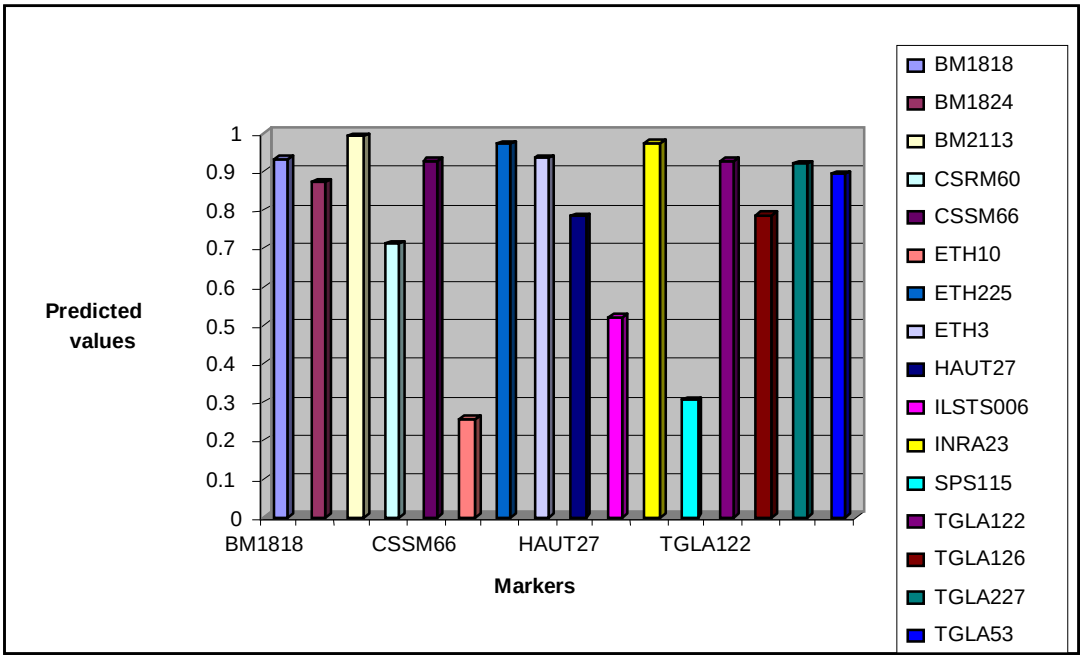

Figure 4.4.1.5. Predicted success rate of markers using *Taq* polymerase from samples placed in the sun on day 5-11 during summer.

By day 12-15 some markers did not amplify at all such as ILSTS006, while activity in markers such as BM2113, ETH3 and INRA23 remained high throughout the experiment.

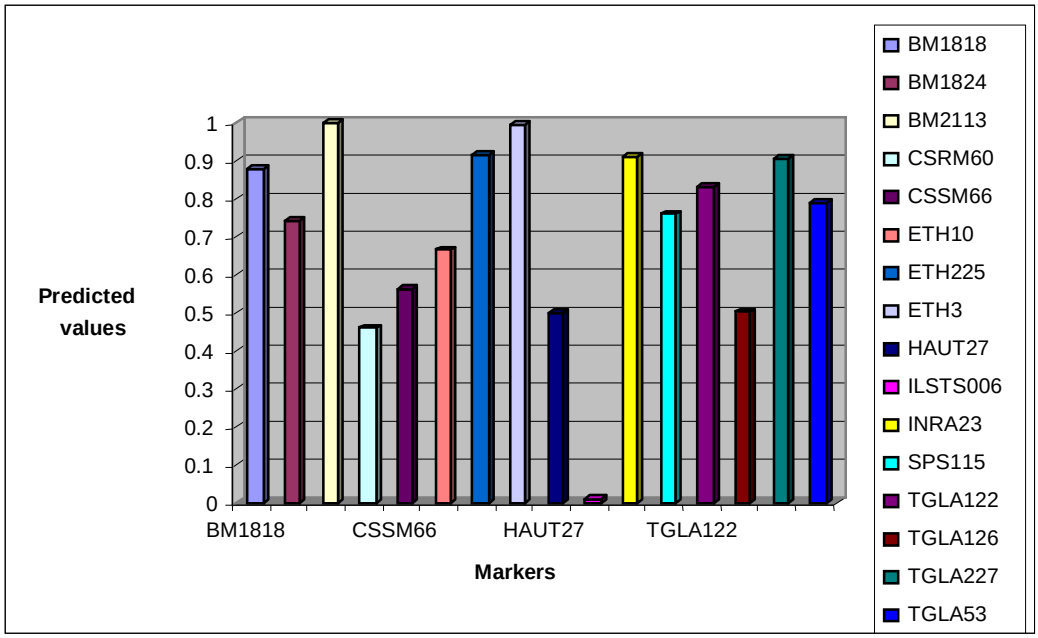

Figure 4.4.1.6. Predicted success rate of markers using *Taq* polymerase from samples exposed to the shade on day 12-15.

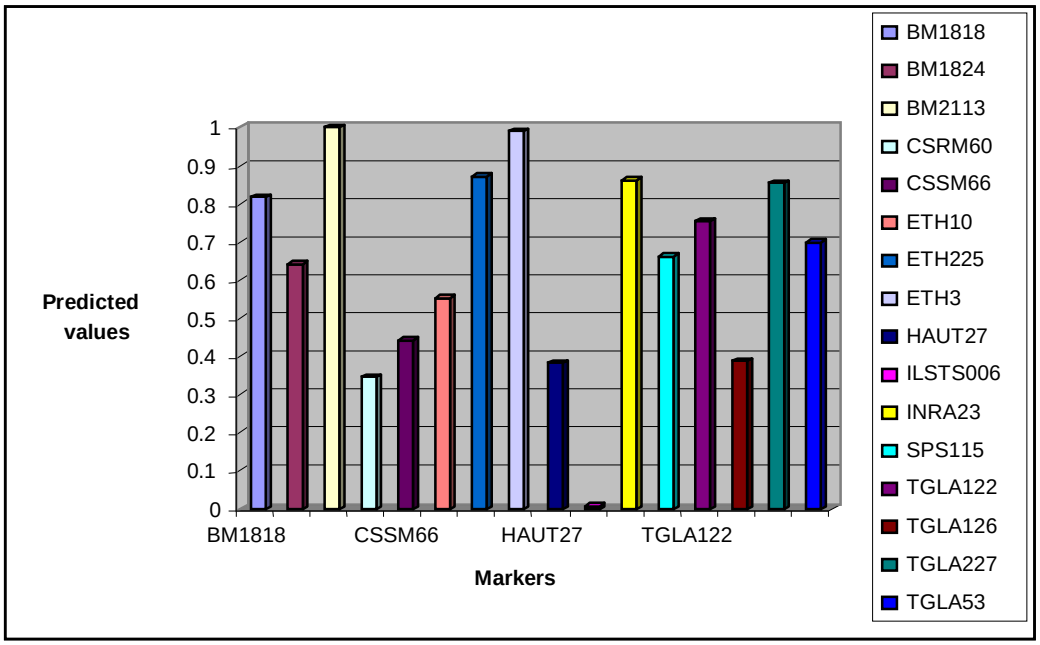

Figure 4.4.1.7. Predicted success rate of markers using *Taq* polymerase from samples exposed to the sun on day 12-15.

4.4.2. Restorase® DNA Polymerase

The amplification of SPS115 and ETH10 from samples placed in the shade during summer is shown in Figure 4.4.2.1. The peaks that were low using *Taq* polymerase (Figure 4.4.1.1) were improved using Restorase® enzyme. The peaks were clear with limited artifacts.

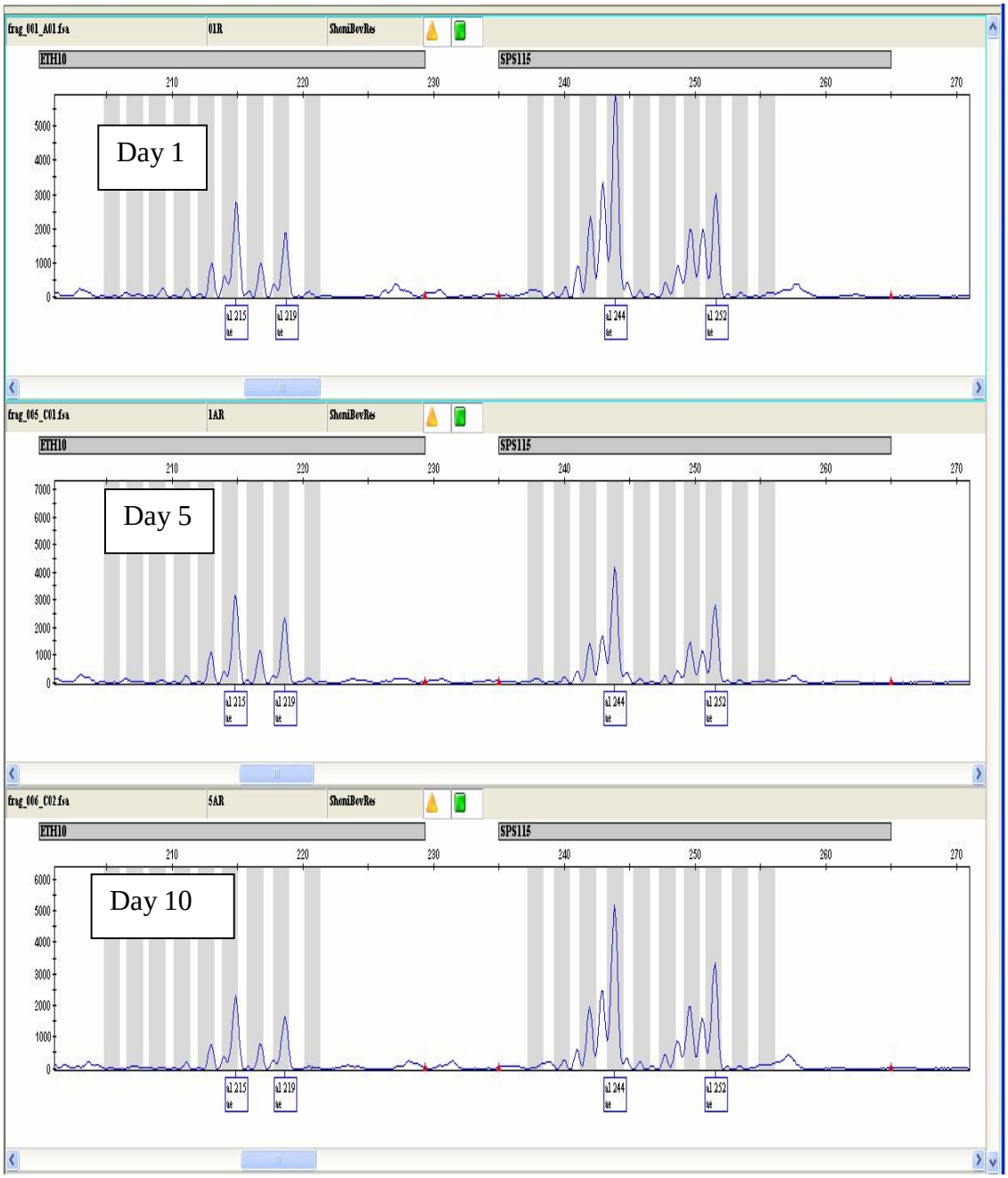

Figure 4.4.2.1. Restorase® enzyme activity of samples in ETH10 and SPS115 markers from day 1, 5 and 10 from samples placed in the shade.

In Figure 4.4.2.2 and 4.4.2.3 the activity of markers using Restorase® from samples exposed to shade from day 0-4 are shown. The activity of the markers from samples placed in the shade was higher as compared to samples from day 0-4 using *Taq* polymerase (Figure 4.4.1.2). From the samples placed in the sun few markers had low activity with the success rate of less than 60%.

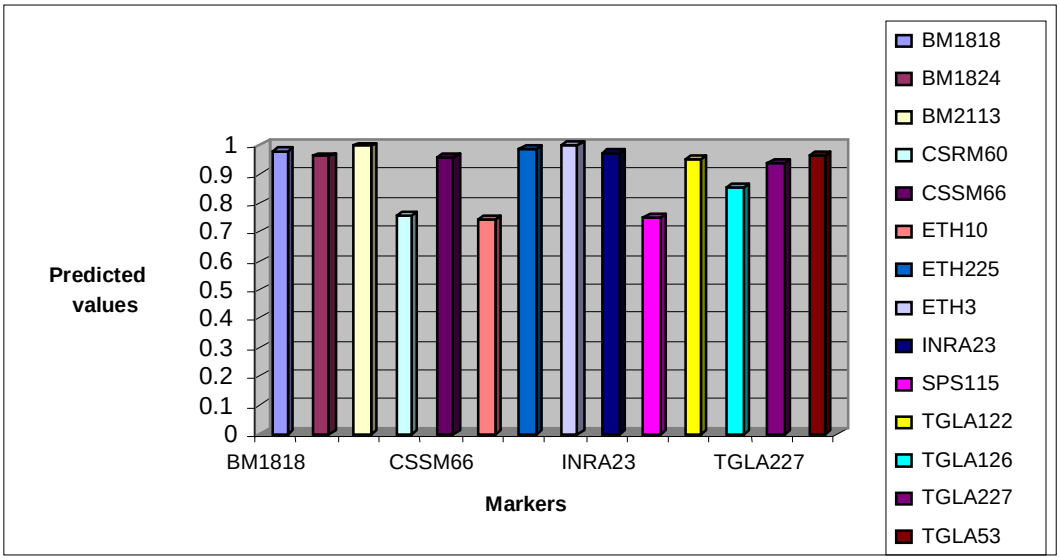

Figure 4.4.2.2. Predicted success rate of markers using Restorase® DNA polymerase from samples placed in the shade on day 0-4.

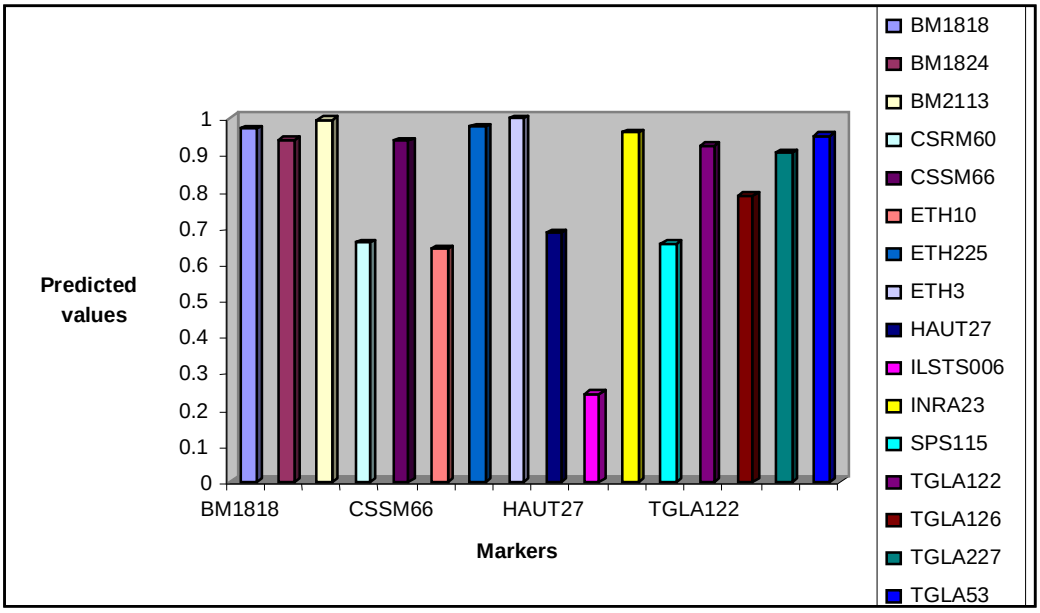

Figure 4.4.2.3. Predicted success rate of markers using Restorase® DNA polymerase from samples placed in the sun on day 0-4.

In Figure 4.4.2.4 and 4.4.2.5 the activity of the markers decreased as the number of days samples exposed to the environment increased. The activity of markers from samples exposed to the sun was lower as compared to the samples exposed to the shade. The amplification of samples exposed to the shade was higher with the success rate of more than 70% in all the markers.

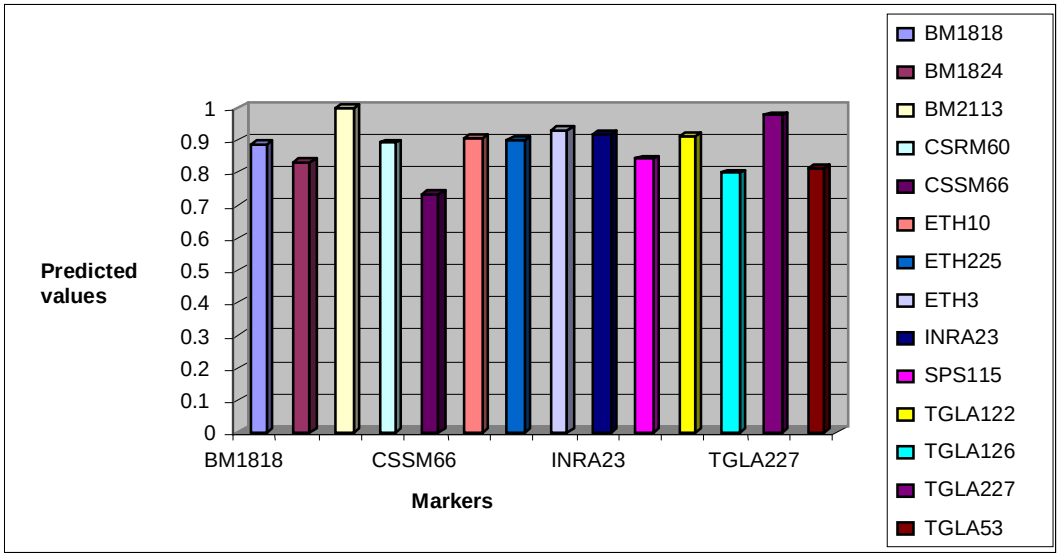

Figure 4.4.2.4. Predicted success rate of markers using Restorase® DNA polymerase from samples placed in the shade on day 5-11.

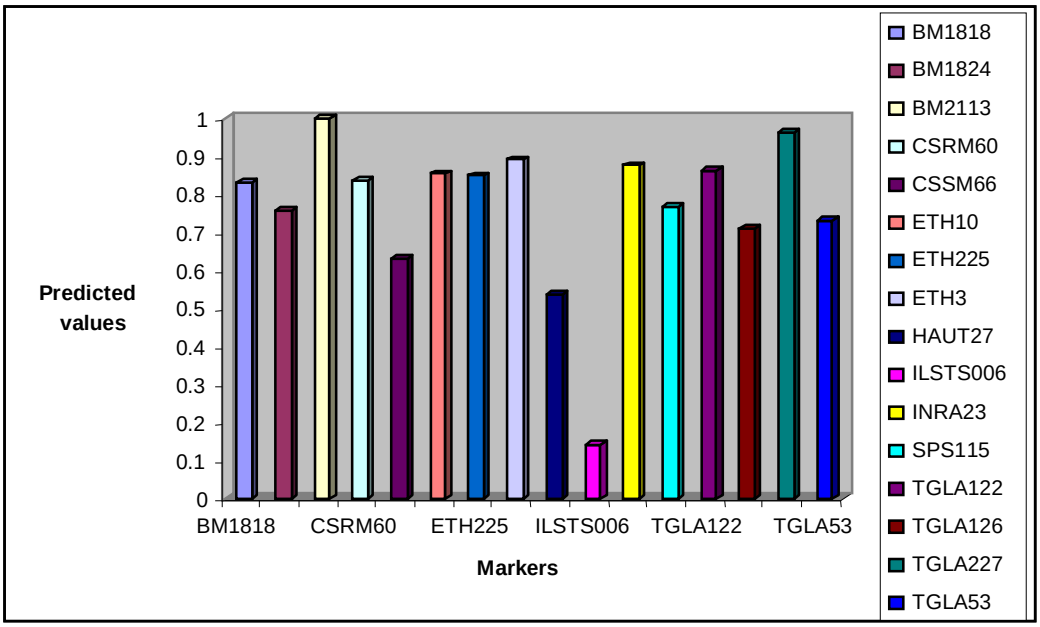

Figure 4.4.2.5. Predicted success rate of markers using Restorase® DNA polymerase from samples placed in the sun on day 5-11.

Figure 4.4.2.6 and 4.4.2.7 shows the Restorase® enzyme activity from samples degraded in the shade. Restorase® performed better even on day 12-15 with the average of 50% success rate as compared to *Taq* polymerase. Marker ILST006 that was missing on day 12-15 using *Taq* polymerase was recovered using Restorase® on day 12-15.

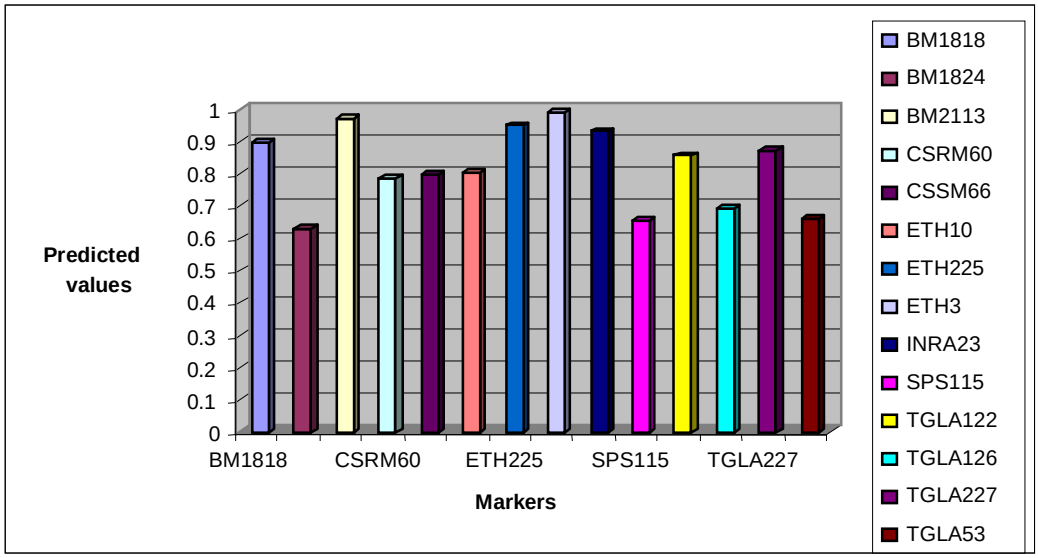

Figure 4.4.2.6. Predicted success rate of markers using Restorase® DNA polymerase from samples degraded in the shade on day 12-15.

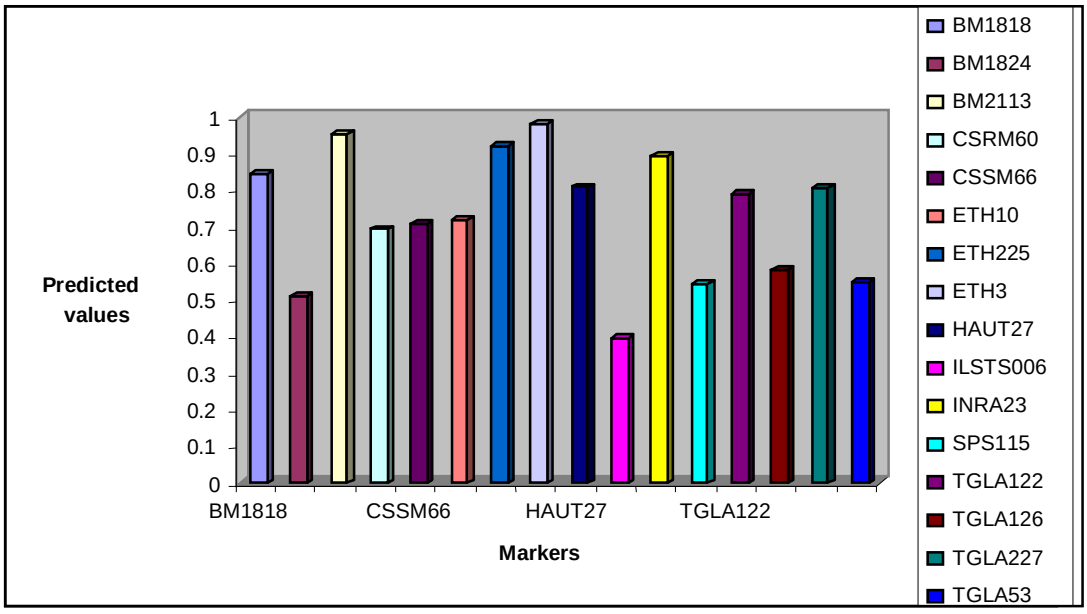

Figure 4.4.2.7. Predicted success rate of markers using Restorase® DNA polymerase from samples degraded in the sun on day 12-15.

4.4.3. FastStart High Fidelity PCR System

Peaks of the samples from day 1, 5 and 10 in summer in the sun using FastStart DNA polymerase are shown in Figure 4.4.3.1. The peaks were clear with no artifacts and few non-specific peaks. The heights of the peaks were higher as compared to *Taq* polymerase using ETH10 and SPS115.

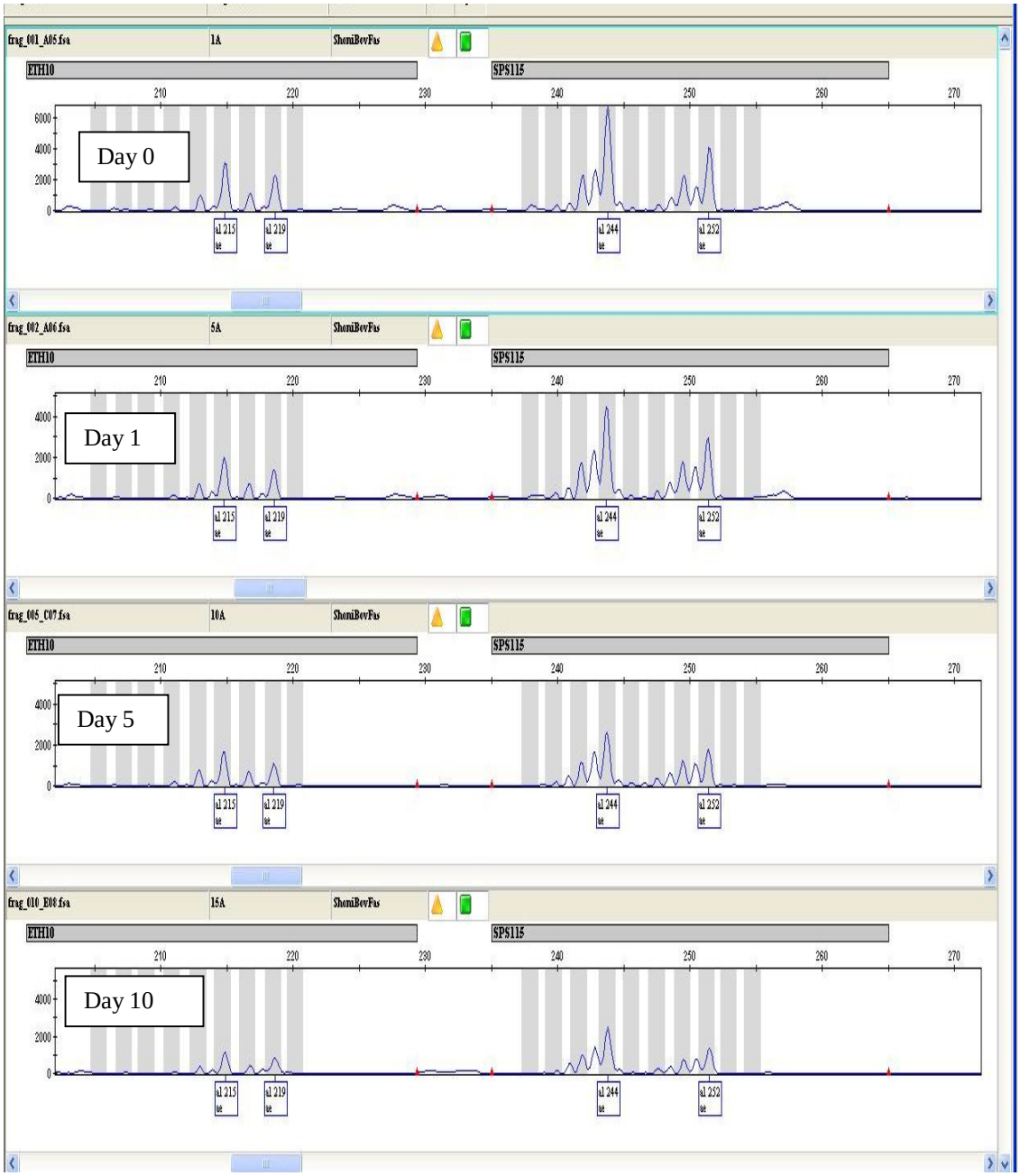

Figure 4.4.3.1. FastStart enzyme activity from samples place in the sun using marker ETH10 and SPS115 markers on day 1, 5 and 10.

The activity of marker ETH10, HAUT27, and SPS115 had a total reduction in activity using FastStart DNA polymerase, even in early days of degradation from samples placed in the shade (Figure 4.4.3.2) as compared to samples placed in the sun (Figure 4.4.3.1). ILSTS006 was very low with the success rate of less than 10% in the shade and also in the sun (Figure 4.4.3.2 and 4.4.3.3).

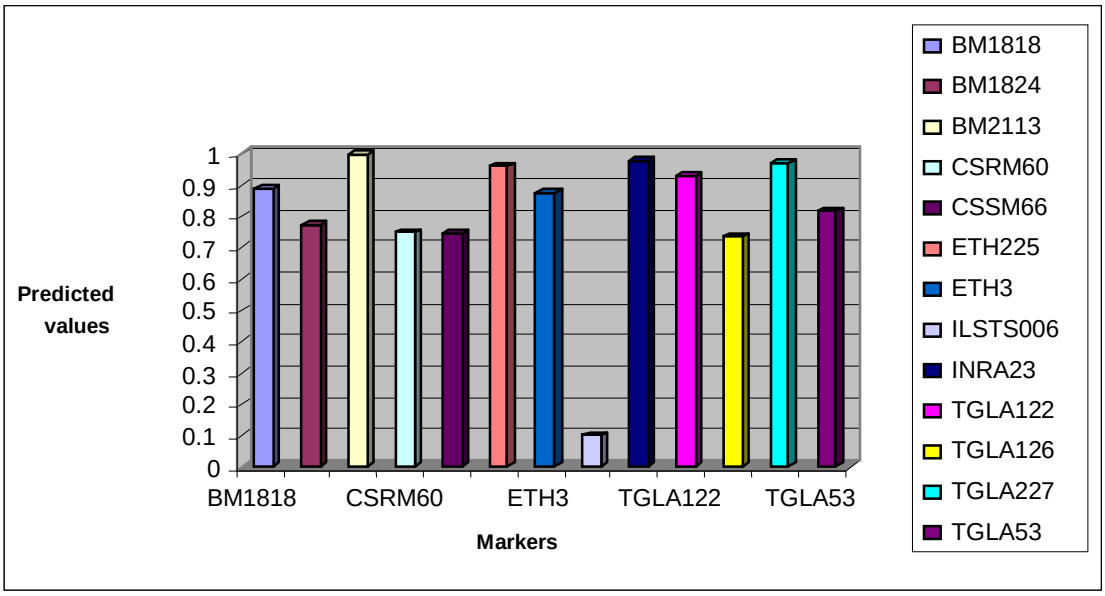

Figure 4.4.3.2. Predicted success rate of markers using FastStart from samples exposed to the shade on day 0-4.

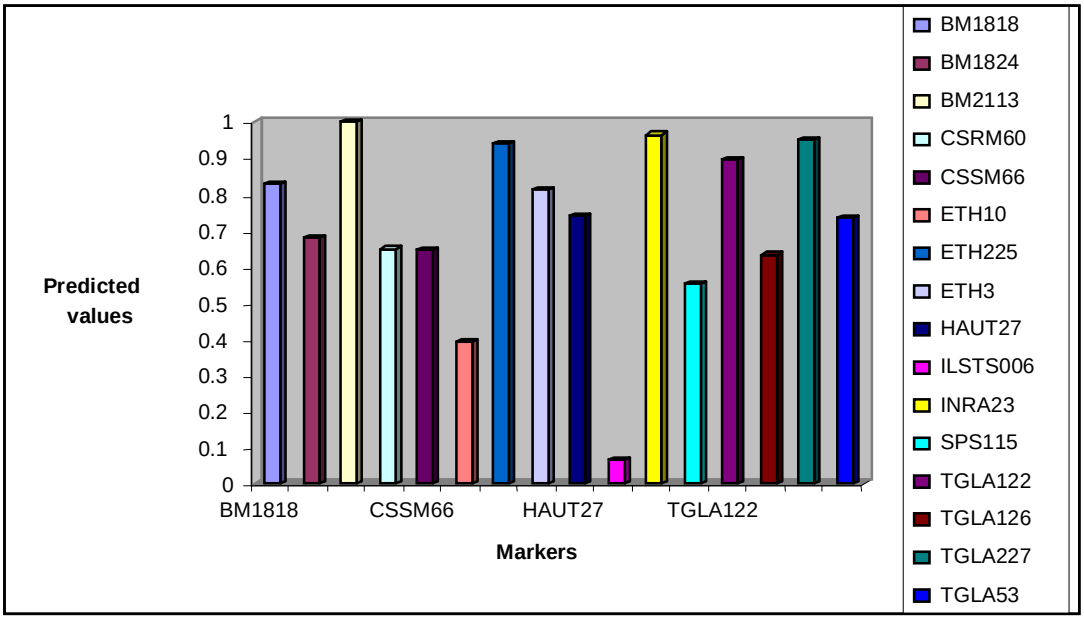

Figure 4.4.3.3. Predicted success rate of markers using FastStart from samples exposed to the sun on day 0-4.

In Figure 4.4.3.4 and 4.4.3.5 there was a little difference in the activity of markers in samples exposed to the sun and samples exposed to the shade.

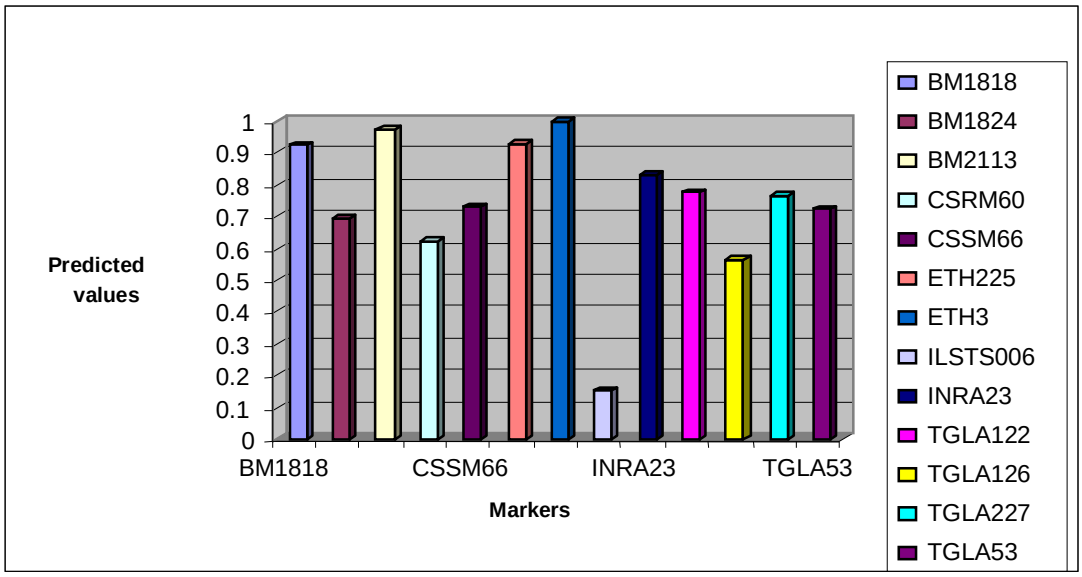

Figure 4.4.3.4. Predicted success rate of markers using FastStart from samples placed in the shade on day 5-11.

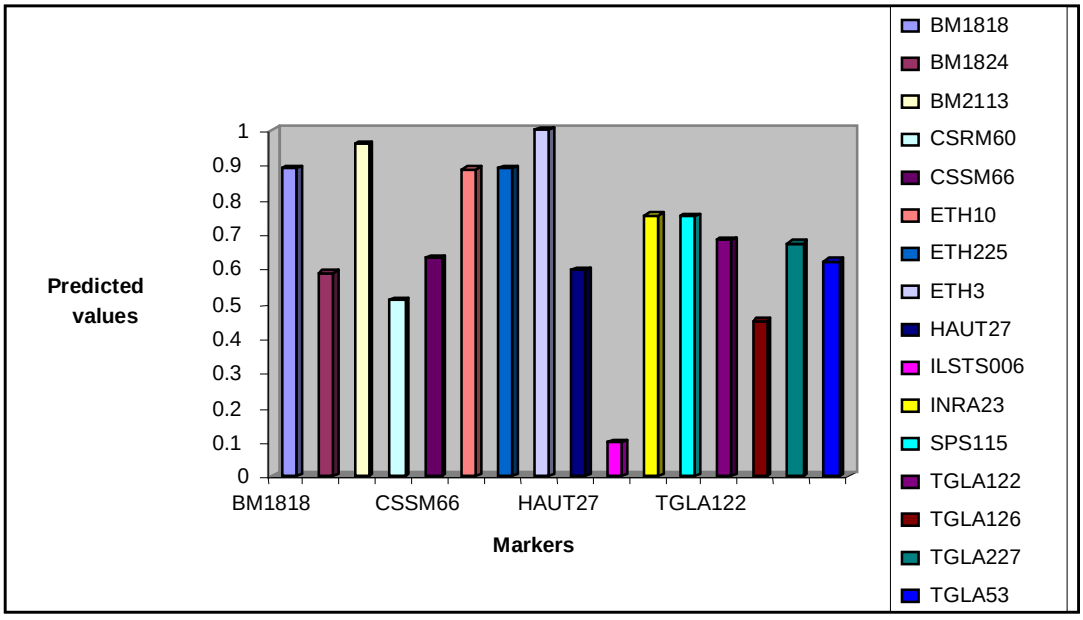

Figure 4.4.3.5. Predicted success rate of markers using FastStart from samples placed in the sun on day 5-11.

It was also found that even on day 12-15 of sample exposure to the environment FastStart gave good peaks on samples exposed to the shade as compared to *Taq* polymerase. This is indicated in Figure 4.4.3.6 and in Figure 4.4.3.7 where marker BM2113 and ETH3 had little higher activity compared to the shade samples.

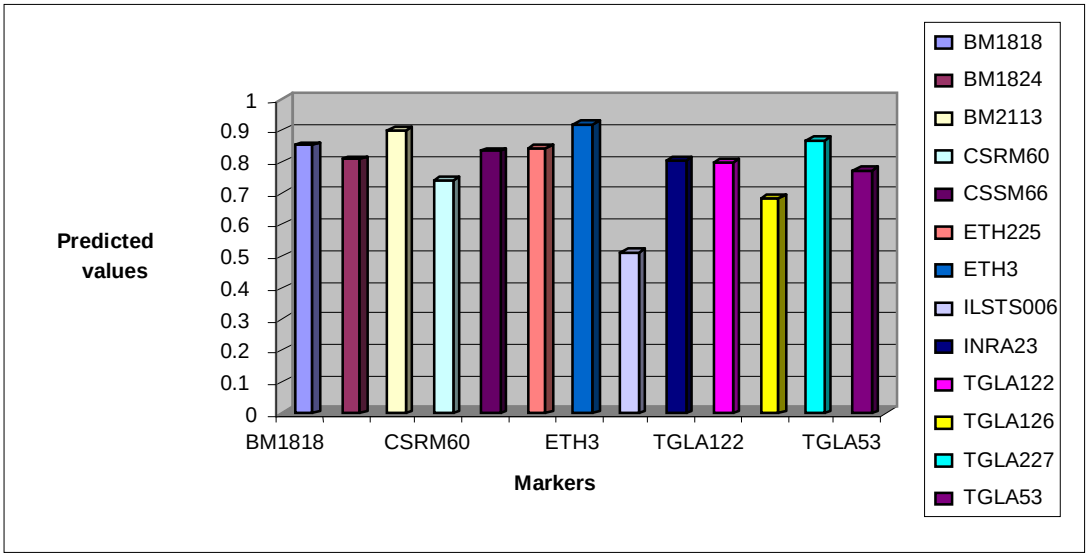

Figure 4.4.3.6. Predicted success rate of markers using FastStart from samples placed in the shade on day 12-15.

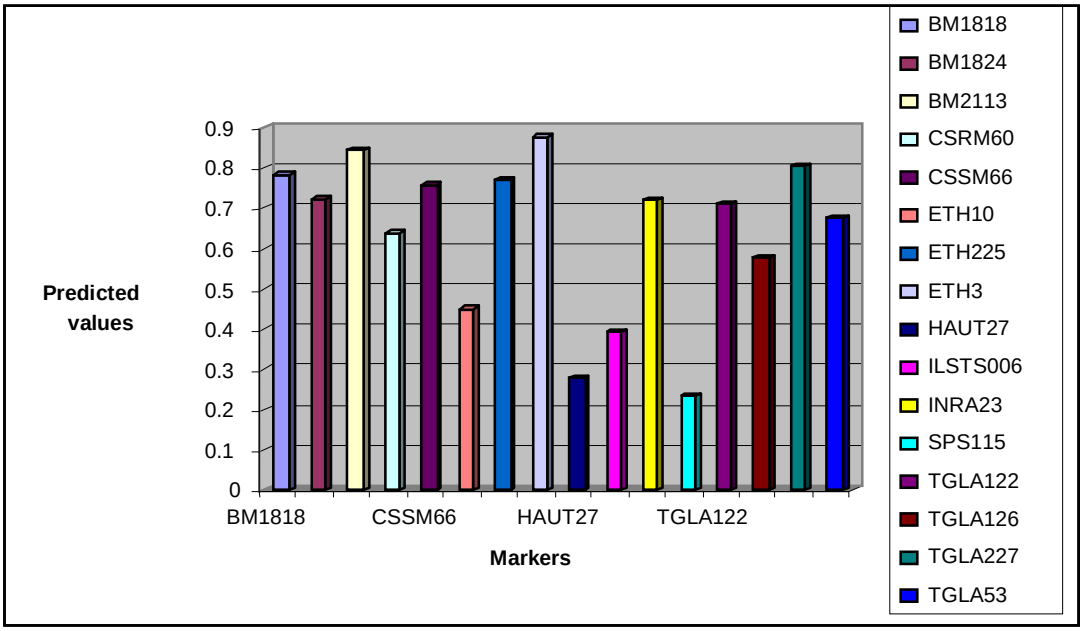

Figure 4.4.3.7. Predicted success rate of markers using FastStart from samples placed in the sun on day 12-15.

In Table 4.4 a summary is provided of the overall success of the markers applied with all three enzymes namely the, *Taq* polymerase, Restorase® and FastStart from the samples placed in both the sun and the shade during summer. An 80% success represents the markers that worked throughout the 15 days period of remaining outside in the sun versus the 60% success where the markers started to have reduced activity.

Table 4.4: Overall panel of markers for all enzymes from samples degraded during summer

Overall Marker Panel												
	***Taq* Polymerase**				**Restorase®**				**FastStart**			
	60% Average		80% Average		60% Average		80% Average		60% Average		80% Average	
Marker	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade	Sun	Shade
BM1818	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**		**X**
BM1824	X	X				X			X	X		X
BM2113	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
CSRM60					X	X			X	X		
CSSM66					X	X			X	X		X
ETH10		X			X	X						
ETH225	X	X	X	X	X	X	X	X	X	X		
ETH3	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
HAUT27					X	X	X					
ILSTS006												
INRA23	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**		**X**
SPS115	X	X				X						
TGLA122	X	X		X	X	X	X	X	X	X		
TGLA126						X			X	X		
TGLA227	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
TGLA53	X	X							X	X		

From Table 4.4 Restorase® showed higher activity in most samples as compared to *Taq* polymerase and FastStart enzymes. Restorase® enzyme activity was high from samples exposed to the shade as compared to samples exposed to the sun. The results also indicate that some of the markers such as BM1818, BM2113, ETH3, INRA23 and TGLA227 remained active throughout the 15 days using all the enzymes. Some markers are easy to obtain whether the DNA is degraded or not, due to the amplification efficiency of the particular marker and also depending on the type of enzyme used during PCR amplification. Markers such as CSRM60, CSSM66 and ILSTS006 were weak markers and did not fall under 60 or 80% average. This can indicate that these markers amplified damaged DNA, but to a very low extent as

compared to other markers. HAUT27 showed to be a very weak marker and cannot be a reliable marker for use on degraded samples.

Chapter 5

Discussion

Sample degradation and DNA quantification

A main concern in forensics using PCR on DNA extracted from ancient or degraded samples is the quality of DNA and the accuracy of the information it contains (Deagle *et al*., 2006; Golenberg *et al*., 1996). An additional concern includes the likelihood that tissue samples of different ages, exposed to different conditions will still provide positive results and reliable genetic information (Golenberg *et al*., 1996). In this study, the difference in sample degradation between summer and winter was distinguished. This was followed by the difference between samples exposed to sunny and shady conditions.

There was rapid sample degradation during summer. Degradation was slower in samples placed in the sun than samples placed in the shade. In the sun most of the samples dried over time. In samples that dried out, sufficient DNA was able to be recovered. The level of degradation was high from samples exposed to the shade because in the shade there is no direct sunlight and if a sample can remain in the shade for a long time, it becomes more degraded than in the sun. In complete degradation the peaks could not be labeled and peak heights could not be determined as well as the allele frequencies.

In winter, samples that were degraded in the shade, even after 15 days of degradation were still in their proper state and DNA was fully recovered. This could be a factor of the low temperatures during the winter season. Samples degraded in the sun, during winter, showed some level of degradation, but the level was low compared to the summer samples. This implies that winter samples have a higher chance for amplification success because the DNA can stay intact for a longer period after exposure to the environment.

The level of degradation of the sample was dependent on the amount of tissue exposed to the environment, and also on the positioning of the sample. In normal

circumstances, if an animal dies during summer, and is exposed to sunlight, after a few days it begins to smell and the carcass will decompose, but if a small piece of meat is exposed to sunlight, it tends to dry out quickly. However in winter if an animal dies, due to low temperatures, it takes time to show the same level of degradation, and depending on the size of an animal, most of the DNA is preserved and DNA analysis becomes simpler.

Samples that remain in a crime scene during winter degrade slowly and DNA becomes damaged over time depending on the period of crime investigation. With higher levels of degradation, more biological material is required to produce a DNA profile because the more the sample degrades, the chance of obtaining sufficient and good quality DNA is limited (Goodwin *et al*., 2007; Budowle and van Daal, 2009). Nucleic acids have an absorption maximum at 260nm. Sometimes samples contain proteins and single stranded DNA/RNA (Ribonucleic Acid) that absorb maximally at 280nm. So the higher the 260/280 ratio (1.8 and 2.0), the purer the DNA sample. The yield and the quality of DNA between summer and winter were slightly different. Samples degraded during winter had a higher DNA yield as compared to the samples degraded during summer with the DNA concentration ranging from 45-163ng/µl for winter and from 26-117ng/µl on summer. Low yields of DNA can be caused by a number of factors such as poor sampling conditions, excessive amount of starting material resulting in insufficient material cell lysis, column loading, sample degradation (Deagle *et al*., 2006), and stochastic factors inherent to the extraction process (Putkonen *et al*., 2010).

However there was a slight variation on DNA concentration especially in samples that were degraded in winter. There was a slight indication of constant level of degradation. The variation may be due to the condition and the positioning of samples during the process of degradation. It could also be because of the size of the sample wherein the inside contents of the sample was not completely degraded and DNA was preserved. From these results it was clear that season, days, temperature and position had an influence on the DNA quality and quantity (Larkin *et al*., 2009; Nelson, 2009).

According to the results, the maximum period the sample remained intact in the field/crime scene during summer was less than five days and the maximum number of

days on which samples remained intact in the field during winter was 10 days, again depending on the size, type, and position of the sample. However, for this project the summer temperature for November 2008 was not very high. The higher degradation will be expected as the temperature increases. During summer, forensic sampling should be done quickly in order to prevent high degradation of samples that lead to damaged DNA, which then lead to poor analysis. Biological material collected for DNA analysis should be stored under conditions which will slow the rate of DNA degradation. A cool and dry environment limits the action of bacteria and fungi that find biological materials as a rich source of food and energy and can thus rapidly degrade biological material. The exact storage condition depends on the nature of the sample and the environment in which samples are to be stored (Goodwin, *et al*., 2007)**.** Therefore, care must be taken when doing DNA analysis because the amount and quality of DNA extracted from the samples will determine the amount of DNA needed for PCR reactions for more efficient amplification.

DNA amplification and fragment analysis

PCR-based analysis has shown to be an essential technology in forensic analysis (Roffey and Harmon, 2003). The lack of techniques for analyzing confiscated samples makes it difficult to enforce the law. Conclusive forensic identification of species requires a complete genetic profile or sequence which is difficult in cases of degraded samples. Although several markers have been developed for species identification, the forensic verification of species remains a challenge (Meganathan *et al*., 2009; Teletchea *et al*., 2005).

In both forensic and paternity analysis, *Taq* DNA polymerase enzyme was found to have certain limitations for analyzing degraded forensic samples (Meganathan *et al*., 2009). In this study it was mostly effective in amplifying degraded samples up to a certain level. Following Genemapper analysis the size of the peaks of samples amplified using *Taq* polymerase was low when compared to Restorase® and FastStart DNA polymerases. Even though the amplification success was high (Table 4.2.1), and all the markers were able to amplify DNA especially for samples in an advanced state of degradation, the peaks in some markers were very low in such that they could not be recorded.

There is concurrent DNA damage that occurs as samples increasingly degrade as a result of sample decomposition. There are a number of double stranded DNA breaks that occur, which cannot be repaired which prevent STR typing (Nelson, 2009). This was noticed when samples gave partial profiles and the loci were not scored correctly. As the damage to samples increased, there was complete failure on STR analysis. When the DNA is repaired the chance of getting the complete amplification is high and the loci can be scored correctly. This means that damaged DNA can be repaired and result in a good analysis for forensic cases (Lynch and Pergolizzi, 2010; Nelson, 2009). Restorase® proved to be excellent in amplifying damaged DNA as compared to FastStart and *Taq* DNA polymerases. It was found in this study that even after 10 days of sample degradation, Restorase® was able to give conclusive results in most markers. This shows that Restorase® DNA polymerase can improve the results of STR analysis, confirming that repair of damaged samples in a forensic environment can indeed improve the analysis and at least partially rescue samples that have been exposed to conditions that result in DNA damage (Nelson, 2009).

Enzymes and markers activity

The statistical significance of forensic DNA profiles in criminal and civil proceedings is an important subject (Loftus, 2005). A judge or jury could appropriately weigh the significance of a DNA match between a defendant and a forensic sample, in the event that it is known what the frequency of occurrence of a specific DNA profile is. If two patterns match without providing any scientifically valid estimate of the frequency with which such matches might occur by chance, it is meaningless (Cummings, 2008). *Taq* polymerase is known to lack exonuclease activity, which limits reproducibility (Bustin, 2002). In this project *Taq* polymerase was used as a reference enzyme in comparison with other available enzymes for amplification of degraded samples, and these were carried out on samples degraded during summer that showed a high level of degradation.

In this study, based on the success model, it was found that some of the markers that failed to be successfully amplified by *Taq* polymerase were recovered by Restorase® DNA polymerase. Markers such as ETH10 and SPS115 were recovered. The peak heights were high compared to those obtained with *Taq* polymerase on day 10. The

peaks were clear with no artefacts or non-specific peaks. The peak heights range of ETH10 with the Restorase® enzymes was higher as compared to ETH10 when using the *Taq* polymerase enzymes. This indicated that Restorase® was able to amplify DNA from the samples that were degraded, but with limitations. Some of the markers that worked using *Taq* polymerase didn't work when using Restorase®. This could be due to the microsatellite instability, insertion or deletion of DNA isolation from target tissues. If the mutation rate is high, any trace of the coalescence is eliminated by subsequent mutations, whereas if the mutation rate is low there is a limited mutation for coalescent events. Analyzing multiple categories of markers with different rates and forms of mutation, for example microsatellites and enzymes, can be beneficial (Estoup *et al*., 2001).

There was a good amplification rate in all the markers in the early days (day 0 to7) of sample degradation with a high success rate from all the polymerase enzymes. This meant that all markers were amplified, but varied on the rate of success and peak sizes. There was a slight difference in the samples degraded in the sun and in the shade with the lowest success rate of less than 5% with ILSTS006 from samples in the shade and in the sun. There was a drop in success rate in the combined days 5-11 with a rate of less than 60% in ETH225, ILSTS006 and SPS115 in both sun and shade. In day 12-15 there was also a drop in success rate from samples in both the sun and the shade with CSRM60, CSSM66, HAUT27 and TGLA126. This showed the reduction in the *Taq* polymerase activity as the samples degraded as also found by Bustin in 2002. It also shows that markers varied in terms of amplification success rate.

The PCR conditions for DNA analysis can be optimized by varying many components such as type and concentration of thermostable polymerase, dNTPs, Mg^{2+} ions, primer and DNA template concentration, and other reactions such as time and temperature of annealing, extension and denaturation. These factors can influence the PCR reaction because not all processes and mechanisms involved in these reactions are as yet fully understood (Bustin 2002, Wolff *et al*., 1993).

The success rate of markers with Restorase® DNA polymerase on combined day 0-4 of sample degradation was done by predicting the success variables in each marker.

The success rate was satisfactory in early days of degradation with the lowest success rate of 75%. It was also found that some markers were always low in peak heights such as INRA23, with the highest peak range of 3500RFU with Restorase®; followed by BM1818 with the highest peak range of 3000RFU (Refer to Table 4.4.1). There was also a difference in the activity of markers with Restorase® from samples in the sun and the shade. Samples that were degraded in the shade didn't have much effect on the marker activity, but there was a decrease in the marker activity in the samples that were degraded in the sun. This can be due to the degradation rate in the sun. There was a decrease in the success rate for markers from samples placed in the sun with the least being 40% in ILSTS006 as compared to a 65% success rate on days 12-15. This showed that in the shade, sample degradation was slow as compared to those samples exposed to sun.

Some markers that were successfully amplified by the FastStart High Fidelity PCR System, such as ETH10, SPS115 and CSRM60, were least amplified by *Taq* DNA polymerase. This indicated that FastStart also had the ability to amplify DNA from degraded samples. The success rate of all the markers with the FastStart High Fidelity PCR System was low compared to the Restorase® DNA polymerase with the lowest success rate being less than 10% with ILSTS006.

The marker variation from the sun-shade samples was high with most of the markers, such as ILSTS006 and HAUT27, using the FastStart High Fidelity PCR System. Most of the samples that were degraded in the sun were successfully amplified. These results indicate that when DNA is damaged, not all markers can be amplified. Microsatellites differ in their ability to amplify consistently, while some markers are sensitive to damaged DNA and are not able to locate the bases to the necessary strands respectively. Success rate for amplification fluctuated considerably for the FastStart System. The power of a specific marker is determined by the level of polymorphism detected during the amplification process. The process can be affected by the mutation rate that occurs at a genomic site involved caused by changes in the number of repeat units of the sequence (Gonzalez-Chavira *et al.*, 2006).

Analysis of degraded or compromised DNA samples often results in dropout of larger molecular weight loci and reduces success of individual identification which is of

concern in resolving forensic cases (Andreasson, 2005). The highest probability value on biological material and a higher discrimination power is obtained when the larger weight genetic marker is successfully amplified, and this is dependent on the quality of the genetic material. The higher the DNA quality, the higher the probability of obtaining accurate results. PCR amplification allows DNA analysis on small samples and the ability to amplify such minute quantities of DNA enabled even highly degraded samples to be analyzed using high proofreading enzymes (Mullis *et al.*, 1986).

Based on the activity of all enzymes on degraded samples, the panel of markers for each enzyme was determined. Restorase® activity was good on degraded samples and it produced more markers in the genetic profile that can contribute in a forensic analysis. Restorase® had eight markers on 80% average followed by *Taq* polymerase with six markers and three markers for the FastStart System. This means that the number of markers that need to be added in the panel markers for Restorase® will be less than the number of markers needed to make a panel for *Taq* polymerase and FastStart. Substantial optimization is still required in order to increase the number of panel markers especially for *Taq* polymerase and FastStart.

Chapter 6

Conclusion

In this study, efficacy of different DNA polymerase enzymes (*Taq* polymerase, Restorase®, the polymerase with the proof reading enzyme, and the FastStart High Fidelity System) on PCR amplification of bovine DNA was evaluated. Factors such as season, sun/shade, sample size and markers used, contributed to the results obtained in this study.

As samples are degraded by exposure to the environment, DNA becomes damaged and some fragments can be repaired while other fragments cannot be repaired. Samples exposed to different seasons had higher degradation. Samples exposed during summer had higher degradation as compared to those degraded during winter and there was a possibility of DNA damage. During winter samples take time to degrade and thus the DNA damage was reduced. In summer, samples exposed to the sun dried over time and samples exposed to the shade became degraded over time.

In the comparison of the three enzymes, *Taq* polymerase was not efficient in amplifying degraded DNA; only samples that are of good quality or partially degraded were amplified. Damaged DNA that could not be amplified by *Taq* DNA polymerase was amplified using Restorase® and the FastStart DNA polymerase with the proofreading protein. There was a significant difference in the activity of these enzymes on a degraded sample and the Restorase® activity was higher as compared to the FastStart System. Markers such as BM1818, BM2113, ETH3, INRA23, and TGLA227 were amplified by all the polymerase enzymes throughout the experiment. Markers, such as ILSTS006 showed weak amplification with all the enzymes. This could be because there was no perfect match at the 3'-end of the primer to the template by the polymerase. A mismatch at this position can result in no or a weak amplification.

Therefore it is recommended that forensic samples should not be left for a long time at crime scenes due to potential DNA degradation. Samples that are dried can be used in forensic analysis because when dry, the DNA is preserved. From this study the

maximum period that a sample should be left at the crime scene can be two weeks depending on the size, season and the location of the sample. In DNA analysis of degraded samples, enzymes with high proof reading capacity should be used, i.e. enzymes with the capacity to repair damaged DNA. This means that Restorase® can be the enzyme of choice in analyzing damaged DNA because of its high proof reading capacity.

Markers such as BM1818, BM2113, ETH3, INRA23, and TGLA227 could form a basis of the bovine marker panel for each enzyme, and a marker such as ILSTS006 need not be included on a bovine marker panel for forensic analysis because of its poor activity. The marker-panel effect should be considered for reliable results and amplification. Optimization of markers and methodology is necessary for successful forensic DNA analysis of bovine samples.

The aim of the study was to determine the efficiency of Restorase®, a novel DNA polymerase blend that is known to repair damaged DNA and the FastStart High Fidelity System, which contains a *Taq* polymerase and a thermostable proof reading protein, on degraded forensic bovine samples using PCR-based methodology. Restorase® was found to be an excellent alternative enzyme for use in bovine forensic analysis.

Future research

This study was limited to bovine samples and only provided a bench line for using different enzymes for forensic analyses. It will be important for future research to also include sheep and goat samples as these are also often part of livestock theft cases in South Africa. It would be ideal if the work could be extended to using degraded whole carcasses so that different tissue types can be analyzed with the different enzymes.

For future studies a wider range of environmental conditions could be included with regard to season, temperature and exposure to sun and insects. This project was limited to two seasons only and a wider range of degradation can be expected from carcass samples in the open field.

In the laboratory different DNA extraction methods could be investigated depending on the sample degradation and different marker parameters could be considered in the selection of the microsatellite markers for forensic analyses. This study however has contributed in providing a reference for working with degraded bovine DNA and Restorase® as an alternative enzyme.

References

Alaeddini, R., Walsh, S.J. and Abbas, A. 2008. Forensic implications of genetic analyses from degraded DNA-A review. *Forensic Science international: Genetics* 2 (4), 301-309

Albretcht, K. and Schultheiss, D. 2004. Proof of paternity: Historical reflections on an andological forensic challenge. *Andrologia* 36, 31-37

Altshuler, D., Daly M.J. and Lander, E.S. 2008. Genetic mapping in human disease. *Science* 322 (5903), 881-888

Anderson, L. 2001. Genetics dissection of phenotypic diversity in farm animals. *Genetics Nature Reviews* 2, 130-137

Andreasson, H. 2005. Sensetive forensic DNA analysis: Application of pyrosequencing and real-time PCR quantification. Acta Univarsitatis Apsaliensis. *Digital comprehensive summaries of Uppsala dissertations from the faculty of Medicine* 33. Uppsalla, USBN 91-554-6234-0. Pg 1-46

Arezi, B., Xing, W., Sorge, J.A. and Hogrefe. 2003. Amplification efficiency of thermostable DNA polymerase. *Analytical Biochemistry* 321, 226-235

Avise, J.C. 1994. *Molecular markers, Natural history and Evolution: Individuality and parentage* (2nd edition). Chapman and Hall, New York. Pg 511

Baldi, P. and Hartfield, G.W. 2002. *DNA microarrays and gene expression: From experiments to data analysis and modeling*. Cambridge University Press, USA. Pg 1-11

Beecher-Monas, E. 2008. Evaluating scientific evidence: An interdisciplinary framework for intellectual due process. *Intermational Comentary on Evidence* 6 (1), 1-4

Bell, S.D. 2006. Molecular biology: Prime-time progress. *Nature* 439, 542-543

Berg, J.M., Tymoczko, J.L. and Stryer, L. 2002. *Biochemistry* (5th edition), Chapter 27. W.H. Freeman and Company, New York. ISBN: 0-7167-3051-0

Beuzen, N.D., Stear, M.J. and Chang, K.C. 2000. Review: Molecular markers and their use in animal breeding. *The Veterinary Journal* 160, 42-52

Bishop, M.D., Kappes, S.M., Keele, J.W., Stone, R.T., Sunden, S.L.F., Hawkins, G.A., Toldo, S.S., Fries, R., Grosz, M.D., Yoo, J. and Beattie, C.W. 1994. A genetic linkage map for cattle. *Genetics* 136 (2), 619-639

Borns, M. and Hogrefe, H. 2000. Unique enhanced DNA polymerase delivers high fidelity and great PCR performance. *Strategies* 13, 1-3

Budowle, B. and van Daal, A. 2008. Forensically relevant SNP classes. *Biotechniques* 44 (5), 603-610

Budowle, B. and van Daal, A. 2009. Review: Extracting evidence from forensic DNA analysis: Future molecular biology directions. *Biotechniques* 46 (5), 339-350

Burns, B. 2007. *DNA fingerprinting: How it works and the application for the beef industry*. Department of Primary Industries and Fisheries, Queensland. Pg 1-5

Burt, D.W., Bumstead, N., Bitgood, J.J. and De Leon, F.A.P. 1995. Chicken genome mapping: a new era in avian genetics. *Trends in Genetics* 11(5), 190-194

Bustin, S.A. 2002. Quantification of mRNA using real time reverse transcription PCR (RT-PCR): Trends and problems. *Journal of Molecular Endocrinology* 29 (1), 23-39

Butler, J.M., Coble, M.D. and Vallone, P.M. 2007. STRs vs SNPs: Thoughts on the future of forensics DNA testing. *Forensic Science, Medicine and Pathology* 3, 200-205

Campbell, M.K. and Farrell, S.O. 2007. Biochemitry (6th edition). Thomson Brooks/Cole, Belmont. ISBN: 9780495390411. Pg 261-286

Campbell, N.A. and Reece, J.B. 2002. *Biology* (6th edition). CA: Pearson Benjamin Cummings, San Fransisco. ISBN 0-8053-6624-5. Pg 1247

Cassidy, B.G. and Ganzales, R.A. 2005. DNA testing in animal forensics. *Journal of Wildlife Management* 69 (4), 1454-1462

Chatterji, S. and Pachter, L. 2006. Reference based annotation with GeneMapper. *Genome Biology* 7 (R29), 1-10

Chial, H. 2008. DNA fingerprinting using Amplified Fragment Length Polymorphism (AFLP). *Nature* 1 (1)

Chistiakov, D.A., Hellemans, B. and Volckaert, F.A.M. 2006. Microsatellites and their genomic distribution, evolution, function and applications: A review with special reference to fish genetics. *Aquaculture* 255, 1-29

Cohen, J.E. 1990. DNA fingerprinting for forensic identification: Potential effects on data interpretation of subpopulation heterogeneity and band number variability. *American Journal of Human Genetics* 46, 358-368

Collins, F.S., Green, E.D., Guttmacher, A.E. and Guyer, M.S. 2003. A vision for the future of genomics research. *Nature* 422, 835-847

Cummings, M.R. 2008. *Human Heredity: Principles and issues* (8th edition). Thomson Brooks/cole, Belmont. ISBN: 0-4955-5445-6, Pg 70-86

Curran, T. 1997. *Forensic DNA analysis: Technology and application.* Science and technology division, Parliamentary Research Branch, Canada. Pg 1-15

Deagle, B.E., Eveson, J.P. and Jarman, S. 2006. Quantification of damage in DNA recovered from highly degraded samples- a case study on DNA in feaces. *Frontiers in Zoology* 3(11), 1-10

Downer, R.G. and Richardson, P.A. 2009. Illustrative logistic regression examples using POC LOGISTIC. Paper SP03-2009, 1-6

Dzimba, J. and Matooane, M. 2005. Stock theft as a threat to human security: A case study in Lesotho. ISS *Monograph* 113, 1-8

Drouin, R., Dridi, W. and Samassekou, O. 2009. Chapter 22: DNA Polymerases for PCR amplifications. *Metapres*, University of Sherbrooke, Canada

Ellegren, H., Moore, S., Robinson, N. and Byrne, K. 1997. Microsatellite evolution-A reciprocal study of repeat lengths at homologous loci in cattle and sheep. *Mol Biol. Evol.* 14 (8), 854-860

Estoup, A, Wilson, I.J., Sullivan, C., Cornuet, J. and Moritz, C. 2001. Inferring population history from microsatellites and enzyme data in serially introduced can toads, Bufo marinus. *Genetics* 159, 1671-1687

Evans, J. and Van Eenennaam, A., 2005. *Livestock identification*. University of California Cooperation Extensive, Carlifonia. Pg. 1-6

Forterre, P., Filee, J. and Myllykallio, H. 2004. Chapter 13: Origin and Evolution of DNA and DNA replication machineries. *The Genetic Code and the Evolution of life*. ISBN: 0-306-47843-7

Foran, D.R. 2006. Ralative degradation of nuclear and mitochondrial DNA: An experimental approach. *Journal of Forensic Sciience* 51 (4), 766-770

Friedman, A.L. 1999. Forensic DNA profiling in the 21st century. *International Journal of Offender Therapy and Comparative Criminolpgy* 43 (168), 168-179

Gama Sosa, M.A., De Gasperi, R. and Elder, G.A. 2010. Animal transgenesis: an overview. *Brain Structure and Function* 214, 91-109

Geldermann, H., 1975. Investigations on inheritance of quantitative characters in animals by gene markers. *Theoretical and Applied Genetics* 46, 319-330

Golenberg, E.M., Bickel, A. and Weihs, P. 1996. Effect of highly fragmented DNA on PCR. *Nucleic Acid Research* 24 (24), 5026-5033

Goodwin, W., Linacre, A. and Hadi, S. 2007. *An introduction to forensic genetics*. John Wiley and Sons Ltd, England. Pg 18-23

Gomez-Raya, L., Okomo-Adhiambo, M., Beattie, C., Osborne, K., Rink, A. and Rauw, W.M. 2007. Modeling inheritance of malignant melanoma with DNA markers in Sinclair Swine. *Genetics* 176, 585-597

Gonzalez-Chavira, M.M., Torres-Pacheco, I., Villordo-Pineda, E., Guevara-Gonzalez, R.G. 2006. DNA Markers: Advances in Agricultural and Food Biotechnology. *Research Signpost* 37/661 (2), 99-134

Grosse, W.M., Kappes, S.M., Laegreid, W.W., Keele, J.W., Chitco-McKown, C.G. and Heaton, M.P. 1999. Single nucleotide polymorphism (SNP) discovery and linkage mapping of bovine cytokine genes. *Mammalian Genome* 10, 1062-1069

Greenhouse, B., Myrick, A., Dokomajilar, C., Woo, J.M., Carlson, E.J, Rosenthal, P.J. and Dorsey, G. 2006. Validation of microsatellite markers for use in genotyping polyclonal Plasmodium Falciparum infections. *American Journal of Tropical Medicine and Hygiene* 75 (5), 836-842

Guido, J.J., Winters, P.C. and Rains, A.B. 2006. Logistic regression basics. NESUG 26 Proceedings, New York. Pg 1-7. http://www.nesug.org/proceedings/nesug06

Hajibabaei, M., de Waard, J.R., Ivanova, N.V., Ratnasingham, S., Dooh, R.T., Kirk, S.L., Mackie, P.M. and Bebert, P.D.N. 2005. Critical factors for assembling a high

volume of DNA barcodes. *Physiological Transactions of Royal Society B*: *Biological Sciences* 360, 1959-1967

Harris, E.J., van Zyl, J.P., Weiermans, J.E. and Parfitt, S.C. 2006. *A comprehensive genetic-based stock-theft prevention system for South Africa*. ARC Press: ARC-Animal Improvement Institute, Irene, South Africa, Pg 1-5

Heaton, M.P., Harhay, G.P., Bennett, G.L., Stone, R.D., Grosse, W.M., Casas, E., Keele, J.W., Smith, T.P.L., Chitko-McKown, C.G. and Laegreid, W.W. 2002. Selection and use of SNP markers for animal identification and paternity analysis in US beef cattle. *Mammalian Genome* 13, 272-281

Hoeijmakers, J.H. 2009. DNA damage, aging and cancer. *The New England Journal of Medicine* 361 (15), 1475-1485.

Hogrefe, H.H. and Borns, M. 2003. High fidelity PCR enzymes. *In: PCR Primer: A laboratory Manual* (2nd edition). Cold Spring Habour Laboratory Press, Coldspring Harbor, NY. Pg 21-34

Jobling, M.A. and Gill, P. 2004. Encoded evidence: DNA in forensic analysis. *Nature Reviews* 5, 739-751

Johnson, M. 2010. Meeting Report: Overview of South Africa by the Department of Agriculture and Forestry and Fisheries. *Parliamentary Monitoring Group*, Parliament of South Africa. http://www.pmg.org.za

Karp, A.H. 2000. Getting started with PROC LOGIC. NESUG 13 Proceedings, Philadelphia. Pg 709-713

Kashi, Y., Lipkin, E., Darvasi, A., Nave, A., Gruenbaum, Y., Beckmann, J.S. and Soller, M. 1990. Parentage identificationin the bovine using deoxyribonucleic acid fingerprints. *Dairy Science* 73, 3306-3311

Kashyap, V.K., Sitalaximi, T., Chattopadhyay, P. and Trivedi, R. 2004. DNA profiling technologies in forensic analysis. *International Journal of Human Genetetics* 4 (1), 11-30

Kobilinsky, L.F., Liotti, T.F., Oeser-Sweat, J. and Watson, J.D. 2004. *DNA: Forensic and Legal Aplications*, Chapter 3. John Wiley and Sons, Hoboken. ISBN: 978-0-471-41478-0

Kunene, N.W. and Fossey, A. 2006. A survey on livestock production in some traditional areas of Northern Kwazulu Natal in South Africa. *Livestock Research for Rural Development* 18 (8), http//www.cipac.org

Kynoch, G. and Ulicki, T. 2001. Cross-border raiding and community conflict in the Lesotho-South Africa border zone. Migration Policy Series No. 21, Southern African Migration Project, Queen's University Press, Canada

Larkin, B., Iaschi, S. Dadour, I. and Tay, G.K. 2009. Using accumulated degree-day to estimate postmoterm interval from the DNA yield of porcine skeletal muscles. *Forensic Science, Medicine and Pathology* 6 (2), 83-92

Li, Z.J., Korolev, S. and Waksman, G. 1998. Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of Thermus aquaticus DNA polymerase I: Structural basis for nucleotide incorporation. *The EMBO Journal* 17 (24) 7514-7525

Liu, Z.J. and Cordes, J.F. 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture* 238, 1-37

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D. and Darnell J. 2000. *Molecular Cell Biology* (4th edition), Chapter 12. W.H. Freeman and Company, New York. ISBN 0-7167-3136-3

Loftus, R., 2005. Traceability of biotech-derived animals: Application of DNA technology. *Revue Scientifique et Technique (International Office of Epizootics)* 24 (1), 231-242

Luftig, M.A and Richey, S. 2001. DNA and forensic science. *New England law review* 35 (3), 609-613

Lynch, K. and Pergolizzi, R.G. 2010. *In vitro* method to quantify UV mediated DNA damage. *Journal of Young Investigators* 20 (2), 1-16

Lynch, M. and Milligan, B.G. 1994. Analysis of population genetics structure with RAPD markers. *Molecular Ecology* 3, 91-99

Mar Alba, M. 2001. Replicative DNA polymerases. *Genome Biology* 2 (1), 3002.1-3002.4

Matthes, M.C., Daly, A. and Edwards, K.J. 1998. Amplified Length Polymorphism (AFLP). *Molecular tools for screening biodiversity: Plants and animals*. Chapman & Hall, London. Pg 183-192

Matukumalli, L.K., Lawley, C.T., Schnabel, R.D., Taylor, J.F., Allan, M.F., Heaton, M.P., O'Connell, J., Moore, S.S., Smith, T.P.L., Sonstegard, T.S. and Van Tassell, C.P. 2009. Development and characterization of high density SNP genotyping assay for cattle. *PloS One* 4 (4), 1-13

Mburu, D. and Hanotte, O. 2005. *A practical approach to microsatellites genotyping with special reference to livestock population*. ILRI Biodiversity project, Nairobi, Kenya. Pg 1-25

McDonald, J.P., Hall, A., Gasparutto, D., Cader, J., Ballantyne, J. and Woodgate, R. 2006. Novel thermostable Y-family polymerases: Applications for the PCR amplification of damaged or ancient DNAs. *Nucleic Acid Research* 34 (4), 1102-1111

Meganathan, P.R., Dubey, B. and Haque, I. 2009. Molecular identification of crocodile species using novel primers for forensic analysis. *Conservation Genetics* 10, 767-770

Michelli, M.R., Bova, R., Pascale, E. and Ambrosio, E. 1994. Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acid Research* 22 (10), 1921-1922

Misner, L.M., Halvorson, M.S., Dreier, J.L., Ubelaker, D.H. and Foran, DR. 2009. The correlation between skeletal weathering and DNA quality and quantity. *Journal of Forensic Science* 54 (4), 822-828

Mitchell, D., Willerslev, E. and Hansen, A. 2005. Review: Damage and repair of ancient DNA. *Mutation Research and Molecular Mechanisms of Mutagenesis* 571 (1-2), 265-276

Mitra, A., Yadav, B.R., Ganai, N.A. and Balakrishnan, C.R., 1999. Molecular markers and their application in livestock improvement. *Current Science* 77 (8), 1045-1053

Mitra, A., 1994. Molecular markers and their different types. PhD Thesis, National dairy Research institute, Deemed University, Karnal, Haryana, India

Mullis, K. Faloona, F., Scharf, S., Saiki, R., Horn G. and Erlich H. 1986. Specific enzymatic amplification of DNA *in vitro*: The Polymerase Chain Reaction. *Cold Spring Harbor Symposium on Quantitative Biology* 51, 263-273

Nelson, J. 2009. Repair of damaged DNA for forensic analysis. PhD Thesis. Molecular and Cellular Biology Laboratory, Biosciences Organization, GE Global Research Center, Niskayuna, New York, 12309. Pg 1-95

Norrgard, K. 2008. Forensics, DNA fingerprinting and CODIS. PhD Thesis. *Nature Education* (1). http://www.nature.com/scitable/topicpage/forensics

Nyren, P. 2006. The history of pyrosequencing. *Methods in Molecular Biology* 373, 1-14

Olivier, N. 2001. The role of DNA in the investigation of crime: A case study of South Africa investigators. Police Practice Group, Technikon SA, Florida, South Africa. Pg 1-9

Pimentel, D and Wilson, A. 2004. World population, agriculture and malnutrition. *World Watch* 17 (5), 22-25

Putkonen, M.K., Palo, J.U., Cano, J.M., Hedman, M. and Sajantila, A. 2010. Factors affecting the STR amplification success in poorly preserved bone samples. *Investigative Genetics* 1 (9), 1-7

Rakoczy-Trojanowska, M. and Bolibok, H. 2004. Characteristics and a comparison of three classes of microsatellite-based markers and their application. *Cellular and Mollecular Biology Letters* 9, 221-238

Roberts, L., Davenport, R.J., Pennisi, E. and Marshall, E. 2001. A history of human genome project. *Science* 291 (5507), 1195-1201

Roffey, E.P. and Harmon, G.J. 1993. The use of PCR technology for routine grouping in the forensic biology laboratory. Australian Institute of Criminology, John Tonge Centre, Queensland. Pg 1-6

Rohrer, G.A., Freking, B.A. and Nonneman, D. 2007. Single nucleotide polymorphisms for pig identification and parentage exclusion. *Animal Genetics* 38, 253-258

Roushdy, K.H., El-Dein, A.Z., Fathi, M.M., Ali, U.M. and Assy, H.M. 2008. Microsatellite genetic differentiation analysis of two local chicken breeds compared with foreign Hy-Line strain. *International Journal of Poultry Science* 7 (11), 1045-1053

Selkoe, K.A. and Toonen, R.J. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite marker. *Ecology Letters* 9, 615-629

Semagn, K., Bjornstad, A. and Ndjiondjop, M.N. 2006. An overview of molecular marker methods for plants. *African Journal of Biotechnology* 5, 2540-2568

Sikorsky, J.A., Primerano, D.A., Fenger, T.W. and Denvir, J. 2007. DNA damage reduces *Taq* polymerase fidelity and PCR amplification efficiency. *Biochemical and Biophysical Research Communications* 355 (2), 431-437

Skage, M. and Schander, C. 2007. DNA from Formalin-fixed tissue: Extraction or repair? *Marine Biology Research* 3 (5), 289-295

Smith, J.J., Scott-Craig, J.S., Leadbetter, J.R., Bush, G.L., Roberts, D.L. and Fulbright, D.W. 1994. Characterization of Rapid Amplified Polymorphic DNA (RAPD) products from *Xanthomonas campestris* and some comments on the use of RAPD products in phylogenetic analysis. *Molecular Phylogenetics and Evolution* 3 (2), 135-145

Sobrino, B., a Brio`n, M. and Carracedo, A. 2005. SNPs in forensic genetics: A review on SNP typing methodologies. *Forensic Science International* 154, 181-194

Swerdlow, H. and Gesteland, R. 1990. Capillary gel electrophoresis for rapid, high resolution DNA sequencing. *Nucleic Acid Research* 18 (6), 1415-1419

Szibor, R., Krawczak, M., Hering, S., Edelmann, J., Kuhlisch, E. and Krause D. 2003. Use of X-linked markers for forensic purposes. *International Journal of Legal Medicine* 117, 67-74

Tamaki, K. and Jeffreys, A.J. 2005. Human tandem repeat sequences in forensic DNA typing. *Legal Medicine* 7, 244-250

Tautz, D. 1989. Hyper variability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* 17, 6463-6471

Teletchea, F., Maudet, C. and Hanni, C. 2005. Food and forensic molecular identification: update and challenges. *Trends in Biotechnology* 23, 359-366

Teneva, A. and Petrovic, M.P. 2010. Application of molecular markers in livestock improvement. *Biotechnology in Animal Husbandry* 26 (3-4), 135-154

Tvedebrink, T., Eriksenal, P.S., Mogensen, H.S. and Morling, N.M. 2009. Estimating the probability of allelic drop-out of STR alleles in Forensic genetics. *Forensic Science International: Genetics* 3 (4), 222-226

Van de Goor, L.H.P., Koskinen, M.T. and Van Haeringen, W.A. 2009. Population studies of 16 bovine STR loci for forensic purposes. *International Journal of Legal Medicine* 125 (1), 111-119. ISSN: 1437-1596

Van Eenennaam, A.L., Weaber, R.L., Drake, D.J., Penedo, M.C.T., Quaas, R.L., Garrick, D.J. and Pollak, E.J. 2007. DNA-based paternity analysis and geneticevaluation in a large commercial cattle ranch settings. *Journal of Animal Science* 85, 3159-3169

Vazquez, J., Perez, T., Urena, F. Gudin, E. Albornoz, J. and Dominguez, A., 2004. Practical application of DNA fingerprinting to trace beef. *Journal of Food Protection* 67, 972-979

Veneroni, G.B., Meirelles, S.L., Grossi, D.A., Gasparin, G., Ibelli, A.M.G., Tizioto, P.C., Oliveira, H.N., Alencar, M.M. and Regitano, L.C.A. 2010. Prospecting candidate SNPs for backfat in Canchim beef cattle. *Genetics and Molecular Research* 9 (4), 1997-2003

Vignal, A., Milan, D., SanCristobal, M. and Eggen, A., 2002. A review on SNP and other types of molecular markers and their use in animal genetics. *Genetic Selection* 34, 275-305

Walker, E.A. and Wyndham, H.U. 1941. A history of South Africa. *Journal of the Royal African Society* 40 (158), 11-18

Williams, J.G.K, Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids research* 18 (22), 6531-6535

Wang, Y., Prosen, D.E., Mei, L., Sullivan, J.C., Finney, M. And Vander Horn, P.B. 2004. A novel strategy to engineer DNA polymerase for enhanced processivity and improved performance in vitro. *Nucleic Acids Research* 32 (3), 1197-1207

Womack, J.E. 2005. Advances in livestock genomics: Opening the barn door. *Genome Research* 15, 1699-1705

Wolff, K., Schoen, E.D. and Van Rijn, P. 1993. Optimizing the generation of random amplified polymorphic DNA in chrysanthemum. *Theory of Applied Genetics* 86, 1033-1037

Wright, J.M and Bentzen, P. 1994. Microsatellites: Genetic markers for the future. Reviews in Fish Biology and Fisheries 4, 384-388

Yang, D.Y. and Speller, C.F. 2006. Co-amplification of cytochrome b and D-loop mtDNA fragments for the identification of degraded DNA samples. *Molecular Ecology Notes* 6, 605-608

http://science.jrank.org
http://www.fermentas.com/catalog
www.nafufarmers.co.za
www.sigmaadrich.com
www.roche-applied-science.com

Appendix 1: The overall odds estimate on enzymes and on markers.

Odds estimates between markers in all enzymes									
Markers	**Day 0-4**			**Day 5-11**			**Day 5-12**		
	Fas	**Res**	**Tpol**	**Fas**	**Res**	**Tpol**	**Fas**	**Res**	**Tpol**
BM1818 vs CSRM60	**2.621**	**17.715**	0.878	**7.623**	0.971	**5.507**	**2.041**	**2.37**	**8.432**
BM1818 vs CSSM66	**2.657**	**2.235**	**2.41**	**4.675**	**2.89**	1.059	1.152	**2.215**	**5.607**
BM1818 vs ETH225	0.316	0.807	1.293	0.97	0.878	0.387	1.076	0.465	0.659
BM1818 vs ETH3	1.128	0.035	0.051	0.004	0.605	0.937	0.502	0.094	0.043
BM1818 vs ILSTS006	**68.852**	**107.503**	**3.671**	**70.993**	**30.692**	**12.471**	**5.559**	**8.235**	**593.535**
BM1818 vs INRA23	0.182	1.453	**2.497**	**2.591**	0.702	0.363	1.4	0.648	0.728
BM1818 vs TGLA122	0.575	**2.768**	1.96	**3.68**	0.779	1.088	1.474	1.446	1.463
BM1818 vs TGLA126	**2.819**	**9.295**	**2.785**	**9.737**	**2.025**	**3.699**	**2.658**	**3.875**	**7.082**
BM1818 vs TGLA227	0.254	**3.59**	0.923	**3.838**	0.184	1.194	0.863	1.277	0.763
BM1818 vs TGLA53	1.744	1.787	**4.626**	**4.81**	**1.84**	1.629	1.718	**4.386**	**1.913**
BM1818 vs ETH10	**7.551**	**19.044**	0.236	1.016	0.84	**39.725**	**4.417**	**2.119**	**3.62**
BM1818 vs SPS115	**3.926**	**17.957**	1.717	**2.605**	1.497	**31.038**	**11.837**	**4.502**	**2.271**
BM1818 vs HAUT27	1.709	**15.686**	**3.176**	**5.357**	**4.273**	**3.719**	**9.298**	1.27	**7.208**
BM1818 vs BM1824		**2.173**	**3.547**	**5.59**	1.585	**1.986**	1.379	**5.109**	**2.498**
BM1818 vs BM2113		0.149	1.551	0.353	<0.001	0.11	0.654	0.261	<0.001
BM1824 vs BM2113	<0.001	0.069	0.437	0.063	<0.001	0.055	0.474	0.051	<0.001
BM1824 vs CSRM60	1.148	**8.153**	0.248	1.364	0.613	**2.773**	1.48	0.464	**3.376**
BM1824 vs CSSM66	1.164	1.028	0.679	0.836	**1.823**	0.533	0.835	0.434	**2.245**
BM1824 vs ETH225	0.138	0.371	0.365	0.174	0.554	0.195	0.78	0.091	0.264
BM1824 vs ETH3	0.494	0.016	0.014	<0.001	0.381	0.472	0.364	0.018	0.017
BM1824 vs ILSTS006	**30.16**	**49.476**	1.035	**12.701**	**19.363**	**6.28**	**4.032**	1.612	**237.622**
BM1824 vs INRA23	0.08	0.669	0.704	0.464	0.443	0.183	1.015	0.127	0.292
BM1824 vs TGLA122	0.252	1.274	0.553	0.658	0.492	0.548	1.069	0.283	0.586
BM1824 vs TGLA126	1.235	**4.278**	0.785	**1.742**	1.278	**1.863**	**1.928**	0.759	**2.835**
BM1824 vs TGLA227	0.111	1.652	0.26	0.687	0.116	0.601	0.626	0.25	0.306
BM1824 vs TGLA53	0.764	0.822	1.304	0.861	1.161	0.82	1.246	0.859	0.766
BM1824 vs ETH10	**3.307**	**8.765**	0.066	0.182	0.53	**20.004**	**3.203**	0.415	1.449
BM1824 vs SPS115	1.72	**8.264**	0.484	0.466	0.945	**15.629**	**8.585**	0.881	0.909
BM1824 vs HAUT27	0.749	**7.219**	0.895	0.958	**2.695**	**1.873**	**6.743**	0.249	**2.886**
BM2113 vs CSRM60	**>999.999**	**118.873**	0.566	**21.572**	**>999.999**	**49.968**	**3.12**	**9.068**	**>999.999**
BM2113 vs CSSM66	**>999.999**	**14.995**	1.553	**13.228**	**>999.999**	**9.609**	**1.761**	**8.477**	**>999.999**
BM2113 vs ETH225	**>999.999**	**5.413**	0.834	**2.745**	**>999.999**	**3.508**	1.644	**1.779**	**>999.999**
BM2113 vs ETH3	**>999.999**	0.237	0.033	0.01	**>999.999**	**8.5**	0.768	0.361	**>999.999**
BM2113 vs ILSTS006	**>999.999**	**721.377**	**2.366**	**200.891**	**>999.999**	**113.16**	**8.497**	**31.513**	**>999.999**
BM2113 vs INRA23	**>999.999**	**9.747**	1.61	**7.333**	**>999.999**	**3.294**	**2.14**	**2.478**	**>999.999**
BM2113 vs TGLA122	**>999.999**	**18.571**	1.263	**10.412**	**>999.999**	**9.87**	**2.253**	**5.534**	**>999.999**
BM2113 vs TGLA126	**>999.999**	**62.372**	1.795	**27.552**	**>999.999**	**33.567**	**4.063**	**14.828**	**>999.999**
BM2113 vs TGLA227	**>999.999**	**24.088**	0.595	**10.861**	**>999.999**	**10.833**	1.32	**4.885**	**>999.999**
BM2113 vs TGLA53	**>999.999**	**11.989**	**2.982**	**13.612**	**>999.999**	**14.783**	**2.626**	**16.784**	**>999.999**
BM2113 vs ETH10	**>999.999**	**127.79**	0.152	**2.875**	**>999.999**	**360.465**	**6.752**	**8.108**	**>999.999**
BM2113 vs SPS115	**>999.999**	**120.497**	1.107	**7.371**	**>999.999**	**281.64**	**18.094**	**17.228**	**>999.999**
BM2113 vs HAUT27	**>999.999**	**105.26**	**2.047**	**15.16**	**>999.999**	**33.746**	**14.213**	**4.86**	**>999.999**
CSRM60 vs CSSM66	1.014	0.126	**2.744**	0.613	**2.975**	0.192	0.564	0.935	0.665
CSRM60 vs ETH225	0.12	0.046	1.473	0.127	0.904	0.07	0.527	0.196	0.078
CSRM60 vs ETH3	0.43	0.002	0.058	<0.001	0.622	0.17	0.246	0.04	0.005

CSRM60 vs ILSTS006	**26.268**	**6.068**	**4.18**	**9.313**	**31.593**	**2.265**	**2.724**	**3.475**	**70.391**
CSRM60 vs INRA23	0.069	0.082	**2.844**	0.34	0.722	0.066	0.686	0.273	0.086
CSRM60 vs TGLA122	0.219	0.156	**2.232**	0.483	0.802	0.198	0.722	0.61	0.173
CSRM60 vs TGLA126	1.075	0.525	**3.17**	1.277	**2.085**	0.672	1.302	1.635	0.84
CSRM60 vs TGLA227	0.097	0.203	1.051	0.503	0.189	0.217	0.423	0.539	0.091
CSRM60 vs TGLA53	0.665	0.101	**5.267**	0.631	**1.894**	0.296	0.842	**1.851**	0.227
CSRM60 vs ETH10	**2.881**	1.075	0.268	0.133	0.864	**7.214**	**2.164**	0.894	0.429
CSRM60 vs SPS115	1.498	1.014	**1.955**	0.342	1.541	**5.636**	**5.8**	**1.9**	0.269
CSRM60 vs HAUT27	0.652	0.885	**3.616**	0.703	**4.398**	0.675	**4.556**	0.536	0.855
CSSM66 vs ETH225	0.119	0.361	0.537	0.207	0.304	0.365	0.934	0.21	0.117
CSSM66 vs ETH3	0.425	0.016	0.021	<0.001	0.209	0.885	0.436	0.043	0.008
CSSM66 vs ILSTS006	**25.912**	**48.108**	1.523	**15.187**	**10.619**	**11.776**	**4.826**	**3.718**	**105.854**
CSSM66 vs INRA23	0.068	0.65	1.036	0.554	0.243	0.343	1.215	0.292	0.13
CSSM66 vs TGLA122	0.216	1.238	0.813	0.787	0.27	1.027	1.28	0.653	0.261
CSSM66 vs TGLA126	1.061	**4.16**	1.156	**2.083**	0.701	**3.493**	**2.308**	**1.749**	1.263
CSSM66 vs TGLA227	0.096	1.606	0.383	0.821	0.064	1.127	0.749	0.576	0.136
CSSM66 vs TGLA53	0.656	0.8	1.92	1.029	0.636	1.538	1.492	**1.98**	0.341
CSSM66 vs ETH10	**2.842**	**8.522**	0.098	0.217	0.29	**37.513**	**3.835**	0.957	0.646
CSSM66 vs SPS115	1.478	**8.036**	0.713	0.557	0.518	**29.31**	**10.276**	**2.032**	0.405
CSSM66 vs HAUT27	0.643	**7.02**	1.318	1.146	1.478	**3.512**	**8.072**	0.573	1.286
ETH225 vs ETH3	**3.573**	0.044	0.039	0.004	0.688	**2.423**	0.467	0.203	0.065
ETH225 vs ILSTS006	**218.066**	**133.279**	**2.838**	**73.191**	**34.944**	**32.253**	**5.168**	**17.712**	**901.106**
ETH225 vs INRA23	0.576	**1.801**	**1.931**	**2.672**	0.799	0.939	1.302	1.393	1.105
ETH225 vs TGLA122	1.821	**3.431**	1.515	**3.794**	0.887	**2.813**	1.371	**3.111**	**2.221**
ETH225 vs TGLA126	**8.927**	**11.524**	**2.153**	**10.038**	**2.306**	**9.567**	**2.472**	**8.334**	**10.752**
ETH225 vs TGLA227	0.804	**4.45**	0.714	**3.957**	0.209	**3.088**	0.803	**2.746**	1.159
ETH225 vs TGLA53	**5.524**	**2.215**	**3.577**	**4.959**	**2.095**	**4.213**	1.598	**9.434**	**2.904**
ETH225 vs ETH10	**23.914**	**23.61**	0.182	1.047	0.956	**102.742**	**4.107**	**4.557**	**5.495**
ETH225 vs SPS115	**12.435**	**22.263**	1.328	**2.685**	1.705	**80.275**	**11.006**	**9.683**	**3.448**
ETH225 vs HAUT27	**5.412**	**19.447**	**2.455**	**5.523**	**4.865**	**9.618**	**8.645**	**2.732**	**10.943**
ETH3 vs ILSTS006	**61.037**	**>999.999**	**72.503**	**>999.999**	**50.761**	**13.313**	**11.071**	**87.18**	**>999.999**
ETH3 vs INRA23	0.161	**41.084**	**49.325**	**726.764**	1.161	0.387	**2.788**	**6.855**	**17.102**
ETH3 vs TGLA122	0.51	**78.272**	**38.71**	**>999.999**	1.289	1.161	**2.936**	**15.31**	**34.359**
ETH3 vs TGLA126	**2.499**	**262.886**	**54.996**	**>999.999**	**3.35**	**3.949**	**5.294**	**41.021**	**166.337**
ETH3 vs TGLA227	0.225	**101.525**	**18.23**	**>999.999**	0.304	1.274	**1.719**	**13.513**	**17.927**
ETH3 vs TGLA53	1.546	**50.532**	**91.364**	**>999.999**	**3.043**	**1.739**	**3.422**	**46.432**	**44.93**
ETH3 vs ETH10	**6.694**	**538.612**	**4.653**	**284.907**	1.389	**42.407**	**8.797**	**22.432**	**85.016**
ETH3 vs SPS115	**3.48**	**507.872**	**33.912**	**730.561**	**2.476**	**33.133**	**23.575**	**47.662**	**53.349**
ETH3 vs HAUT27	1.515	**443.65**	**62.718**	**>999.999**	**7.066**	**3.97**	**18.518**	**13.445**	**169.293**
ILSTS006 vs INRA23	0.003	0.014	0.68	0.037	0.023	0.029	0.252	0.176	0.001
ILSTS006 vs TGLA122	0.008	0.026	0.534	0.052	0.025	0.087	0.265	0.471	0.002
ILSTS006 vs TGLA126	0.041	0.086	0.759	0.137	0.066	0.297	0.478	0.155	0.012
ILSTS006 vs TGLA227	0.004	0.033	0.251	0.054	0.006	0.096	0.155	0.533	0.001
ILSTS006 vs TGLA53	0.025	0.017	1.26	0.068	0.06	0.131	0.309	0.257	0.003
ILSTS006 vs ETH10	0.11	**0.177**	0.064	0.014	0.027	**3.185**	0.795	0.547	0.006
ILSTS006 vs SPS115	0.057	0.167	0.468	0.037	0.049	**2.489**	**2.129**	0.154	0.004
ILSTS006 vs HAUT27	0.025	0.146	0.865	0.075	0.139	0.298	1.673		0.012
INRA23 vs TGLA122	**3.16**	**1.905**	0.785	1.42	1.11	**2.997**	1.053	**2.234**	**2.009**
INRA23 vs TGLA126	**15.491**	**6.399**	1.115	**3.757**	**2.886**	**10.191**	**1.899**	**5.985**	**9.726**

INRA23 vs TGLA227	1.395	**2.471**	0.37	1.481	0.262	**3.289**	0.617	**1.971**	1.048
INRA23 vs TGLA53	**9.587**	1.23	**1.852**	**1.856**	**2.621**	**4.488**	1.227	**6.774**	**2.627**
INRA23 vs ETH10	**41.498**	**13.11**	0.094	0.392	**1.196**	**109.442**	**3.155**	**3.273**	**4.971**
INRA23 vs SPS115	**21.578**	**12.362**	0.688	1.005	**2.134**	**85.509**	**8.455**	**6.953**	**3.119**
INRA23 vs HAUT27	**9.392**	**10.799**	1.272	**2.067**	**6.088**	**10.246**	**6.641**	**1.962**	**9.899**
TGLA122 vs TGLA126	**4.902**	**3.359**	1.421	**2.646**	**2.599**	**3.401**	**1.803**	**2.679**	**4.841**
TGLA122 vs TGLA227	0.441	1.297	0.471	1.043	0.236	1.098	0.586	0.883	0.522
TGLA122 vs TGLA53	**3.034**	0.646	**2.36**	1.307	**2.361**	1.498	1.166	**3.033**	1.308
TGLA122 vs ETH10	**13.132**	**6.881**	0.12	0.276	1.077	**36.52**	**2.996**	1.465	**2.474**
TGLA122 vs SPS115	**6.828**	**6.489**	0.876	0.708	**1.921**	**28.534**	**8.03**	**3.113**	1.553
TGLA122 vs HAUT27	**2.972**	**5.668**	1.62	1.456	**5.483**	**3.419**	**6.307**	0.878	**4.927**
TGLA126 vs TGLA227	0.09	0.386	0.331	0.394	0.091	0.323	0.325	0.329	0.108
TGLA126 vs TGLA53	0.619	0.192	1.661	0.494	0.908	0.44	0.646	1.132	0.27
TGLA126 vs ETH10	**2.679**	**2.049**	0.085	0.104	0.415	**10.739**	1.662	0.547	0.511
TGLA126 vs SPS115	1.393	**1.932**	0.617	0.268	0.739	**8.39**	**4.453**	1.162	0.321
TGLA126 vs HAUT27	0.606	1.688	1.14	0.55	**2.109**	1.005	**3.498**	0.328	1.018
TGLA227 vs TGLA53	**6.871**	0.498	**5.012**	1.253	10.022	1.365	**1.99**	**3.436**	**2.506**
TGLA227 vs ETH10	**29.744**	**5.305**	0.255	0.265	4.574	33.274	**5.117**	1.66	**4.742**
TGLA227 vs SPS115	**15.466**	**5.002**	**1.86**	0.679	8.157	25.997	**13.712**	**3.527**	**2.976**
TGLA227 vs HAUT27	**6.732**	**4.37**	**3.44**	1.396	23.277	3.115	**10.771**	0.995	**9.444**
TGLA53 vs ETH10	**4.329**	**10.659**	0.051	0.211	0.456	24.384	**2.571**	0.483	**1.892**
TGLA53 vs SPS115	**2.251**	**10.05**	0.371	0.541	0.814	19.052	**6.889**	1.026	1.187
TGLA53 vs HAUT27	0.98	**8.78**	0.686	1.114	2.322	2.283	**5.412**	0.29	**3.768**
ETH10 vs SPS115	0.52	0.943	**7.287**	2.564	1.783	0.781	**2.68**	**2.125**	0.628
ETH10 vs HAUT27	0.226	0.824	**13.478**	5.274	5.089	0.094	**2.105**	0.599	**1.991**
SPS115 vs HAUT27	0.435	0.874	**1.849**	2.057	2.853	0.12	**0.786**	0.282	**3.173**

Appendix 2: Odds estimate ratio of enzymes and markers.

Odd estimates ratios on enzymes	0- 4 days	5-11 days	12-15 days
FSTARTX5 vs RESTX7 MARK=BM1818	0.143	1.584	0.673
FSTARTX5 vs TPOL1 MARK=BM1818	0.303	0.581	0.808
RESTX7 vs TPOL1 MARK=BM1818	2.122	0.367	1.201
FSTARTX5 vs RESTX7 MARK=BM1824	0.136	0.449	2.492
FSTARTX5 vs TPOL1 MARK=BM1824	0.471	0.207	1.463
RESTX7 vs TPOL1 MARK=BM1824	3.465	0.46	0.587
FSTARTX5 vs RESTX7 MARK=BM2113	>999.999	<0.001	0.269
FSTARTX5 vs TPOL1 MARK=BM2113	>999.999	0.181	<0.001
RESTX7 vs TPOL1 MARK=BM2113	22.093	506.586	<0.001
FSTARTX5 vs RESTX7 MARK=CSRM60	0.965	0.202	0.781
FSTARTX5 vs TPOL1 MARK=CSRM60	0.102	0.42	3.337
RESTX7 vs TPOL1 MARK=CSRM60	0.105	2.081	4.274
FSTARTX5 vs RESTX7 MARK=CSSM66	0.12	0.979	1.293
FSTARTX5 vs TPOL1 MARK=CSSM66	0.275	0.132	3.932
RESTX7 vs TPOL1 MARK=CSSM66	2.289	0.134	3.04
FSTARTX5 vs RESTX7 MARK=ETH225	0.365	1.434	0.291
FSTARTX5 vs TPOL1 MARK=ETH225	1.241	0.232	0.495
RESTX7 vs TPOL1 MARK=ETH225	3.403	0.162	1.702
FSTARTX5 vs RESTX7 MARK=ETH3	0.004	268.564	0.127
FSTARTX5 vs TPOL1 MARK=ETH3	0.014	152.749	0.069
RESTX7 vs TPOL1 MARK=ETH3	3.039	0.569	0.541
FSTARTX5 vs RESTX7 MARK=ILSTS006	0.223	0.685	0.996
FSTARTX5 vs TPOL1 MARK=ILSTS006	0.016	0.102	86.251
RESTX7 vs TPOL1 MARK=ILSTS006	0.072	0.149	86.567
FSTARTX5 vs RESTX7 MARK=INRA23	1.14	0.429	0.311
FSTARTX5 vs TPOL1 MARK=INRA23	4.159	0.081	0.42
RESTX7 vs TPOL1 MARK=INRA23	3.649	0.19	1.351
FSTARTX5 vs RESTX7 MARK=TGLA122	0.687	0.335	0.66
FSTARTX5 vs TPOL1 MARK=TGLA122	1.033	0.172	0.802
RESTX7 vs TPOL1 MARK=TGLA122	1.503	0.512	1.215
FSTARTX5 vs RESTX7 MARK=TGLA126	0.471	0.329	0.98
FSTARTX5 vs TPOL1 MARK=TGLA126	0.299	0.221	2.152
RESTX7 vs TPOL1 MARK=TGLA126	0.636	0.67	2.195
FSTARTX5 vs RESTX7 MARK=TGLA227	2.019	0.076	0.995
FSTARTX5 vs TPOL1 MARK=TGLA227	1.102	0.181	0.714
RESTX7 vs TPOL1 MARK=TGLA227	0.546	2.388	0.718
FSTARTX5 vs RESTX7 MARK=TGLA53	0.146	0.606	1.717
FSTARTX5 vs TPOL1 MARK=TGLA53	0.804	0.197	0.899

RESTX7 vs TPOL1 MARK=TGLA53	5.495	0.325	0.524
FSTARTX5 vs RESTX7 MARK=ETH10	0.36	1.309	0.323
FSTARTX5 vs TPOL1 MARK=ETH10	0.009	22.736	0.662
RESTX7 vs TPOL1 MARK=ETH10	0.026	17.37	2.052
FSTARTX5 vs RESTX7 MARK=SPS115	0.653	0.91	0.256
FSTARTX5 vs TPOL1 MARK=SPS115	0.133	6.928	0.155
RESTX7 vs TPOL1 MARK=SPS115	0.203	7.61	0.606
FSTARTX5 vs RESTX7 MARK=HAUT27	1.311	1.263	0.092
FSTARTX5 vs TPOL1 MARK=HAUT27	0.563	0.404	0.626
RESTX7 vs TPOL1 MARK=HAUT27	0.43	0.32	6.816