Genetic diversity of *Aloe* species in Kenya and the efficacy of *Aloe secundiflora*, *Aloe lateritia* and *Aloe turkanesis* on *Fusarium oxysporum* and *Pythium ultimum*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University or any other institution of higher learning.

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DEDICATION

This thesis is dedicated to my loving mum Elyjoy Muthoni Micheni and dad Isaac Micheni Nkari who have gone out of their way to support my education. I also dedicate this work to my brothers Maurice Murimi and Brian Muchiri Micheni who have been a constant source of encouragement.

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ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immuno Deficiency Syndrome
AFLPs	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
CLSI	Clinical and Laboratory Standards Institute
CTAB	Cetyl Trimethyl Ammonium Bromide
DCM	Dichloromethane
°C	Degrees centigrade
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
ESTs	Expressed Sequence Tags
EDTA	Ethylene diamine tetra acetic acid
GBK	Gene Bank of Kenya
H_E	Expected heterozygosity
KALRO	Kenya Agricultural and Livestock Research Organization
KAPAP	Kenya Agricultural Productivity and Agribusiness Project
kb	Kilobase(s)
MICs	Minimum Inhibitory Concentrations
NILs	Near Isogenic Lines
ORFs	Open Reading Frames
PCR	Polymerase Chain Reaction
SCARs	Sequence Characterized Amplified Regions
SNPs	Single Nucleotide Polymorphisms

S-SAPS Sequence-Specific Amplification Polymorphisms

SSRs Single Sequence Repeats

THSDT Tukeys Honest Significant Difference Test

ABSTRACT

Aloe species are common in Kenya and have many health benefits which include promotion of wound healing, antifungal activity, hypoglycemic or anti-diabetic effects, anti-inflammatory and anticancer properties. However, little has been done to study the genetic variability of *Aloe* species in Kenya. *Fusarium oxysporum* and *Pythium ultimum* are economically important plant pathogens with a wide host range causing several diseases including root rots and damping off. The objectives of this study were to genetically characterize *Aloe* species found in Kenya and assess the efficacy of their extracts on *Fusarium oxysporum* and *Pythium ultimum*. Thirty one morphologically diverse *Aloe* species were sampled from ex-situ and in-situ collections. Morphological characterization was done using leaf color, presence or absence of variegation and color of spines. DNA extraction was carried out using cetyl trimethyl ammonium bromide (CTAB) method. Sequence related amplified polymorphism (SRAP) markers were used to determine genetic diversity of the *Aloe* species Forty eight primer pairs were screened to select those that had the highest number of polymorphisms. Eight primer pairs, which were polymorphic, were selected to genotype the *Aloe* species. Crude extracts of the leafs were made by extracting with dichloromethane: methanol in the ratio of 1:1 and made into concentrations of 100mg/100µl, 50mg/100µl, 25mg/100µl, 12.5mg/100µl and 6.25mg/100µl using sterile distilled water. Similar concentrations were done with the antifungal drug triazole as the positive control. Assessment of the efficacy of different concentrations of the *Aloe* species leaf extracts against *Fusarium oxysporum* and *Pythium ultimum* was carried out using the agar disc diffusion method. Paper discs impregnated with different concentrations of the *Aloe* extracts were placed in the petri plates and zones of inhibition measured in millimeters for 11 days. SRAP primers produced 893 genetic loci with percentage polymorphic loci of 56.8%. Nei genetic distance for the populations ranged from 0.094 to 0.362 with 9% and 91% variation among

populations and within individuals, respectively. Organic crude extracts of *A. Secundiflora* showed the highest activity (Mean inhibition = 16.01 mm) against the test fungi compared to *A. lateritia* (Mean inhibition = 13.09 mm) and *A. turkanensis* (Mean inhibition = 11.04 mm). The crude extracts showed higher activity against *F. oxysporum* than *P. ultimum*; but there was no activity for all the crude extracts at 6.25mg/ml. None of the crude extracts showed higher activity than the commercially used fungicide triazole. The findings of this study could form basis for better conservation of the genus which has potential to control fungi of agricultural importance alongside other control measures.

Key words: *Aloe* species, Sequence Related Amplified Polymorphism (SRAP), *Fusarium oxysporum*, *Pythium ultimum*.

CHAPTER ONE: INTRODUCTION

1.1 Background of the study

Aloe genus is a perennial succulent herb found in tropical and subtropical parts of the world. The genus consists of about 500 species of flowering and succulent plants. The different species have different concentrations of active ingredients (Das *et al.*, 2011). A quarter of *Aloe* genera is valued for traditional medicine (Grace *et al.*, 2009), while a small number is harvested from the wild or cultivated for natural products prepared from the bitter leaf exudates or gel-like leaf mesophyll. *Aloe* gel is 99% water with a pH of 4.5 and is a common ingredient in many non-prescription skin salves. *Aloe* extracts have been used to treat canker sores and stomach ulcers. The gel contains an emollient polysaccharide, glucomannan, which is a good moisturizer utilized in many cosmetics. Acemannan, the major carbohydrate fraction in the gel demonstrates antineoplastic and antiviral effects.

In nature, *Aloe* species is propagated through lateral buds (Rahimi-Dehgolan, 2012). There are morphological variations (Darokar *et al.*, 2003) as well as leaf phenolic constitution (Van *et al.*, 1995; Viljoen, 1999; Das *et al.*, 2011) in some economically important Aloe species. However, due to lack of expressions for reproductive characters in some of the species, it is impossible to distinguish them (Reynolds, 1990). The success of any genetic conservation and breeding program depends largely on the identification of the amount and distribution of genetic diversity in the gene pool of the concerned plant. Knowledge on the genetic diversity and relationships among plant varieties is important to recognize gene pools, to identify gaps in germplasm collections and to develop effective conservation and management strategies (Nayanakantha *et al.*, 2010).

DNA- based assays have revolutionized and modernized the ability to characterize genetic variation. The first advantage of molecular techniques is their capacity to detect genetic diversity at a higher level of resolution than other methods. They are also robust and speedy, and information may be obtained from small amounts of plant material at any stage of development. In addition, they are not affected by environmental conditions (Lanteri *et al.*, 2001). Examples of markers that have been used for molecular characterization include: Restriction Fragment Length Polymorphism (RFLPs), and PCR-based markers such as Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLP), Microsatellites/ Simple Sequence Repeats (SSRs), Sequence Characterized Amplified Regions (SCARs) and Sequence Related Amplified Polymorphism (SRAP) markers.

Sequence-related amplified polymorphism (SRAP) genetic marker system, reveals genetic variation in the open reading frames (ORFs) of genomes among related organisms (Li *et al.*, 2003). SRAP has been used in the construction of genetic maps, comparative genetic studies and examination of genetic diversities in many plant species due to its simplicity and efficiency (Li *et al.*, 2003). It has also been used to study genetic variations in fungi (Yu *et al.*, 2008). Given its effectiveness and reliability in detecting genetic variation, this technique may provide a useful alternative for studying genetic variability in *Aloe* species.
Medicinal plants are a rich source of antimicrobial agents and normally produce bioactive secondary metabolites and many of them exhibit activity, hence can be used in antimicrobial drugs (Mahesh *et al.*, 2008). *Aloe vera* contains various biochemical components including

phenol, saponin, anthraquinones, which are classified as anti-bacterial, antiviral and antifungal agents.

Fusarium oxysporum and *Pythium ultimum* are plant pathogens that cause detrimental effects to a wide host range of crops. They cause a number of diseases including root rots and damping off (Ayanardi *et al.*, 2012). These pathogens are soil borne and colonize senescing tissues of the diseased plant surviving in the soil for many years.

Various approaches currently being used to control the pathogens include: physical, chemical, biological and cultural methods. Effective and efficient management of crop diseases has been generally achieved by use of synthetic pesticides (Kiran *et al.*, 2006). However due to increased awareness on the risks involved in use of pesticides, increased attention has been focused on alternative methods of pathogen control. The cost of chemical fungicides particularly in countries where pesticides are imported is quite inhibitive for resource poor farmers. Pollution to soil, water and air due to accumulation of obnoxious chemical residues following continuous use of fungicides and development of resistant pathogen races are additional factors that are making scientists to search for alternative control methods which are ecologically friendly, safe and pathogen specific. The recurrent and indiscriminate use of fungicides poses a serious threat to human health and to the existing human ecogeographical conditions as some of them have already been proved to be either mutagenic or carcinogenic. The use of plant extracts in the management of plant diseases is therefore gaining importance (Kiran *et al.*, 2006).

1.2 Problem Statement and Justification

Throughout the world, biodiversity is changing at an unprecedented rate. The most important drivers of this change are climate change, pollution, unsustainable harvesting of natural resources and the introduction of exotic species (Brooks *et al.*, 2002).

In Kenya *Aloe* species are used by local communities for medicinal, cultural and aesthetic purposes. Industrial uses include pharmaceuticals, cosmetics, nutraceuticals among others. Some *Aloe* species found in Kenya are currently being exploited for commercial bitter gum (Wabuyele, 2006). However, concern has been raised locally and internationally on the level and impact of this exploitation to wild populations. Unsustainable harvesting of wild *Aloe* species poses many threats ranging from overexploitation to ecological imbalance and possible loss of the species. Overexploitation of *Aloe* species in Kenya prompted a Presidential decree in 1986 banning harvesting of *Aloe* species from the wild for commercial purposes.

It is important to determine the genetic diversity of *Aloe* species in Kenya. Knowledge on the genetic diversity and relationships among plant varieties is important to recognize gene pools, to identify gaps in germplasm collections and to develop effective conservation and management strategies (Nayanakantha *et al.*, 2010). The success of any genetic conservation and breeding program depends largely on the identification of the amount and distribution of genetic diversity in the gene pool of the concerned plant. Molecular markers may provide a powerful tool for determining genetic diversity of *Aloe* species thereby contributing to better conservation of the genus.

Fusarium oxysporum and *Pythium ultimum* are fungal pathogens that cause damping off and root rots. They infect a wide range of plants and cause serious losses to agricultural crops. Antimicrobial activities of *Aloe* species have been reported against *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisae* (Kawai *et al.*, 1988; Kahlon *et al.*, 1991; Tizard *et al.*, 1994).

Majority of the fungicides that are based on synthetic chemicals cause severe and long-term environmental pollution, are highly and acutely toxic, and may cause cancer in humans and wild animals (Manuel *et al.*, 2008). There is also risk of development of resistance by the targeted pathogens (Manuel *et al.*, 2008). Consequently, there is need for new strategies that aim at developing fungicides that combine sustainability, high efficacy, reduced toxicity, safety for humans, animals, host plants and ecosystems with low production cost. Since fungicides of biological origin have been demonstrated to be specifically effective on target organisms and are also biodegradable, biological control has become popular worldwide (Kiran *et al.*, 2006).

Scientific evidence has demonstrated the possibility of utilising plant extracts in the treatment of fungal infections, and development of anti-fungal products that control phyto-pathogens that cause huge economic losses (Farnsworth, 1994; Fox, 1999). Thus the second aim of this study was to conduct efficacy studies of *Aloe* species against *Fusarium oxysporum* and *Pythium ultimum*.

1.3 Objectives

1.3.1 Broad objective

The broad objective of this study was to genetically characterize *Aloe* species found in Kenya and to evaluate the efficacy of *Aloe turkanensis*, *A. secundiflora* and *A.lateritia* crude extracts on *Fusarium oxysporum* and *Pythium ultimum*.

1.3.2 Specific objectives

i. To evaluate the diversity of *Aloe* species in Kenya based on morphological characteristics.

ii. To evaluate the pattern of genetic diversity and population structure among *Aloe* species in Kenya using Sequence Related Amplified Polymorphism (SRAP) PCR – based marker system.

iii. To determine the efficacy of *Aloe turkanensis*, *A. secundiflora* and *A.lateritia* on *Fusarium oxysporum* and *Pythium ultimum*.

1.4 Statement of hypotheses

i. *Aloe* species found in Kenya are not morphologically different.

ii. There is no genetic variation between and within *Aloe* species found in Kenya.

iii. The crude extracts of the *Aloe turkanensis*, *A. secundiflora* and *A.lateritia* have no antimicrobial activity against *Fusarium oxysporum* and *Pythium ultimum*.

CHAPTER TWO: LITERATURE REVIEW

2.1 The genus *Aloe*

2.1.1 Taxonomy and botany of genus *Aloe*

The *Aloe* genus contains about 500 species of flowering succulent plants. These species are perennial herbs found in tropical and subtropical parts of the world. The most common and well known of these is *Aloe vera*, or "true *Aloe* ". The genus is native to Africa, and is common in South Africa's Cape Province, the mountains of tropical Africa, and neighboring areas such as Madagascar and the Arabian Peninsula (Stevens, 2001).

In the past it has also been assigned to families Aloaceae and Liliaceae or lily family and currently Asphodelaceae (Stevens, 2001). Members of the closely allied genera *Gasteria*, *Haworthia* and *Kniphofia*, which have a similar mode of growth, are also popularly known as *Aloe* species Most *Aloe* species have a rosette of large, thick, fleshy leaves. The leaves are often lance-shaped with a sharp apex and a spiny margin. *Aloe* flowers are tubular, frequently yellow, orange, pink or red, and are borne, densely clustered and pendant, at the apex of simple or branched, leafless stems. Many species of *Aloe* appear to be stem less, with the rosette growing directly at ground level; other varieties may have a branched or unbranched stem from which the fleshy leaves spring. They vary in color from grey to bright-green and are sometimes striped or mottled. Some *Aloe* species native to South Africa are arborescent (Stevens, 2001).

The *Aloe* leaf can be divided into two major parts, namely the outer green rind, including the vascular bundles, and the inner colorless parenchyma containing the *Aloe* gel. Description of the inner central part of the aloe leaf may sometimes be confusing, due to the different terms

that are used interchangeably such as inner pulp, mucilage tissue, mucilaginous gel, mucilaginous jelly, inner gel and leaf parenchyma tissue. Technically, the term 'pulp' or 'parenchyma tissue' refers to the intact fleshy inner part of the leaf including the cell walls and organelles, while 'gel' or 'mucilage' refers to the viscous clear liquid within the parenchyma cells (Ni *et al.*, 2004).

The three structural components of the *Aloe vera* pulp are the cell walls, the degenerated organelles and the viscous liquid contained within the cells. These three components of the inner leaf pulp have been shown to be distinctive from each other both in terms of morphology and sugar composition (Ni *et al.*, 2004). The raw pulp of *A. vera* contains approximately 98.5% water, while the mucilage or gel consists of about 99.5% water (Eshun *et al.*, 2004). The remaining 0.5 – 1% solid material consists of a range of compounds including water-soluble and fat-soluble vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids (Boudreau *et al.*, 2006). It has been hypothesized that this heterogenous composition of the *Aloe vera* pulp may contribute to the diverse pharmacological and therapeutic activities which have been observed for aloe gel products (Talmadge *et al.*, 2004).

2.1.2 Economic importance of *Aloe* species

At least, a quarter of *Aloe* genera is valued for traditional medicine (Grace *et al.*, 2009) while a small number is harvested from the wild or cultivated for natural products prepared from the bitter leaf exudates or gel-like leaf mesophyll. *Aloe* gel which is 99% water with a pH of 4.5 is a common ingredient in many non-prescription skin salves (Rajasekaran *et al.*, 2006). Other important pharmacological activities of *Aloe vera* are anti-diabetic, antiseptic,

anti-tumor, wound and burn healing effect (Rajasekaran *et al.*, 2006). The sticky latex liquid is derived from the yellowish-green pericyclic tubules that line the leaf (rind); which is the part that yields the laxative anthraquinones. The leaf lining (latex, resin or sap) contains anthraquinone glycosides (aloin, *Aloe* -emodin and barbaloin) and these are potent stimulant laxatives (Rajasekaran *et al.*, 2006). The different species have somewhat different concentrations of active ingredients (Yagi *et al.*, 1998).

Some species of *Aloe* have been grown in gardens and homes as flowers and live fences. In Kenya, many locals have traditionally used the sap and leaves for cure of various ailments including malaria, fungal and bacterial diseases. Other uses include, rehabilitation of bare lands, bee forage, browse for wild animals, livestock fodder in dry season and as live fence and ornamental plants (Mukonyi *et al.*, 2007).

2.1.3 Distribution of *Aloe* species in the world

About 350 species of *Aloe* are found in Africa majority of which are concentrated in eastern and southern Africa including Madagascar (Knapp, 2006; Klopper *et al.*, 2007; Crouch *et al.*, 2009). The centre of highest diversity lies south of the Kunene, Okavango and Limpopo Rivers in southern Africa (Smith *et al.*, 1991). With approximately 140 Aloe taxa, South Africa has the largest number of *Aloe* species compared to any other African country (Klopper and Smith, 2007; Crouch *et al.*, 2009; Klopper *et al.*, 2009). Other centers of diversity include parts of West Africa, the East-Afro Arc of tropical southern Africa, Saudi Arabia and Yemen (Smith *et al.*, 2000). *Aloe* species are also found on several Indian Oceanic islands such as the Mascarene Islands (Mauritius and Réunion), Comoros,

Seychelles, Pemba and Socotra (Grace *et al.*, 2009). The cosmopolitan *Aloe vera*, which is thought to have originated in Saudi Arabia, has been traded for leaf exudate since the fourth century BC, resulting in the species' movement along trade routes from the Arabian Peninsula to the Mediterranean, Indian subcontinent, the Americas and the Caribbean, where it has become naturalized. Apart from *A.vera*, there do not appear to be many *Aloe* species that have become alien invaders outside their natural distribution (Grace, 2011).

Aloe species are generally associated with dry habitats. However, they are also well-represented in subtropical summer rainfall and winter rainfall regions (Van Jaarsveld, 1989). The majority of species occur in arid savanna, which is widespread over subtropical southern and eastern Africa (Van Jaarsveld, 1989). *Aloe* species occupies a wide array of habitats, from closed-canopy forests to desert shrublands, and occur from sea level to altitudes of 2700M above sea level (Sachedina and Bodeker, 1999). Individual species, however, tend to be geographically restricted (Holland, 1978; Sachedina and Bodeker, 1999). The three primary factors influencing distribution of *Aloe* species are fire tolerance, moisture (rainfall and soil moisture), and temperature (frost tolerance) (Jordan, 1996). It has also been suggested that their distribution is affected by the presence of specific pollinators and by seed morphology (Jordan, 1996). Some *Aloe* seeds such as those of *Aloe excelsa* have large, efficient wings that aid dispersal, and may account for their widespread distribution (Kamstra, 1971; Jordan, 1996). Others such as *Aloe aculeata* produce wingless seeds, presumably limiting their dispersal, thus resulting in dense stands of plants in localised areas (Kamstra, 1971; Jordan, 1996).

2.1.4 Distribution of *Aloe* species in Kenya

About 83 species of *Aloe* occur in East Africa with about 60 species having been identified in Kenya, many of which grow naturally in dry lands (Carter, 1994; Wabuyele, 2006). *Aloe* species are used by local communities for medicinal, cultural and aesthetic purposes. However only five (*Aloe turkanensis, A. scabrifolia, A. secundiflora, A. calidophia* and *A. rivae*) are exploited commercially. Many of the commercial *Aloe* species grow naturally in Baringo, Samburu, West Pokot, Turkana and Laikipia regions in abundance. Other areas known to have significant populations of *Aloe* include Nakuru, Koibatek, Kajiado, Isiolo, Meru, Marsabit, Moyale, Narok and Wajir. Some parts of Nyeri, Kiambu, Thika, Machakos, Kitui and Mwingi and western and coast regions of the country are also known to have some naturally growing commercial Aloe species (Figure 1) (Mukonyi *et al.*, 2007).

2.1.5 Phytochemistry of *Aloe* species

There are more than 200 compounds found in *Aloe* species with about 75 of them having biological activity (Shelton, 1991). The prominent components are anthraquinones, aloin (Shelton, 1991), *Aloe* emodine polysaccharides (Atherton, 1997), enzymes (Atherton, 1998), reducing sugars (Ro *et al.*, 2000), organic acids and metallic cations (Ro *et al.*, 2000). The *Aloe* gel or fillet, which is stored in the inner portion of the leaf, contains 99.5% water and 0.5% solid matter (Hutter *et al.*, 1996).

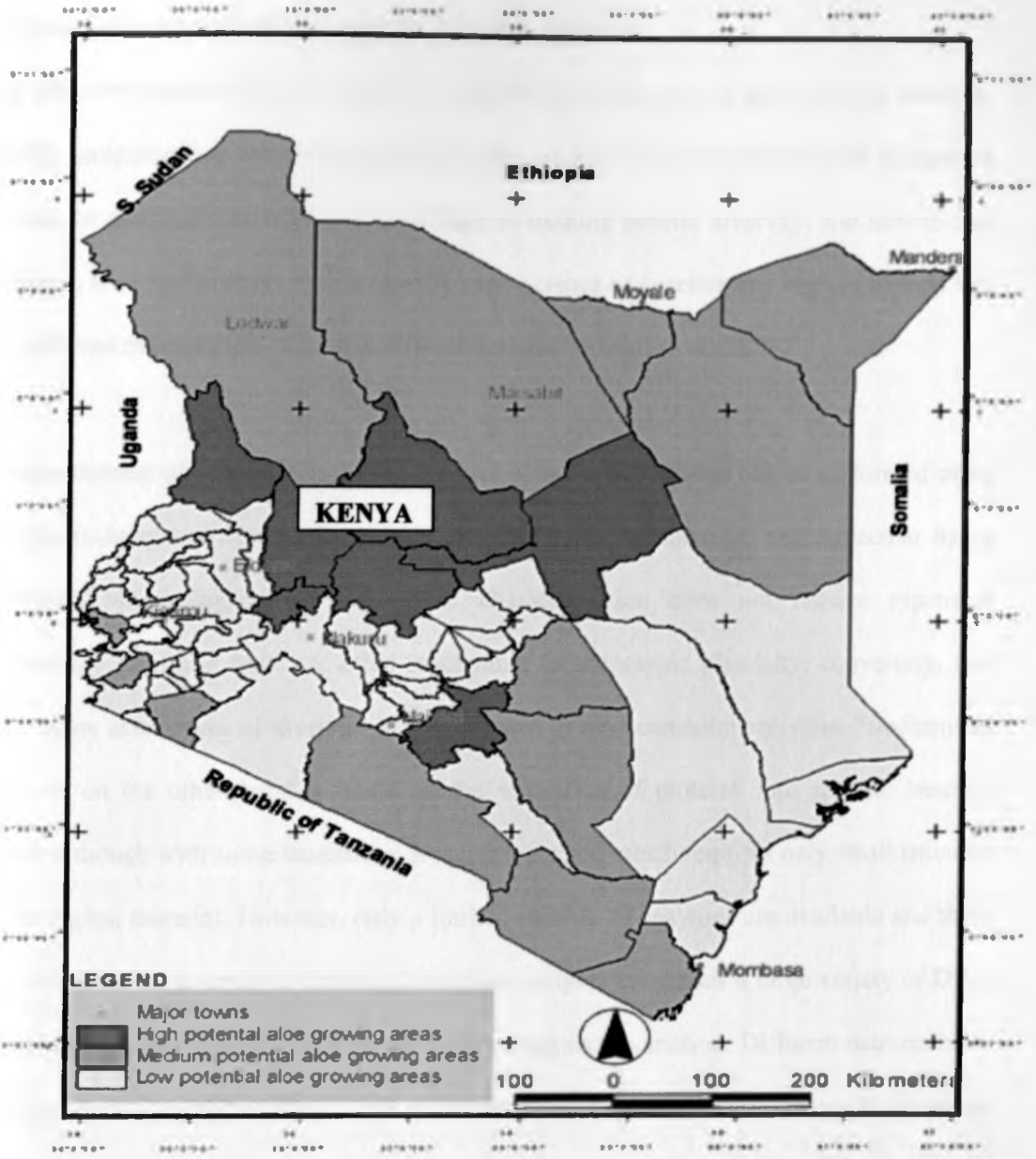


Figure 1: Distribution of *Aloe* species in Kenya.

Source: Mukonyi *et al.* (2007).

2.2 Role and methods of assessing plant genetic diversity

For effective conservation, management, and efficient utilization of plant genetic resources (PGR), understanding the molecular basis of the essential biological phenomena in plants is crucial. In particular, an adequate knowledge of existing genetic diversity, and how to best utilize it, is of fundamental interest for the basic science of genetics and applied aspects like the efficient management of crop genetic resources (Mondini *et al.*, 2009).

The assessment of genetic diversity within and between populations can be performed using various techniques including morphological, agronomic, biochemical and molecular based methods. Morphological and agronomic characterization does not require expensive technology but these traits are often susceptible to phenotypic plasticity; conversely, this may allow assessment of diversity in the presence of environmental variation. Biochemical analysis on the other hand is based on the separation of proteins into specific banding patterns though with some limitations. It is a fast method which requires only small amounts of biological material. However, only a limited number of enzymes are available and thus, the resolution of diversity is limited. Molecular analyses comprises a large variety of DNA molecular markers, which can be employed for analysis of variation. Different markers have different genetic qualities; they can be dominant or co-dominant, can amplify anonymous or characterized loci and can contain expressed or non-expressed sequences (Mondini *et al.*, 2009).

2.2.1 Morphological and agronomic characterization of crops.

Morphological and agronomic descriptors are reliable, easy to study and relatively inexpensive to evaluate. However, the use of morphological descriptors present some

limitations, such as: limited polymorphism, lowering the potential success of an extended classification approach, which would require a high number of descriptors in order to compensate for the small number of morphotypes; potential environmental influence on the phenotype, making even more complex the process of evaluation and information exchange. Care should be taken with false positives when the environment affects specific morphotypes or impact of a morphological descriptor in the viability of the individual (Ferreira, 2005).

2.2.2 Use of biochemical markers in the study of crop genetic diversity

The use of biochemical markers involves the analysis of seed storage proteins and isozymes (enzymes that differ in amino acid sequence but catalyze the same chemical reaction). (Mondini *et al.*, 2009). This technique utilizes enzymatic functions and is a comparatively inexpensive yet a powerful method of measuring allele frequencies for specific genes (Mondini *et al.*, 2009). Allozymes (variant forms of an enzyme that are coded by different alleles at the same locus) provide an estimate of gene and genotypic frequencies within and between populations. This information can be used to measure population subdivision, genetic diversity, gene flow, genetic structure of species, and comparisons among species out-crossing rates, population structure and population divergence, such as in the case of crop wild relatives (Spooner *et al.*, 2005).

Major advantages of these types of markers are: assessing co-dominance, absence of epistatic and pleiotrophic effects, ease of use, and low costs. Disadvantages of isozymes include: the few isozyme systems available per species (no more than 30) with

correspondingly few markers; the limited number of polymorphic enzymatic systems available, a small and non-random representation of the genome (the expressed part) - therefore, the observed variability may not be representative of the entire genome; although these markers allow large numbers of samples to be analyzed. Comparisons of samples from different species, loci, and laboratories are problematic, since they are affected by extraction methodology, plant tissue, and plant stage (Spooner *et al.*, 2005).

2.2.3 Use of molecular markers in the study of crop genetic diversity

In the last decade, the use of DNA markers for the study of crop genetic diversity has become routine and has revolutionized the studies of biological systems. Molecular markers have proven useful for assessment of genetic variation in germplasm collections (Mohammadi and Prasanna, 2003). Increasingly, techniques are being developed to assess genetic variation more accurately, quickly and cheaply (Spooner *et al.*, 2005). Due to the rapid developments in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Schlotterer, 2004). Genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost (Aggarwal *et al.*, 2008). No marker is superior to all others for a wide range of applications. The selection of the most appropriate marker technology will depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints and financial limitations (Spooner *et al.*, 2005).

An ideal molecular marker technique should: be polymorphic and evenly distributed throughout the genome; provide adequate resolution of genetic differences; generate multiple, independent and reliable markers; simple, quick and inexpensive; need small amounts of tissue and DNA samples; have linkage to distinct phenotypes and require no prior information about the genome of an organism. The major molecular markers that have been used in diversity studies include RFLPs, RAPDs, AFLPs, S-SAPs, SSR, SNPs and SRAP.

2.2.3.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a molecular marker analysis method based on restriction-hybridization techniques. DNA polymorphism is detected by hybridizing a chemically-labeled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile. The RFLP markers are relatively highly polymorphic, codominantly inherited and highly replicable allowing the simultaneous screening of numerous samples (Mondini *et al.*, 2009). DNA blots can be analyzed repeatedly by stripping and reprobing (usually eight to ten times) with different RFLP probes.

Nevertheless, this technique is not widely used as it is time-consuming, involves expensive and radioactive/toxic reagents and requires large quantities of high quality genomic DNA. Moreover, the pre-requisite of prior sequence information for probe construction contributes to the complexity of the methodology (Mondini *et al.*, 2009). RFLP can be applied in diversity and phylogenetic studies ranging from individuals within populations or species, to

closely related species. Restriction Fragment Length Polymorphisms have also been used in gene mapping studies (Neale and Williams, 1991) to investigate relationships of closely related taxa (Miller and Tanksley, 1990) as fingerprinting tools (Fang *et al.*, 1997), for diversity studies (Dubreuil *et al.*, 1996), and for studies of hybridization and introgression, including studies of gene flow between crops and weeds (Brubaker and Wendel 1994; Clausen and Spooner, 1998; Desplanque *et al.*, 1999).

2.2.3.2 Random amplified polymorphic DNA (RAPD)

The basis of Random Amplified Polymorphic DNA (RAPD) technique is differential PCR amplification of genomic DNA. It deduces DNA polymorphisms produced by "rearrangements or deletions at or between oligonucleotide primer binding sites in the genome" using short random oligonucleotide sequences (mostly ten bases long) (Williams *et al.*, 1991). As the approach requires no prior knowledge of the genome that is being analyzed, it can be employed across species using universal primers. The major drawback of the method is that the profiling is dependent on the reaction conditions so may vary within two different laboratories and as several discrete loci in the genome are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001). Due to the speed and efficiency of RAPD analysis, high-density genetic mapping in many plant species such as alfalfa (Kiss *et al.*, 1993), faba bean (Torress *et al.*, 1993) and apple (Hemmat *et al.*, 1994) were developed in a relatively short time. The RAPD analysis of near isogenic lines (NILs) has been successful in identifying markers linked to disease resistance genes in tomato (*Solanum lycopersicum*) (Martin *et al.*, 1991), lettuce

(*Lactuca sativa*) (Paran *et al.*, 1991) and common bean (*Phaseolus vulgaris*) (Adam-Blondon *et al.*, 1994).

2.2.3.3 Amplified fragment length polymorphism (AFLP)

To overcome the limitation of reproducibility associated with RAPD, AFLP technology was developed (Vos *et al.*, 1995). It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers. The primer pairs used for AFLP usually produce 50–100 bands per assay. The amplified fragments are detected on denaturing polyacrylamide gels using an automated ALF DNA sequencer with the fragment option (Huang and Sun, 1999). Number of amplicons per AFLP assay is a function of the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size and complexity. The AFLP technique generates fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping. The technique can be used to distinguish closely related individuals at the sub-species level (Althoff *et al.*, 2007) and can also map genes. Applications for AFLP in plant mapping include establishing linkage groups in crosses, saturating regions with markers for gene landing efforts (Yin *et al.*, 1999) and assessing the degree of relatedness or variability among cultivars (Mian *et al.*, 2002). For high-throughput screening approach, fluorescence tagged primers are also used for AFLP analysis.

Sequence specific PCR based markers with the advent of high-throughput sequencing technology and abundant information on DNA sequences for the genomes of many plant species has been generated (Goff *et al.* 2002 and Arabidopsis (Yu *et al.*, 2002). Expressed Sequence Tags of many crop species have been generated and thousands of sequences have been annotated as putative functional genes using powerful bioinformatics tools.

2.2.3.4 Sequence-Specific Amplification Polymorphism (S-SAP)

In order to correlate DNA sequence information with particular phenotypes, sequence-specific molecular marker techniques have been designed. Sequence-Specific Amplification Polymorphism was first used to investigate the location of BARE-1 retrotransposons in the barley genome (Waugh *et al.*, 1997). In principle, it is a simple modification of the standard AFLP (Amplified Fragment Length Polymorphism) protocol (Vos *et al.*, 1995). The final amplification is performed with retrotransposon-specific and MseI-adaptor-specific primers. S-SAP has been extensively used to generate markers to study genetic diversity and to prepare linkage maps in several plants, including the pea, medicago, wheat, and the cashew (Pearce *et al.*, 2000; Porceddu *et al.*, 2002; Queen *et al.*, 2004; Syed *et al.*, 2005). All S-SAP systems described to date utilize Long Terminal Repeats (LTRs) of Ty1-copia or Ty3-gypsy retrotransposons. They were all proved to be as efficient as or even more efficient than the original AFLP technique. S-SAP can be used for mapping, fingerprinting, marker-assisted selection and evolutionary studies (Leigh *et al.*, 2003).

2.2.3.5 Microsatellite-based marker technique (Simple Sequence Repeats -SSRs)

Microsatellite or short tandem repeats or simple sequences repeats are monotonous repetitions of very short (one to five) nucleotide motifs, which occur as interspersed repetitive elements in all eukaryotic genomes (Tautz and Renz, 1984). Variation in the number of tandemly repeated units is mainly due to strand slippage during DNA replication where the repeats allow matching via excision or addition of repeats (Schlotterer and Tautz, 1992). As slippage in replication is more likely than point mutations, microsatellite loci tend to be hypervariable. Microsatellite assays show extensive inter-individual length polymorphisms during PCR analysis of unique loci using discriminatory primers sets (Powell *et al.*, 1996).

The PCR amplification protocols used for microsatellites employ loci-specific either unlabelled primer pairs or primer pairs with one radiolabelled or fluorolabelled primer. Analysis of unlabelled PCR products is carried out using polyacrylamide or agarose gels. The employment of fluorescent labelled microsatellite primers and laser detection (e.g., automated sequencer) in genotyping procedures has significantly improved the throughput and automatisation (Wenz *et al.*, 1998). However, due to the high price of the fluorescent label, which must be carried by one of the primers in the primer pair, the assay becomes costly. Schuelke (2000) introduced a novel procedure in which three primers are used for the amplification of a defined microsatellite locus: a sequence-specific forward primer with M13 (–21) tail at its 5' end, a sequence-specific reverse primer and the universal fluorescent-labelled M13 (–21) primer which proved simple and less expensive.

Microsatellites are highly popular genetic markers because of their codominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing SSR size variation by PCR with pairs of flanking primers. The reproducibility of microsatellites is such that they can be used efficiently by different research laboratories to produce consistent data (Saghai *et al.*, 1994). Locus-specific microsatellite-based markers have been reported from many plant species such as lettuce (*Lactuca sativa* L.) (van de Wiel *et al.* 1999), barley (*Hordeum vulgare* L.) (Maroof *et al.* 1994) and rice (*Oryza sativa* L.) (Wu and Tanksley 1993).

2.2.3.6 Single Nucleotide Polymorphism (SNP)

Single nucleotide variations in genome sequences of individuals of a population are known as SNPs. They result from changes in a single nucleotide position (point mutations) and are the most abundant molecular markers in the genome (Mondini *et al.*, 2009). They are widely dispersed throughout genomes with a variable distribution among species. The SNPs are usually more prevalent in the non-coding regions of the genome. SNPs and flanking sequences can be found by library construction and sequencing or through the screening of readily available sequence databases (Spooner *et al.*, 2005). Genotyping methods, including DNA chips, allele-specific PCR and primer extension approaches based on SNPs, are particularly attractive for their high data throughput and for their suitability for automation. They are used for a wide range of purposes, including rapid identification of crop cultivars and construction of ultra-high-density genetic maps (Mondini *et al.*, 2009).

2.2.3.7 Sequence Related Amplified Polymorphism (SRAP)

The aim of SRAP technique (Li and Quiros, 2001) is the amplification of open reading frames (ORFs). It is based on two-primer amplification. The technique uses primers of arbitrary sequence, which are 17–21 nucleotides in length. It uses pairs of primers with AT- or GC- rich cores to amplify intragenic fragments for polymorphism detection. The primers consist of the following elements: Core sequences, which are 13–14 bases long, where the first 10 or 11 bases starting at the 5'-end, are sequences of no specific constitution (''filler'' sequences), followed by the sequence CCGG in the forward primer and AATT in the reverse primer. The core is followed by three selective nucleotides at the 3'-end. The filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long. For the first five cycles the annealing temperature is set at 35°C. The following 35 cycles are run at 50°C.The amplified DNA fragments are fractionated by denaturing acrylamide gels and detected by autoradiography. Sequence-related amplified polymorphism (SRAP) combines simplicity, reliability, moderate throughput ratio and facile sequencing of selected bands (Li and Quiros, 2001). Sequencing demonstrated that SRAP polymorphism results from two events, fragment size changes due to insertions and deletions, which could lead to co-dominant markers, and nucleotide changes leading to dominant markers. Many comparative studies have found SRAP markers provide comparable levels of variation to AFLP markers, but with significantly less technical effort and cost for similar levels of band-pattern variability and reproducibility (Li and Quiros, 2001; Liu *et al.*, 2007; Wang *et al.*, 2007; Lou *et al.*, 2010). Further, codominance has been identified in up to 20% of SRAP markers examined (Li and Quiros, 2001), which is a higher rate than previously described for AFLP (Mueller and Wolfenbarger, 1999).

The SRAP marker system has been adapted for a variety of purposes in different crops, including map construction, gene tagging and genetic diversity studies (Gulsen *et al.* 2006). Sequence Related Amplified Polymorphisms targets coding sequences in the genome and results in a moderate number of co-dominant markers. Like other dominant markers, SRAPs have demonstrated the ability to elucidate genetic variation at a variety of taxonomic levels (Uzun *et al.*, 2009), but are often used for analyses of populations of inter- and intraspecific hybrids (Liu *et al.*, 2008). Analysis of SRAP data has frequently been employed for the construction of linkage maps (Lin *et al.*, 2003, 2009; Yeboah *et al.*, 2007; Levi *et al.*, 2011) and identification of quantitative trait loci (Yuan *et al.*, 2008; Zhang *et al.*, 2009). Consequently, this system has been valuable for the improvement of agronomic crops (Zhang *et al.*, 2005; Zhao *et al.*, 2010; Wright and Kelly, 2011).

2.3 Plant pathogens and their control

The development of disease in cultivated crops has long been known to depend on the complex interrelationship between host, pathogen and prevailing environmental conditions (De *et al.*, 2001). In the case of soil borne pathogens, further opportunities exist for interactions with other microorganisms occupying the same ecological niche. The natural soil environment harbours a multitude of microorganisms (De *et al.*, 2001). As many as 10^6-10^8 bacterial cells,10^6-10^7actinomycete cells, 5 x 10^4-10^6 fungal colony forming units (CFU), 10^5-10^6 were estimated to be present in a gram of field soil taken from the surface (Gottlieb, 1976), while Richards (1976) found $1x10^7$ nematodes in an area of 1 m^2 of fertile soil. Although many of these organisms are saprophytic, having little, if any effect on

cultivated crops, the moist soil environment is favourable for the activities of plant-parasitic nematodes (PPN) and for the growth and multiplication of pathogenic fungi (De *et al.*, 2001).

The principles of plant disease management and control usually involve pathogen exclusion, pathogen eradication and reduction of inocula, plant protection, use of resistant varieties and integrated management (Jacobsen, 2001). There are many alternative strategies to reduce the use of synthetic fungicides, including the use of resistant plant varieties, health certified seed, phytosanitary measures, good cultural practices, water management, biofumigations, soil solarisation, biological control and the use of natural fungicides obtained from plants (Satish *et al.*, 2009). This last aspect would seem the most effective to reduce the use of synthetic fungicides (Satish *et al.*, 2009). The substitution of synthetic fungicides with ecofriendly products holds great potential because the use of pesticides will be increasingly restricted due to their undesirable side effects such as recalcitrance to degradation, accumulation in the food chain and interference with soil microbiology (Rongai *et al.*, 2012).

Natural plant products are important sources of new agrochemicals for the control of plant diseases (Maya *et al.*, 2013). Their use in controlling diseases is considered an interesting alternative to synthetic fungicides due to their less negative impacts on the environment, as they do not leave toxic residues and therefore can effectively replace synthetic fungicides (Cao *et al.*, 2002) .These natural products or plant extracts can be exploited either as leads for chemical synthesis of new agrochemicals, or as commercial products in their own right, or as a source of inspiration to biochemists for the development of new bioassays capable of

detecting other, structurally simpler compounds with the same mode of action. The use of plants may offer a new source of antimicrobial agents with significant activity (Munoz *et al.*, 2003). Plant preparations have been used for centuries in medicine and pest control. Farmers in India use neem leaves to protect their stored grain from insects. Herbs and spices, such as basil and clove, have been used by many cultures to protect food from spoilage, as both have antimicrobial properties (Manohar *et al.*, 2001).

In recent times, focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potential of plants used in various traditional systems. More than 13,000 plants have been studied during the last 5 year period (Bensky *et al.*, 2004). Dahanukar has reviewed the research on plant based antifungal compounds as a scientific approach and innovative scientific tool from 1994-1998 (Dahanukar, 2000). Antifungal activity of plant extracts against a wide range of fungi has also been reported by many workers (Abd-Alla *et al.*, 2001, Yanar *et al.*, 2011, Talibi *et al.*, 2012).

2.3.1 *Fusarium oxysporum*

Fusarium oxysporum affects a wide variety of hosts during different growth stages. Tomato (*Fusarium oxysporum* f.sp radicis-lycopersici), tobacco (*Fusarium oxysporum* **f. sp.** *nicotianae*), sweet potatoes (*Fusarium oxysporum* f. sp. *batatas*) and banana (*Fusarium oxysporum* f.sp. *cubense*) are a few of the most susceptible plants. However it will also infect other herbaceous plants. *F. oxysporum* generally produces symptoms such as wilting, chlorosis, necrosis, premature leaf drop, browning of the vascular system, stunting, and damping-off (Agrios, 1988). The most important of these is vascular wilt. *F.oxysporum* is

the most widely dispersed of the *Fusarium* species and is found worldwide. The pathogen has no known sexual stage, but produces three types of asexual spores: microconidia, macroconidia, and chlamydospores with the microconidia being the most abundantly produced spores. They are oval, elliptical or kidney shaped and produced on aerial mycelia. Macroconidia, which have three to five cells and have gradually pointed or curved edges, are found on sporodochia on the surface of diseased plant (in culture the sporodochia may be sparse or nonexistent). Chlamydospores are usually formed singly or in pairs, but can sometimes be found in clusters or in short chains. They are round thick walled spores produced within or terminally on an older mycelium or in macroconidia. Chlamydospores unlike the other spores can survive in the soil for a long period of time (Agrios, 1988).

The coloration of *F. oxysporum* mycelium is initially white but later becomes purple, with discrete orange sporodochia (mass of hyphae) present in some strains. They can also be hyaline (glass-like, transparent), dark blue, or dark purple. Their conidiophores, the means through which *F. oxysporum* asexually reproduce, are short, single, lateral monophialides (flask-shaped projections) in the aerial mycelium, later arranged to densely branched clusters. Their macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm. Microconidia are abundant, never in chains, mostly non-septate, ellipsoidal or cylindrical, straight or curved, 5-12 x 2.3-3.5 µm. Chlamydospores are terminal or intercalary, hyaline, smooth or rough-walled, 5-13 µm (Smith *et al.*, 1988).

Rosca-Casian *et al.*, (2007) reported that hydroalcoholic plant extract obtained from *A. vera* fresh leaves harvested from the greenhouses of "Alexandru Borza" Botanical Garden in Cluj-Napoca, Romania had antifungal activity against the mycelial growth of *B. gladiolorum* and *F. oxysporum* f.sp. *gladioli*.

2.3.2 *Pythium ultimum*

Pythium ultimum is an ubiquitous soilborne pathogen which causes damping-off and root rot on plants. Originally, the genus Pythium was placed in the Family Saprolegniaceae by Pringsheim in 1858 (Hendrix and Campbell, 1973). Currently, Pythium species are placed in the Family of Pythiaceae, Class of Oomycota. Isolation of P. ultimum was first reported in 1931 by Wager in the Union of South Africa (Wager, 1931).

Pythium ultimum is widely distributed throughout the world and have a wide range of hosts including many important crops. The genus is distributed in Africa, Australia, Brazil, Canada, China, Japan, Korea and many other countries in the world. Studies have shown that the genus is common in most cultivated soils and forests, for example, *P. ultimum* the causal agent of the *Pythium* blight of turfgrass, which causes serious damage to golf courses. (Allen *et al.* 2004). *P. ultimum* can also infect other crops such as tomato, cabbage, carrot, cucumber, melon, turfgrass and wheat (Allen *et al.*, 2004). In general, abundant soil moisture and high soil temperature are the two most important environmental factors that affect the distribution of the genus (Allen *et al.*, 2004).

Pythium species can grow saprophytically and survive as resistant resting structures in the soil and in root residues. When conditions are favorable, the fungi begin to infect the seeds

and/or root tips of plants. Vegetative hyphae can directly penetrate plant cells. Mycelial growth and the movement of zoospores can facilitate the spread to other susceptible plants. The genus can reproduce both sexually and asexually. For asexual reproduction, sack-like sporangia will be formed. Sporangia can directly germinate as hyphae. For sexual reproduction, an oogonium and a club-shaped antheridium will be produced. When they contact with each other, the nuclei of this two structures will form a zygote, then a thick-wall oospore will be formed. Both sporangia and zoospores are short-lived in soils, while oospores can be survived in the soil for longer periods (Allen *et al.* 2004).

Haouala *et al.*, (2008) and Suleiman and Emua (2009) reported that an aqueous extract of *Aloe* could inhibit mycelial growth of *P. aphanidermatum*.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Source of the *Aloe* accessions

A total of 31 *Aloe* accessions that were morphologically diverse were collected. Of these 13 *Aloe* accessions were collected as seed from the Kenya Agricultural and Livestock Research Organization (KALRO) Gene bank of Kenya (Table 1). They were planted at the KALRO Biotechnology centre for a period of 3 months. An additional four whole plant *Aloe* accessions previously collected from Taita Taveta County were obtained from the Medicinal and Aromatic Plants (MAPs) project at the KALRO Biotechnology center. The rest of the whole plant samples were collected from four areas in the Rift Valley namely Baringo, Naivasha, Nakuru and Narok. All the accessions were established in the screen house at KALRO Biotechnology centre and were watered on a daily basis (Figure 2). Table 1 shows details of each collection the accessions where identified and source.

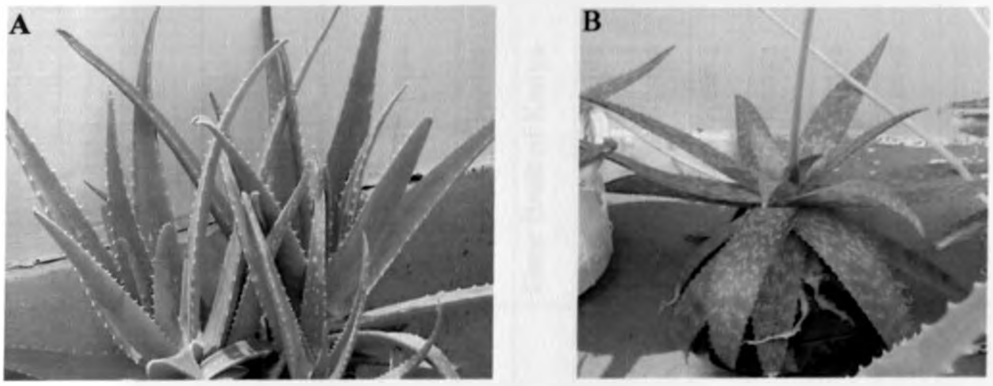


Figure 2: *Aloe* species established in the screen house. *Aloe barbidensis* (A), *Aloe lateritia* (B)

Table 1: Sources of *Aloe* accessions used in the study

Entry Number	Entry code/Name	Source	Entry Number	Entry code/Name	Source
1	GBK 001	Gene Bank of Kenya	17	GBK 012	Gene Bank of Kenya
2	NVS001 – *Aloe lateritia*	Naivasha	18	GBK 013	Gene Bank of Kenya
3	NVS002 – *Aloe lateritia lateritia*	Naivasha	19	NKR 001 – *Aloe barbidensis*	Nakuru
4	NRK 001	Narok	20	BRG 001 – *Aloe chinensis*	Baringo
5	NRK 002	Narok	21	BRG 002 – *Aloe secundiflora*	Baringo
6	NRK 003	Narok	22	NKR 002	Nakuru
7	GBK 002	Gene Bank of Kenya	23	BRG 003 – *Aloe confuse*	Baringo
8	GBK 003	Gene Bank of Kenya	24	NKR 003 – *Aloe lateritia*	Nakuru
9	GBK 004	Gene Bank of Kenya	25	BRG 004 – *Aloe turkanensis*	Baringo
10	GBK 005	Gene Bank of Kenya	26	KBC 001	Taita
11	GBK 006	Gene Bank of Kenya	27	KBC 002	Taita
12	GBK 007	Gene Bank of Kenya	28	KBC 003	Taita
13	GBK 008	Gene Bank of Kenya	29	KBC 004	Taita
14	GBK 009	Gene Bank of Kenya	30	NKR 004	Nakuru
15	GBK 010	Gene Bank of Kenya	31	NKR 005	Nakuru
16	GBK 011	Gene Bank of Kenya			

BRG – Baringo, GBK – Gene Bank of Kenya, KBC – KALRO Biotechnology Centre, NVS – Naivasha, NKR – Nakuru, NRK – Narok

3.2 Source of test pathogens

Medicicnal and aromatic plants project sought to provide biological remedies for the control of *Fusarium oxysporum* and *Pythium ultimum* using locally available materials.

Fusarium oxysporum and Pythium *ultimum* were retrieved from the culture collection center, Plant Pathology laboratory, University of Nairobi. The cultures had been isolated from soil samples collected from the University of Nairobi (Upper Kabete) field station and maintained at -20°C. The identity of the test pathogens was confirmed through cultural and morphological characteristics of cultures grown on potato dextrose agar.

3.2.1 Preparation of culture media and retrieval of test pathogens

Potato dextrose agar medium (39 grams) was suspended in one liter of sterilized distilled water. The agar was sterilized by autoclaving at a temperature of 121 °C and pressure of 15 psi for 15 minutes and about 15 ml of the agar poured on sterile plates. The pathogens were then retrieved from -20°C and inoculated on fresh PDA and incubated at 25 °C.

3.3 Morphological characterization of the *Aloe* accessions

Morphological studies of the 31 accessions were carried out using the following descriptors; Color, leaf variegation and spine color: There were three types of color variants dark green, green and light green. Leaf variegation was scored for presence or absence while spine color was either green or red.

3.4 Molecular characterization of the *Aloe* accessions

3.4.1 Extraction of *Aloe* DNA

Genomic DNA was extracted using a modified CTAB method (Doyle and Doyle, 1987). Small sized young leaves (200mg) of *Aloe* species were harvested from the greenhouse and washed with sterile distilled water followed by washing with 70% ethanol. A slurry was made with 200 milligrams of leaf sample ground in liquid nitrogen to fine powder and 1 ml of 2% CTAB buffer added. The mixture was transferred to a microcentrifuge tube, mixed well and incubated at 60 ℃ for 1 hour on a water bath with occasional mixing by inverting the tubes. After incubation, the mixture was cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) added and mixed gently for at least 30 min. The mixture was centrifuged at 10,000 rpm for 10 min at room temperature. The aqueous phase (supernatant) was transferred to a fresh and sterile microcentrifuge tube and the DNA precipitated by adding an equal volume of ice-cold isopropanol. The tube was stored overnight at -20 ℃. The precipitated DNA was collected out by centrifugation at 13,000 rpm for 5 minutes at room temperature and the upper layer discarded. The pellet was air dried and washed twice using 70% v/v ethanol and air dried. The dried pellet was dissolved in low salt Tris-EDTA (1M Tris (Hydroxymethyl) amino methane; 0.5M Diaminoethane tetraacetic acid).

3.4.2 Purification of genomic DNA

The DNA sample was treated with 10 µl RNase (10 mg/ml stock) and incubated in water bath for 30 min at 37 ℃. An equal volume of the mixture of phenol: chloroform: isoamyl

alcohol (25:24:1) was added and centrifuged at 8000 rpm for 10 min at room temperature. The aqueous phase was taken and an equal volume of the mixture of chloroform: isoamylalcohol (24:1) added. The aqueous phase was again collected and to it a 1/10 volume of 3M sodium acetate added, followed by 3 volumes of chilled absolute ethanol for precipitation of DNA. The precipitated DNA was spooled out, washed with 70% ethanol twice and dried at room temperature (25 °C) in a laminar air flow for 30 min. Finally, the DNA pellet was dissolved in 50 µl of low salt Tris EDTA (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) and stored at -20 °C for future use.

3.4.3 Quantification of *Aloe* DNA

A one percent (1% w/v) agarose gel was made by adding agarose powder to 0.5×TBE buffer (45 mM Tris-borate and 1 mM EDTA, pH 8.0) and heated to dissolve. Two micro liters of ethidium bromide was added in a fume chamber. This was poured in a gel tray and combs placed. After solidification of the gel, it was transferred to the electrophoresis tank having 0.5×TBE buffer. Two microlitres sample loading dye and 5 µl DNA were mixed well and loaded into the wells using a micropipette. The electrophoresis was run at 90V for 30 minutes. Under UV transilluminator the quality of DNA was checked by presence of a single compact band. The DNA was quantified by using a molecular ladder. The remaining DNA sample was then diluted with an appropriate amount of sterile distilled water to yield a working concentration of 100 µg/ml of DNA and stored at 4 °C awaiting SRAP PCR analysis.

3.4.4 Sequence Related Amplified Polymorphism (SRAP) Analysis

Sequence Related Amplified Polymorphism (SRAP) analysis was conducted according to previously established protocols (Li and Quiros, 2001). A total of 6 forward primers and 8 reverse primers (Bioneer, Inc Almeda United States) identified by Li and Quiros, 2001 were used (Appendix A). The SRAP-PCR reaction mixture was 20µL in volume, which consisted of 1× bulk PCR Premix (1.5mM $MgCl_2$, 250 µM, dNTPs, 1U Taq polymerase, 10mM Tris-HCl, 30mM KCl, stabilizer and tracking dye), sterile distilled water, 0.5-µM of each primer (forward and reverse) and 30-ng DNA template. The amplifications were carried out in a Techne (TC- 412) thermo-cycler (Keison Products, Essex, England) under the following block temperature cycle:- after an initial heat denaturation at 94°C for 5 min, the first five cycles were run at 94°C for 1 min, 35°C for 1 min and 72°C for 1 min for denaturing, annealing and extension, respectively. Then the annealing temperature was raised to 50°C for another 35 cycles.

In this assay, 48 different primer combinations were employed using 6 forward and 8 reverse primers (Appendix B). Primer combinations that showed polymorphism were used for genotyping the *Aloe* species. The PCR products were separated by electrophoresis of 1.7% agarose gels in 0.5× TBE buffer for about 60 min with running voltage of 100 V. The gels were then visualized using Enduro GDS UV Gel imaging system (BioExpress, Utah, United States) and photographs of the images captured.

3.5 Assessment of the efficacy of *Aloe* extracts on *Fusarium oxysporum* and *Pythium ultimum*

3.5.1 Preparation of crude *Aloe* extracts

Leaves (100 grams) of *Aloe* species were freeze-dried for forty eight hours after which they were ground using a laboratory mortar and pestle. The powder was put in a mixture of 100% ethanol and dichloromethane (DCM) in the ratio of 1:1 for 24 hours according to standard extraction methods (Harborne, 1998). The procedure was repeated for another 24 hours and the decants combined and put in a conical flask. The crude extracts were then filtered using Whatman filter paper No.1 and Buchner funnel to get a uniform extract. Dry organic crude extracts were obtained after evaporating dichloromethane and ethanol using a rota-evaporator at 80 °C. The aqueous extract filtrates were put in sterile bottles and left to dry after which they were tightly capped and stored at 4 °C.

3.5.2 Dilution of *Aloe* crude extracts and preparation of paper discs

A 2 ml stock solution at a concentration of 1000 mg/ ml was prepared from the crude extracts by adding 2 grams of the extract to 2 ml of sterilized distilled water. Two fold serial dilutions were prepared from each stock solution by first preparing 100 mg/100µl concentration f rom the stock solution and subsequently halving the concentration until 6.25mg/100µl was achieved.

Paper discs were prepared from Whatman filter paper No.1 using a paper punch, put into a 100ml media bottle and autoclaved at a temperature of 121 °C and pressure of 15 psi for 15 minutes. From each concentration prepared, 100µl was pipetted onto individually placed

sterile paper discs (0.6cm) on flat-bottomed glass plates drop by drop using a micropipette in a laminar flow cabinet under aseptic conditions. The paper discs were allowed to dry before the release of the next drop, and kept at (4 °C) waiting for antimicrobial assays. The potency for each paper disc per extract for the concentrations prepared were; 100mg/100µl, 50mg/100µl, 25mg/100µl, 12.5mg/100µl, 6.25mg/100µl. The standard fungicide triazole was also prepared in the same manner. Two grams of triazole powder was dissolved in 2ml of sterile distilled water to make a stock solution of 1000mg/ml. Two fold serial dilutions were prepared from the stock solution. This was done by first making 100 mg/100µl from the stock solution and subsequently halving the concentration upto 6.25mg/100µl.

3.5.4 Preparation of standard inoculum of test pathogens

Spore suspension of *Fusarium oxysporum* was prepared from young 7-day old cultures grown on potato dextrose agar. The colonies were covered with 5 ml of sterile distilled water. The suspensions were obtained by scraping of the culture surface with a sterile microscope slide. Using a haemocytometer, the spore suspension of *F. oxysporum* was adjusted to 10^5 spore s/ml. The suspension was then filtered using a Muslin cloth and collected in a sterile media bottle.

For P. ultimum 7-day old cultures were harvested by scraping sporangia from the surface of the plate and blending them for 30 sec in a Tissuemiser (Tekmar, Cincinnati, OH). Sporangia were counted with the aid of a hemacytometer and diluted in 100 ml of sterile distilled water to provide 300 sporangia per square centimeter.

3.5.5 Determination of antimicrobial activity of *Aloe* plant extracts

The disc diffusion technique recommended by the Clinical and Laboratory Standards Institute (CLSI) was used (CLSI, 2012). The suspensions were first shaked after which one milliliter of the spore and mycelia suspension was introduced into Petri dishes containing PDA and evenly spread. This was done in a laminar flow cabinet under aseptic conditions. Paper discs impregnated with crude extracts, were aseptically transferred onto plates inoculated with 1ml of standard inocula for each test organism. The plates were labeled, sealed with parafilm, and incubated at 25°C for both *Fusarium oxysporum* and *Pythium ultimum*. Triazole a standard fungicide (Grant *et al.*, 1990) was used at similar concentrations as the positive control, while discs with extraction solvent (ethanol and Dichloromethane) only were used as negative controls. The bioassay was carried out in four replicates under sterile conditions.

The antimicrobial activity of the *Aloe* extracts was determined by measuring in millimeters the diameter of inhibition zones (including diameter of paper discs) formed using a transparent ruler. The size of the inhibition zone was recorded on daily basis for a period of eleven days. Minimum inhibitory concentrations (MICs) were determined by recording the lowest concentration of the active extracts that inhibited growth of the micro-organisms (Ochei and Kolhatkar, 2000).

3.6 Data Analysis

Only bands that could be unambiguously scored across all the sampled populations of *Aloe* species were used in the analysis. SRAP-amplified fragments were scored manually for band presence (1) or absence (0). The resulting presence/absence data matrix was analyzed using GenAlex 6.5 (Peakall and Smouse, 2012), assuming Hardy-Weinberg equilibrium, to estimate the following three genetic diversity parameters: the percentage of polymorphic loci (PPB), Shannon's information index of diversity (*I*) and Nei's gene diversity (*H*). The analysis of molecular variance (AMOVA) was used to partition the total SRAP variation into within-population and between-population (Excoffier *et al.*, 1992). Dendrograms were constructed with a genetic identity matrix to display population relationships using the unweighted pair-group mean algorithm (UPGMA) of Darwin software, version 5 (Perrier *et al.*, 2006).

GenStat for Windows 14th Edition (VSN International, 2011) was used to analyze data on mean inhibition zones. Multivariate ANOVA was used to determine significant factors in formation of inhibition zones. Tukey's Honest Significant Difference test (THSDT) was used for mean comparison within 95% confidence level.

CHAPTER FOUR: RESULTS

4.1 Characterization of *Aloe* accessions in Kenya

4.1.1 Morphological characterization

Morphological groupings were based on leaf color, presence or absence of variegation and color of spines (Table 2). A total of 8 morphological groups were identified. All accessions from Gene Bank of Kenya had no variegation and were in two main groups. The other accessions were distributed in the remaining six groups (Table 2).

Table 2: Morphological characteristics of *Aloe* accessions used in the study

Entry code/Name	COLOR	VARIEGA-TION	SPINES	Entry code/Name	COLOR	VARIEGA-TION	SPINES
BRG 001 *Aloe chinensis*	Dark green	Absent	Red	GBK 005	Green	Absent	Red
BRG 002 *Aloe secundiflora*	Dark green	Absent	Red	GBK 006	Green	Absent	Red
NKR 002	Dark green	Absent	Red	GBK 009	Green	Absent	Red
BRG 004 *Aloe turkanensis*	Dark green	Present	Green	GBK 010	Green	Absent	Red
NRK 001	Dark green	Present	Green	GBK 012	Green	Absent	Red
NRK 003	Dark green	Present	Green	GBK 013	Green	Absent	Red
KBC 003	Dark green	Present	Red	KBC 002	Green	Present	Green
NRK 002	Dark green	Present	Red	NKR 001	Green	Present	Green
NVS 001	Dark green	Present	Red	KBC 001	Green	Present	Red
NVS 002	Dark green	Present	Red	NKR 003	Green	Present	Red
BRG 003 *Aloe confuse*	Green	Absent	Green	GBK 002	Light green	Absent	Red
GBK 007	Green	Absent	Green	GBK 003	Light green	Absent	Red
GBK 011	Green	Absent	Green	GBK 004	Light green	Absent	Red
KBC 004	Green	Absent	Green	GBK 008	Light green	Absent	Red
NKR 004	Green	Absent	Green	GBK 001	Light green	Present	Red
				NKR 005	Light green	Present	Red

BRG – Baringo, GBK – Gene Bank of Kenya, KBC – KALRO Biotechnology Centre, NVS – Naivasha, NKR – Nakuru, NRK – Narok

4.2 Molecular characterization of *Aloe* species

4.2.1 Purity and integrity of genomic DNA

The genomic DNA extracted from leaves had acceptable integrity with 1% agarose gel electrophoresis, as the DNA bands were distinct and showed no dispersed appearance (Figure 3).

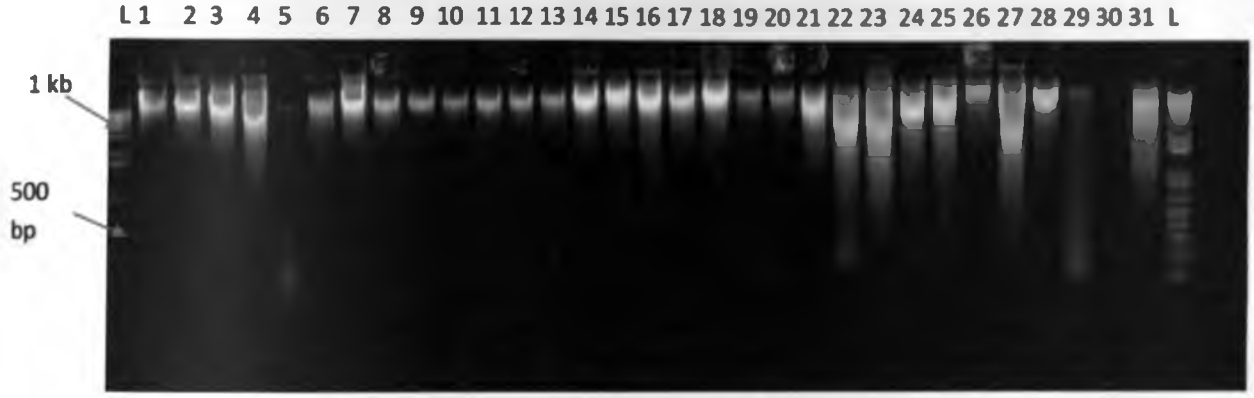


Figure 3: DNA profile – Agarose gel electrophoresis of *Aloe* DNA
Key: Lane 1- 31: *Aloe* accessions as shown in Table 1, L: Ladder

4.2.2 SRAP genotyping of *Aloe* genomic DNA

An initial screen of the 48 primer combinations yielded 10 polymorphic ones. A subsequent screening was done of the 10 polymorphic primer combinations and 7 demonstrated high levels of polymorphism with consistent banding patterns (Appendix B). Seven SRAP primer combinations that showed high levels of polymorphism and consistent banding patterns were used to genotype the 31 *Aloe* accessions (Figure 4). Seven (26%) of the 31 samples were done in duplicate to test for reproducibility for all the primer combinations (Figure 4).

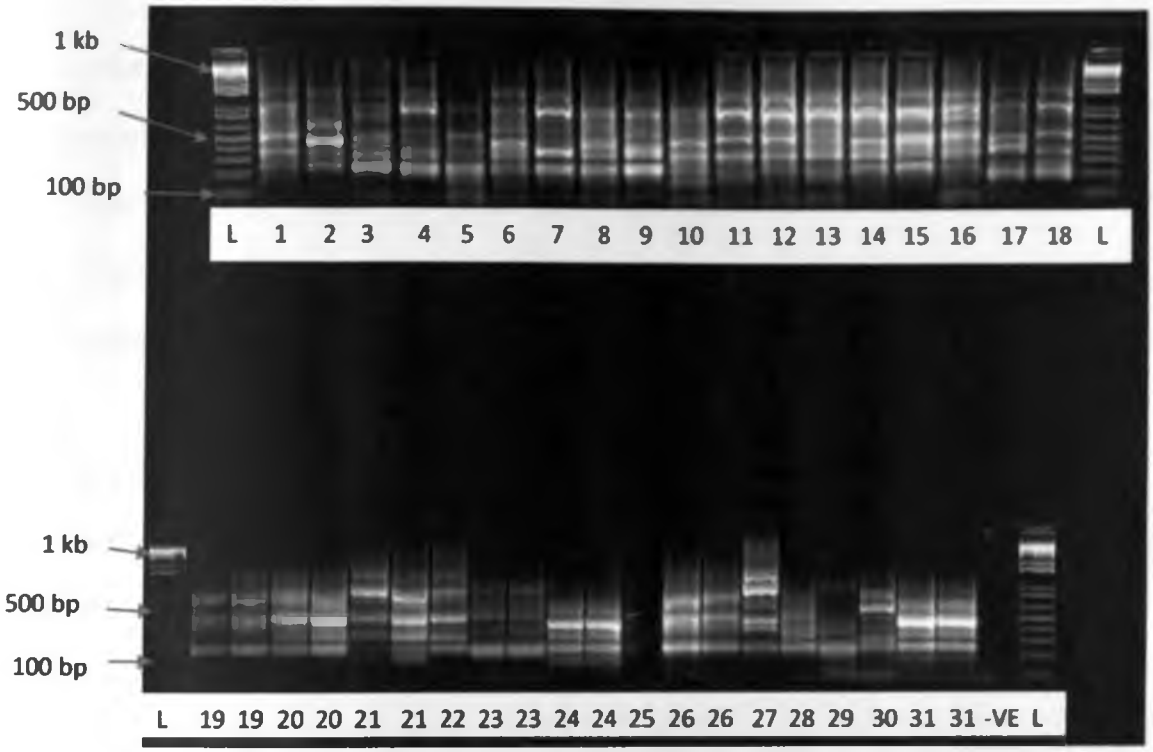


Figure 4: Agarose gel electrophoresis of SRAP primer combination ME1 and EM5 used in genotyping *Aloe* accessions

Key: Lane 1- 31: *Aloe* accessions as shown in Table 1, L: Ladder

4.2.2 Genetic relationships between *Aloe* species using SRAP markers

In total, 4525 amplified fragments were scored with an average of 282.8 fragments per primer pair across the accessions, thereby confirming the high multiplex ratio expected for the SRAPs.

Genetic diversity among *Aloe* accessions populations was estimated using different statistics: Percentage of polymorphic fragments within populations, ranged from 33.3% for 'Gene bank of Kenya' to 73.8% for the 'Narok' population, with an average of 56.8% (Table 3). Following the same order, the genetic diversity coefficients based on Nei's (1978)

genetic diversity (H) ranged from 0.063 to 0.362 for 'Baringo' and 'Gene Bank of Kenya' populations and Shannon's information index (I) ranged from 0.183 to 0.369 (Table 3). Banding patterns across the populations ranged from 0.122 to 0.236 for 'Narok' and 'Nakuru' populations according to expected heterozygosity (Table 3). One private band was unique to the 'Gene bank of Kenya' population (Figure 5).

Pair-wise comparison of genetic distance between the populations ranged from 0.063 for KALRO Biotech by Baringo to 0.362 for Naivasha by KALRO Biotechnology centre (Table 4).

Table 3: Genetic diversity indices of *Aloe* accessions using SRAP markers

Population	n	I	HE	% Polymorphic Loci
Baringo	8	0.318	0.214	57.1
Gene Bank of Kenya	13	0.316	0.201	73.8
KALRO Biotechnology Centre	5	0.265	0.169	57.1
Nakuru	8	0.369	0.236	81.0
Narok	3	0.183	0.122	33.3
Naivasha	2	0.230	0.158	38.1

(n) Sample size, (I) Shannon's information index, (He) Expected heterozygosity and % polymorphic loci

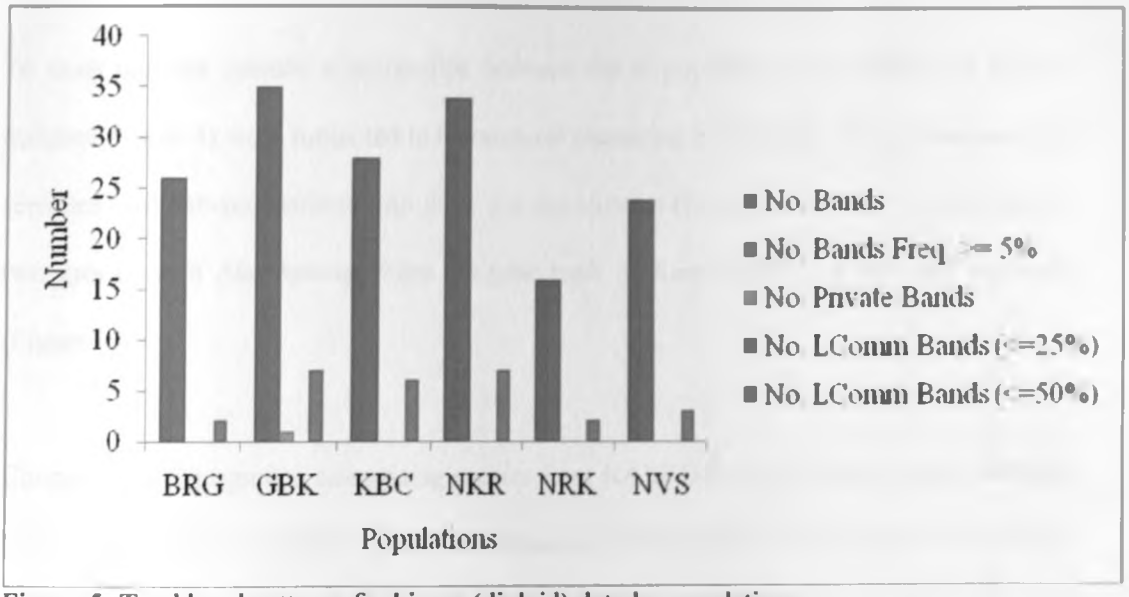


Figure 5: Total band patterns for binary (diploid) data by populations
BRG – Baringo, GBK – Gene Bank of Kenya, KBC – KALRO Biotechnology Centre, NKR – Nakuru, NRK – Narok, NVS – Naivasha.

Table 4: Nei's genetic distance of the six populations.

	BRG	GBK	KBC	NKR	NRK	NVS
BRG	0.000					
GBK	0.278	0.000				
KBC	0.063	0.326	0.000			
NKR	0.065	0.200	0.080	0.000		
NRK	0.160	0.107	0.170	0.094	0.000	
NVS	0.240	0.157	0.362	0.254	0.183	0.000

BRG – Baringo, GBK – Gene Bank of Kenya, KBC – KALRO Biotechnology Centre, , NKR – Nakuru, NRK – Narok, NVS – Naivasha

To construct the genetic relationships between the 6 populations, the values of genetic distance (Table 4) were subjected to hierarchical clustering by UPGMA. The cluster analysis separated the sub-populations into three distinct clusters (Figure 6). Cluster 1 comprised of two groups with *Aloe* species from the gene bank of Kenya (GBK), Narok and Naivasha (Figure 6).

Cluster 2 had four groups comprising species from KALRO Biotechnology Centre, Baringo, Nakuru, Narok and Naivasha. Cluster 3 comprised of *Aloe* species. from Nakuru and Narok. he three clusters reveal that there was distinct population clustering for the Gene bank of Kenya and Baringo populations but individuals of the other populations were wide spread and not restricted to specific clusters (Figure 6).

Samples that were done in duplicates to show for reproducibility also showed 100% similarity in the genetic diversity tree (Figure 6). This included *Aloe* accessions 19, 20, 21, 23, 24, 25, 26 and 31.

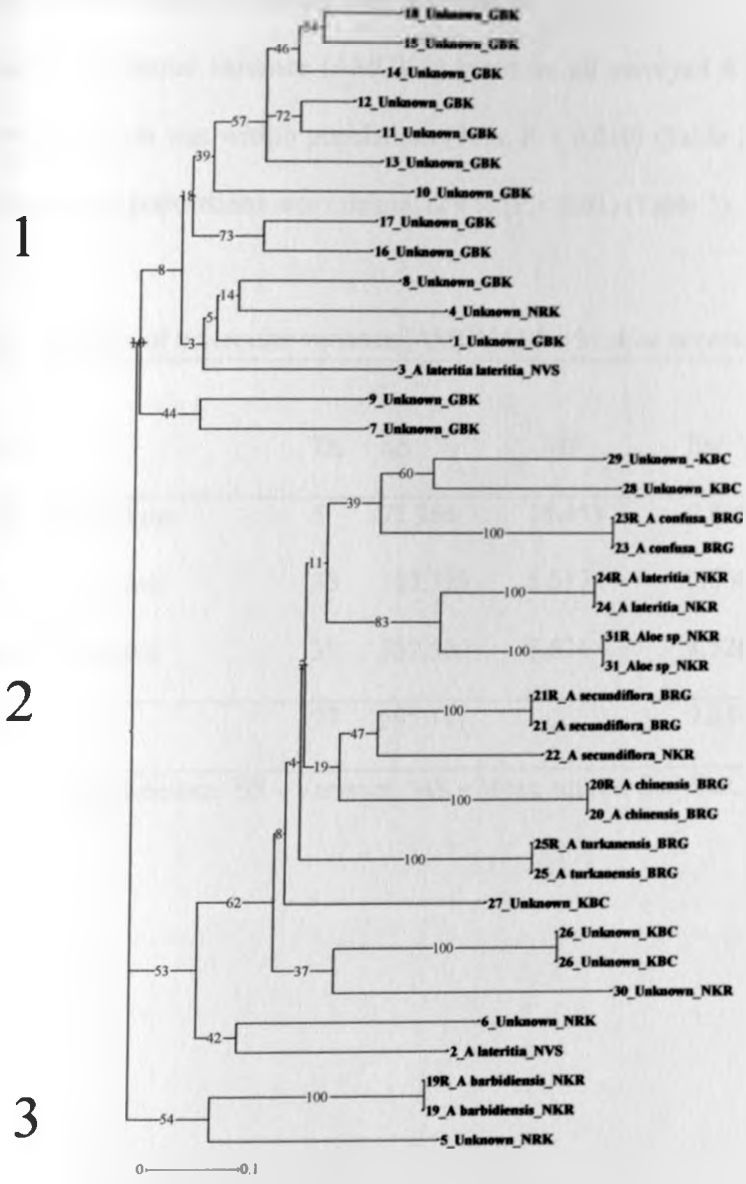


Figure 6: UPGMA dendograms of 31 accessions of *Aloe* in six populations

BRG – Baringo, GBK – Gene Bank of Kenya, KBC – KALRO Biotechnology Centre, NVS – Naivasha, NKR – Nakuru, NRK – Narok

4.2.3 Molecular variance among *Aloe* populations

Analysis of molecular variance (AMOVA) based on all surveyed 6 populations indicated that most variation was within populations (91%, $P < 0.010$) (Table 5). On the other hand, variation among populations was minimal at 9 % ($P < 0.01$) (Table 5).

Table 5: Analysis of molecular variance (AMOVA) for 31 *Aloe* accessions

Source	Df	SS	MS	Est. Var.	%
Among Populations	5	77.266	15.453	0.809	9%
Among Individuals	33	185.375	5.617	0.000	0%
Within Individuals	39	332.500	8.526	8.526	91%
Total	77	595.141		9.334	100%

Df – Degrees of freedom, SS - Variance, MS – Mean square, Est. Var. – Estimated Variance

4.3 Activity of crude extracts of *Aloe* species on *Fusarium oxysporum* and *Pythium ultimum*

4.3.1 Morphological and cultural characteristics of *Fusarium oxysporum* and *Pythium ultimum*

Plate cultures of *F. oxysporum* had purple mycelia while *P. ultimum* had white creamish mycelia in color (Figure 7). Morphologically *F. oxysporum* produced non septate kidney shaped microconidia while *P. ultimum* had spherical sporangia.

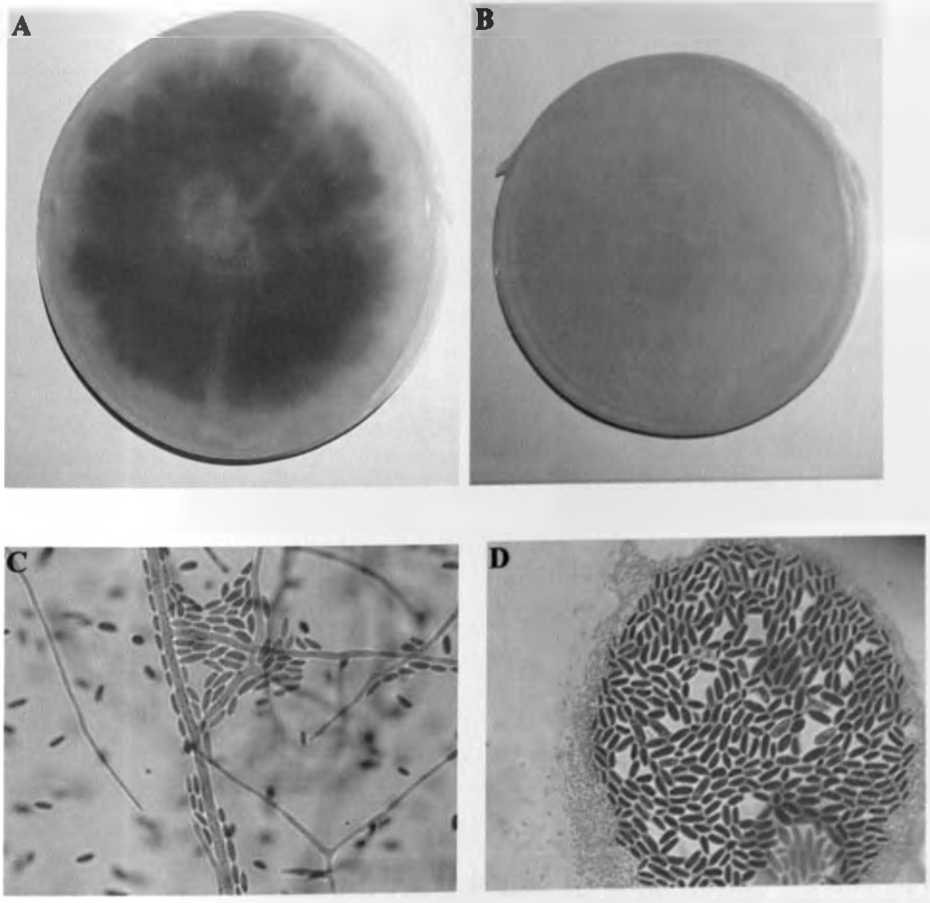


Figure 7: Cultural and morphological characteristics of *Fusarium oxysporum* and *Pythium ultimum*.Culture plates of *F. oxysporum* (A), culture plate of *Pythium ultimum* (B) and microconidia of *Fusarium oxysporum* (C, D).

4.3.2 Efficacy of *Aloe* crude extracts on *Fusarium oxysporum* and *Pythium ultimum*

The crude extracts inhibited both test pathogens forming various sizes of inhibition zones at different concentrations (Figure 8).

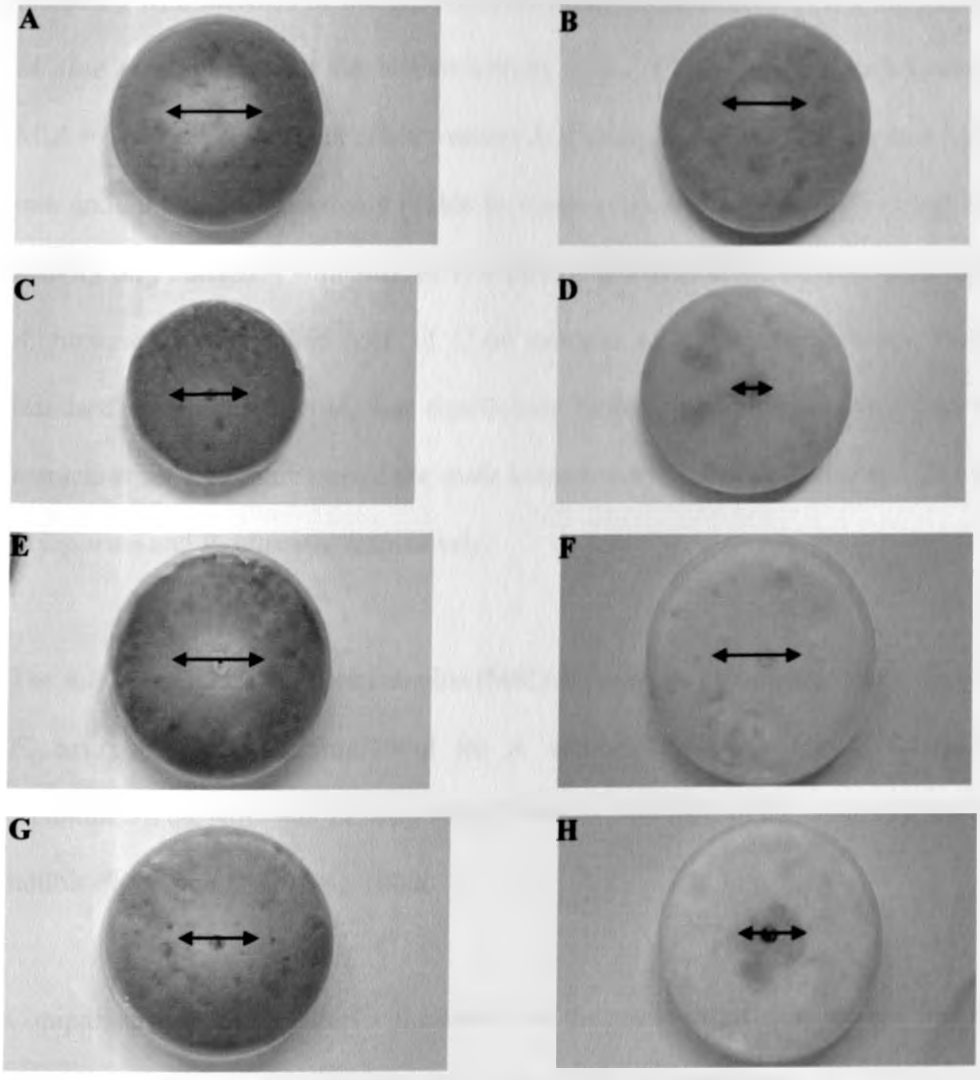


Figure 8: Zones of inhibition (mm) formed by crude extracts of *Aloe* species and the standard fungicide Triazole on *F. oxysporum* and *P. ultimum* after 10 days of incubation
Triazole on *F. oxysporum* (A) and *P. ultimum* (B) at 50mg/100µl; crude extract of *A. turkanensis* on *F. oxysporum* (C) and *P. ultimum* (D) at 25mg/100µl; crude extract of *A. secundiflora* on *F. oxysporum* (E) and *P. ultimum* (F) at 100mg/100µl; Crude extract of *A. lateritia* on *F. oxysporum* (G) and *P. ultimum* (H) at 100mg/100µl.
The arrows indicate the zones of inhibitions formed by the crude extracts against the test pathogens.

The crude extracts of *Aloe* species had significant ($p \leq 0.05$) fungistatic activity against *F. oxysporum* and *P. ultimum* with varying levels of activity (Figure 9). The zones of inhibition increased with increase in the concentration of the crude extracts (Table 6). Crude extracts of *Aloe secundiflora* had the highest activity against *F. oxysporum* (Mean inhibition zone, MIZ = 16.7 mm) whereas crude extracts *A. lateritia* and *A. turkanensis* had MIZ of 12.3 mm and 10.8 mm, respectively (Table 6). Crude extracts of *A. secundiflora* had the highest activity on *P. ultimum* with MIZ of 15.3 mm compared to crude extracts of *A. lateritia* and *A. turkanensis* which had MIZ of 13.84 mm and 11.32 mm, respectively. However the standard fungicide, triazole, had significantly higher ($p \leq 0.05$) activity than the crude extracts at all concentrations of the crude extracts with MIZ of 26.1 mm and 23.1 mm on *F. oxysporum* and *P. ultimum*, respectively.

The minimum inhibitory concentration (MIC) of the crude extracts that was effective against *F. oxysporum* was 6.25mg/100µl for *A. secundiflora* while for *A. lateritia* and *A. secundiflora* the MIC was 12.5mg/100µl (Table 6). The MIC of the crude extracts that could inhibit *P. ultimum* was 25mg/100µl.

Comparison was also made for the activity of the crude extracts against the test pathogens over time (Figure 10, 11 and 12) except for concentrations of 6.25mg/100µl and 12.5mg/100µl as the crude extracts had no activity against *P. ultimum* at these concentrations. Crude extracts of *A. secundiflora* had significant higher activity against *F. oxysporum* than the standard antifungal drug triazole at 25mg/100µl for the first four days and had similar activity six days after onset of the bioassay (Figure 12 B).

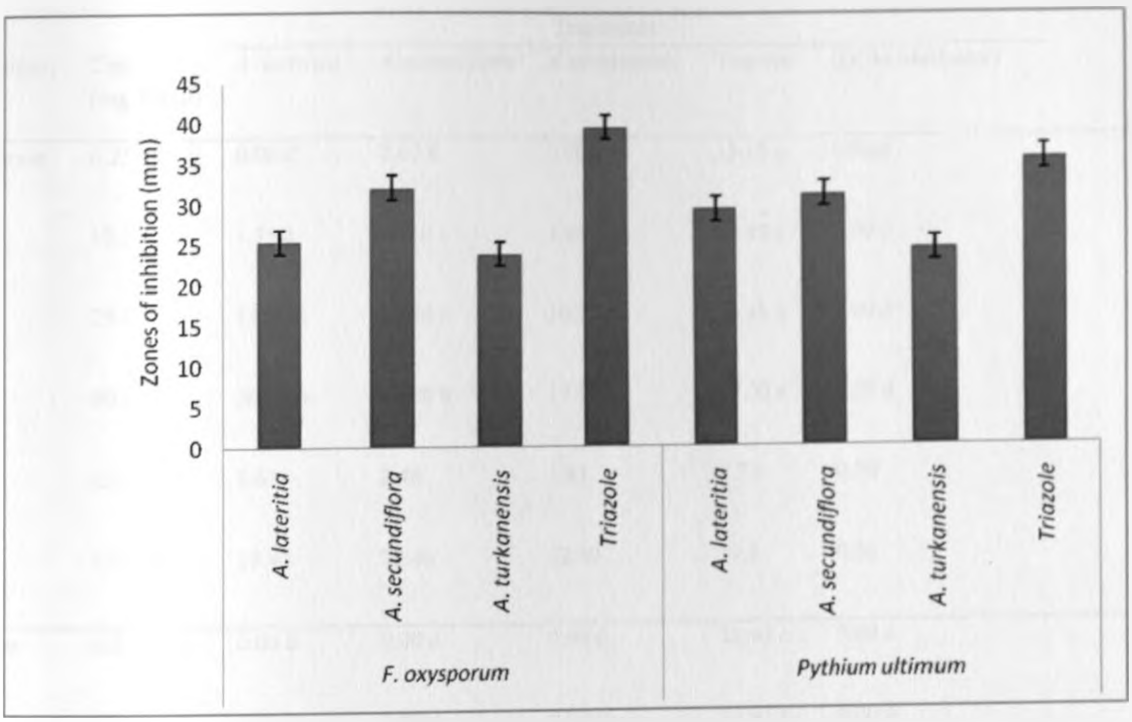


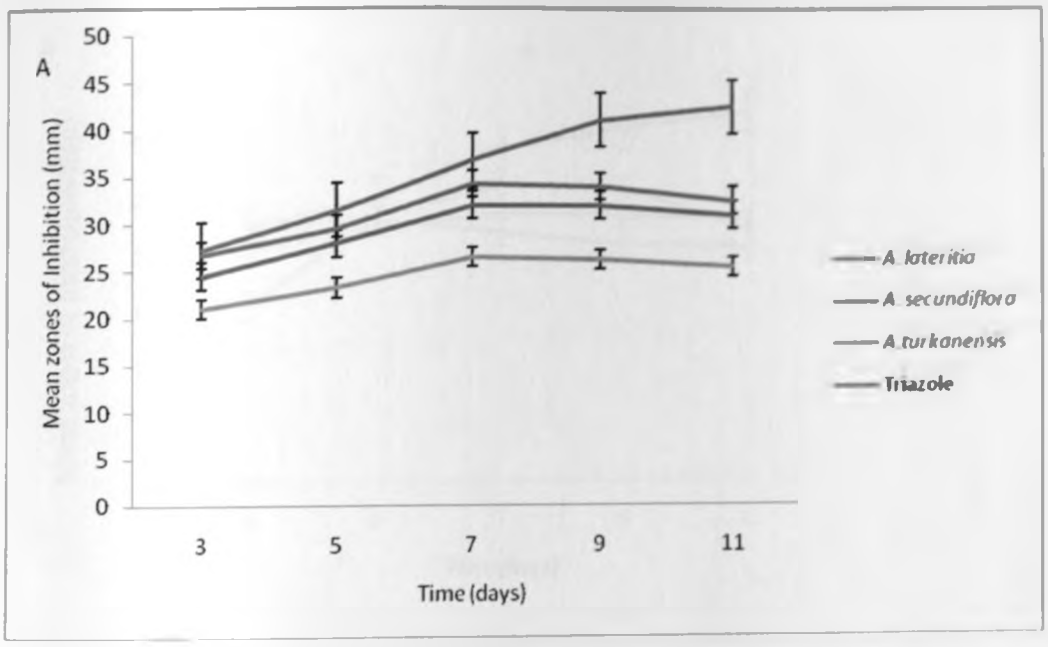
Figure 9: Mean inhibition (mm) of growth of *F. oxysporum* and *P. ultimum* by crude extracts of different *Aloe* species

*Error bars denote the standard error of the mean

Table 6: Mean inhibition (mm) of growth of *F. oxysporum* and *P. ultimum* by crude extracts of different *Aloe* species

Test Pathogen	Conc (mg/100µl)	Treatments				
		A. lateritia	*A.secundiflora*	*A. turkanensis*	Triazole	(DCM:Methanol)
F. oxysporum	6.25	0.00 d	2.60 d	0.00 d	15.15 c	0.00 d
	12.5	1.75 d	10.10 c	1.95 d	19.85 c	0.00 d
	25.0	14.00 c	15.40 c	10.95 c	24.45 b	0.00 d
	50.0	20.52 b	22.80 b	17.10 c	32.00 a	0.00 d
	LSD	1.63	2.48	1.82	2.72	0.00
	CV (%)	25.40	28.40	32.40	19.8	0.00
P. ultimum	6.25	0.00 d	0.00 d	0.00 d	12.40 c	0.00 d
	12.5	0.00 d	0.00 d	0.00 d	16.95 c	0.00 d
	25.0	16.75 c	20.15 b	12.65 c	21.75 b	0.00 d
	50.0	22.95 b	24.80 b	19.45 b	28.45 b	0.00 d
	100.0	29.50 a	31.40 a	24.50 b	35.80 a	0.00 d
	LSD	1.1	1.26	1.26	2.33	0.00
	CV (%)	15.20	15.80	15.80	19.30	0.00

Mean inhibition zones (mm) followed by the same letter along the column for each of the test pathogens are not significantly different ($p \leq 0.05$).



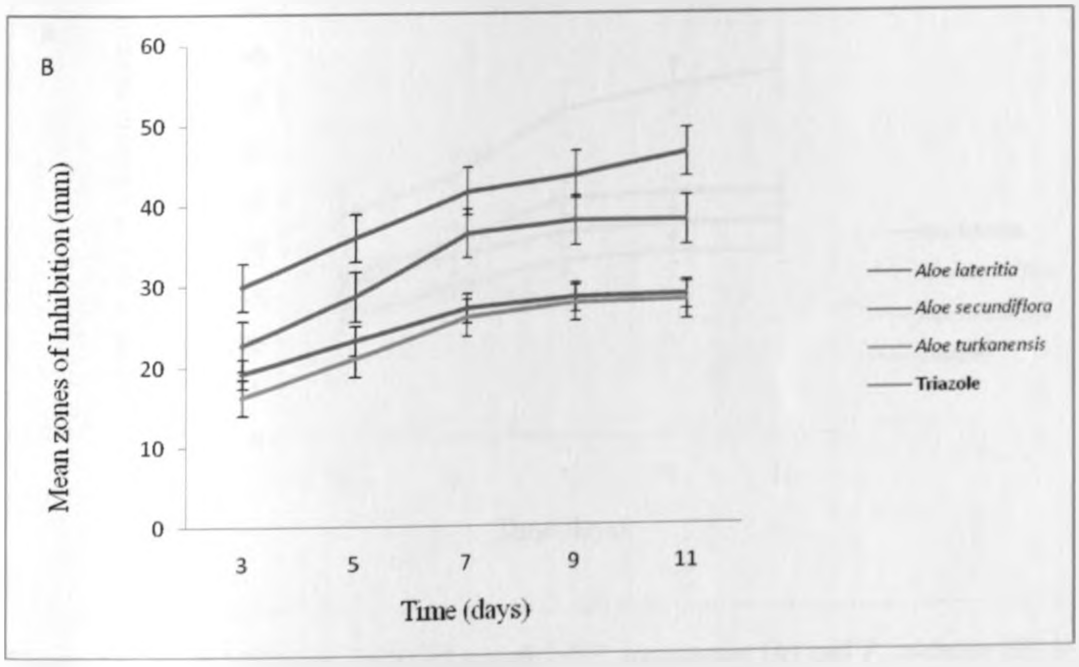


Figure 10: Mean inhibition (mm) of growth of *F. oxysporum* (A) and *P. ultimum* (B) by crude extracts of different *Aloe* species at a 100mg/100µl concentration over time.

*Error bars denote the standard error of the mean

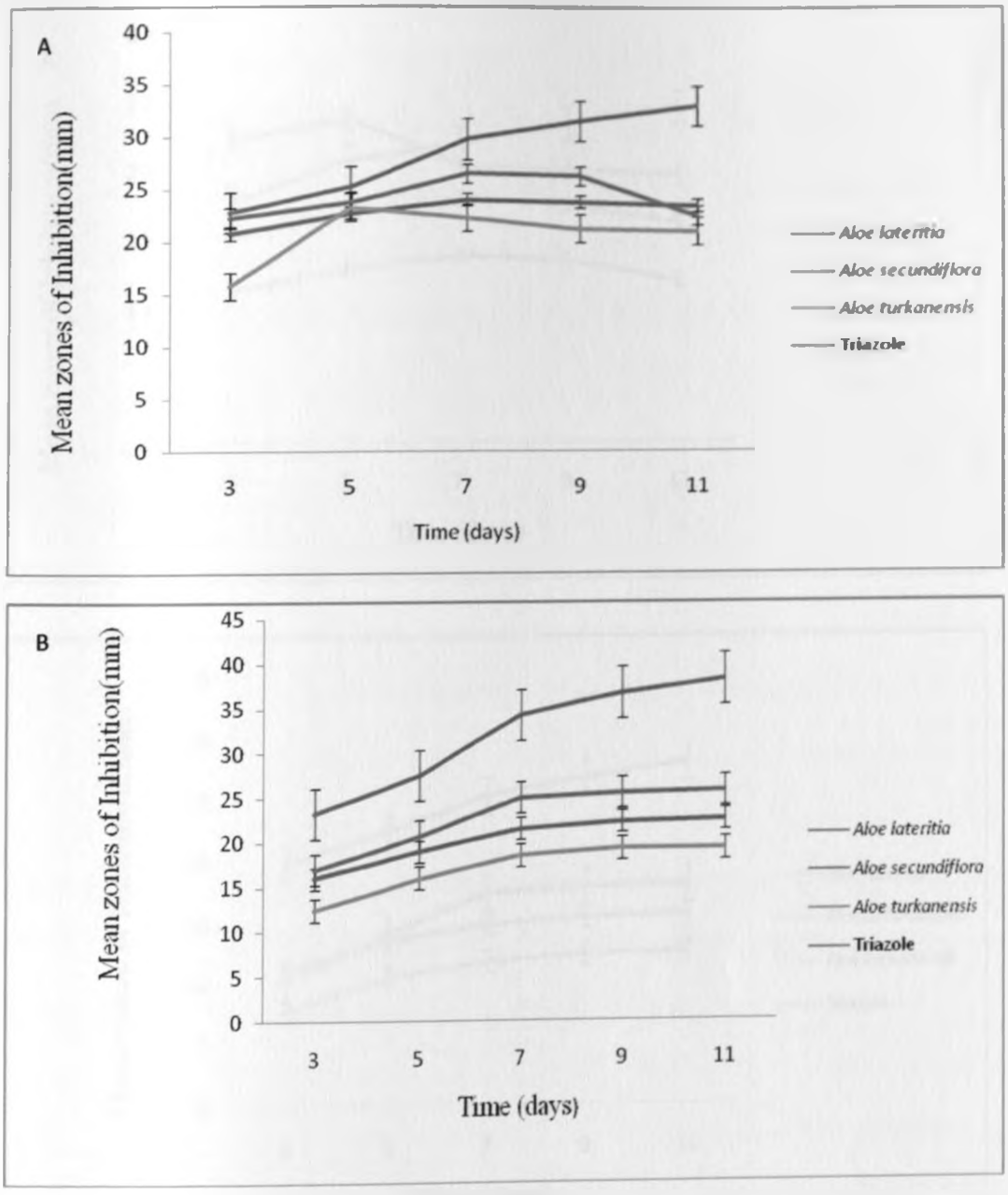
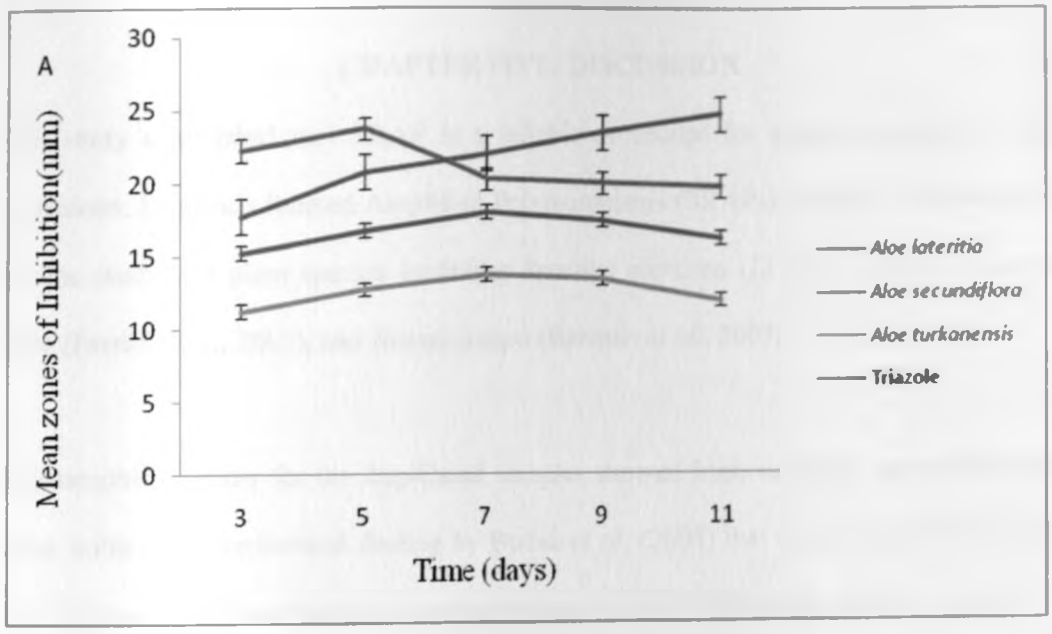


Figure 11: Mean inhibition (mm) of growth of *F. oxysporum* (A) and *P. ultimum* (B) by crude extracts of different *Aloe* species at a 50mg/100µl concentration over time.

*Error bars denote the standard error of the mean



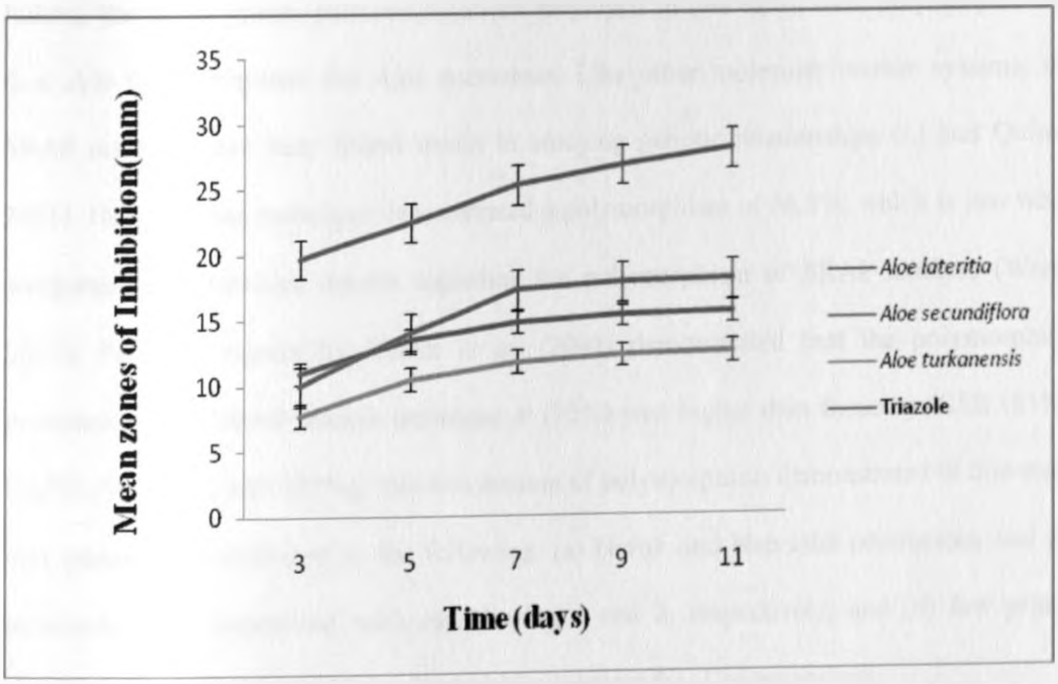


Figure 12: Mean inhibition (mm) of growth of *F. oxysporum* (A) and *P. ultimum* (B) by crude extracts of different *Aloe* species at a 25mg/100µl concentration over time.

*Error bars denote the standard error of the mean

CHAPTER FIVE: DISCUSSION

This study established that SRAP is a reliable procedure for genetic analyses of *Aloe* accessions. Sequence Related Amplified Polymorphisms (SRAPs) has been used widely in genetic studies of plant species including *Brassica oleracea* (Li *et al*., 2003), *Cucurbita pepo* (Ferriol *et al*., 2003), and *Brassica rapa* (Rahman *et al*., 2007).

Polymorphic patterns for the duplicated samples showed high similarity and results from these initiatives corroborated finding by Budak *et al*. (2004) that showed that SRAPs were highly reproducible and therefore were appropriate for the *Aloe* genetic diversity study. Indeed, the seven primer pair combinations employed in this study were polymorphic and thus able to discriminate the *Aloe* accessions. Like other molecular marker systems, the SRAP markers have been found useful in studying genetic relationships (Li and Quiros, 2001). However this technique demonstrated a polymorphism of 56.8%, which is low when compared with previous reports regarding the polymorphism of SRAP markers (Wang, 2012). Previous reports by Budak *et al*. (2004) demonstrated that the polymorphism produced by the SRAP marker technique at (95%) was higher than those by ISSR (81%), RAPD (79%) and SSR (87%). The low amount of polymorphism demonstrated in this study may partially be attributed to the following: (a) Narok and Naivasha populations had the least number of accessions with each having 3 and 2, respectively; and (b) few primer combinations were screened for polymorphism against the *Aloe* accessions.

Accessions from the Gene Bank of Kenya grouped in one cluster and similarly had the same phenotypic characteristics. However, molecular and phenotypic marker-based analysis did

not show the same similarity in the other populations. This may be caused by quantitative control of phenotypic traits studied and/or fluctuations in environmental conditions, having potential effect on phenotypic performances (Gulsen *et al.*, 2007). Fufa *et al.* (2005) also, reported no significant correlation coefficients among distance estimates of morphological and SRAP markers. Sequence Related Amplified Polymorphism markers tended to have low correlation with the other genetic diversity estimates, they may provide different and unique insights into genetic diversity.

This study hypothesized that there was no genetic variation between and within *Aloe* species in Kenya. The 31 accessions of *Aloe* were grouped into 3 major clusters. Cluster B comprised mainly of *Aloe* accessions from Baringo population while cluster A comprised mainly of *Aloe* accessions from the gene bank of Kenya. Cluster C comprised of two *Aloe* accessions from Nakuru and Narok populations. Each of the three accessions from Narok was distributed in all the three clusters as well as accessions from the Nakuru population. *Aloe* accessions from KALRO Biotechnology were all in cluster A.

Cluster analysis showed that populations from Nakuru and Narok were more divergent than all the other populations with populations from gene bank of Kenya and KALRO Biotechnology Centre being closely related. This relatedness may suggest that they may have similar origins but were grown in different geographical localities. Nayanakantha *et al.* (2010) reported similar findings for *Aloe* accessions collected from the Defense Agricultural Research Lab in India.

Genetic distance and similarity within populations based on pair-wise comparison revealed moderate genetic diversity within the populations studied. The populations had low genetic distance between them and this could be attributed to the proximity of the sampled areas. Wu *et al.* (2004) suggested that germplasm from all major geographic regions where the species occurs should be collected, to fully cover the available variation.

It was not possible to conduct a full diversity analysis using morphological characteristics due to the small number of descriptors. It was observed that there was no correlation between the morphological and molecular characteristics. However the majority of accessions from the KALRO Gene Bank of Kenya using morphological characteristics were found only in cluster A using molecular characterization.

Results from this study imply that there is genetic diversity within the accessions of *Aloe* sampled from the 6 populations. Conservation efforts of this genus are necessary to preserve the already endangered species and in particular *Aloe* species that are commercially exploited for bitter gum.

This study showed that crude extracts of *A. secundiflora, A. turkanensis* and *A. lateritia* significantly inhibited mycelial growth of *Pythium ultimum* and *Fusarium oxysporum*. This suggests that the extracts possess fungicidal potential capable of controlling the two fungal pathogens *in vitro*. It was also observed that activity of the crude extracts against the test pathogens was higher at increased concentrations.

The crude extract of *A. secundiflora* had significant activity against both fungi than crude extracts of *A. lateritia* and *A. turkanensis*. Antifungal activity of the crude extracts increased with concentration with the highest activity at concentrations of 100mg/100µl followed by 50mg/100µl and 25mg/100µl, respectively. This is similar to the findings by Wokocha and Wekereke, (2005) who observed that the activity of the crude extracts increased with increase in concentration.

Aloe vera has been reported to contain six antiseptic agents that have inhibitory effects against fungi, bacteria and viruses. They are lupeol, salicylic acid, urea nitrogen, cinnamonic acid, phenols and sulphur (Ozsoy *et al.*, 2009). The use of methanol to prepare the crude extracts may also have contributed to the antifungal effects of the *Aloe*. Leelaprakash *et al.* (2011) reported that methanolic extracts contain higher concentration of antimicrobial agents. These may include alkaloids, glycosides, carbohydrates, proteins and amino acids phytochemicals which the *Aloe* genus is rich in.

Cell-wall synthesis is the target for various groups of drugs (Gooday, 1993). The ability of the crude extracts and the fungicide triazole to control *Fusarium oxysporum* more than *Pythium ultimum* can be attributed to the fact that in addition to chitin, *P. ultimum* also contains cellulose in its cell wall structure. Cherif *et al.* (1993) reported that both chitin and cellulose are found in the cell wall of the oomycete *P. ultimum* whereas only chitin is present in the cell wall of *F. oxysporum*. Depending on composition, the cell wall can act as a barrier preventing fungicides from reaching the site of action (Kariba, 2000).

There was no significant difference between 6.25 mg/100µl and 12.5mg/100µl of crude extracts against the test pathogens. Since integrated pest management advocates the use of minimal dosages of chemicals (Hill and Waller, 1982), 25 mg/100µl would be appropriate in combination with other options available for control of *F. oxysporum* and *P. ultimum*.

Results from the activity of the crude extracts against the test pathogens over time revealed that there was an increase in activity for the first seven days and a decrease in subsequent days of incubation on PDA. This concurs with findings by Ogechi *et al.* (2006) where activity of ginger against *F. oxysporum* decreased from 6 – 12 days of incubation on PDA. This might indicate that the breakdown of active compounds maybe slower over time or their stability was compromised during the experiment. Therefore there is need to purify and refine the active compounds of the crude extracts to increase its activity.

This study has revealed the potential of *Aloe* crude extracts in the control of root rot and damping off pathogens *in vitro*. However, it is pertinent to note that this activity is one among many other possibilities in control of plant diseases. More effort is required in integrating the study to other related technologies. The use of plant products in integrated fungi management could reduce over reliance on one control strategy among farmers, as well as cut down the cost of agricultural production. The fact that *Aloe* species used in this study are easily available, with easy method of extraction, implies that they can be exploited in the control of root rot and damping off pathogens of economically important crops.

5.1.1 Conclusions

i. The SRAP methodology developed in this study successfully discriminated the *Aloe* accessions from in situ and ex situ collections.

ii. Most of the diversity in *Aloe* accessions is partitioned within population with Nakuru and Narok populations being the most diverse on the genetic distances obtained.

iii. Crude extracts of *Aloe* species had activity against *F. oxysporum* and *P. ultimum* at varying concentrations. The minimum inhibitory concentration of *A. secundiflora* was 25 mg/100ul against *P. ultimum* and 6.25mg/100ul for *A. secundiflora* against *F. oxysporum*.

iv. Active compounds in *A. secundflora, A. turkanensis* and *A. lateritia* can be exploited for development of fungicides for control of *F. oxysporum* and *P. ultimum*.

5.1.2 Recommendations

i. Findings of this study could form basis for better conservation efforts and utilization of *Aloe* in Kenya

ii. Future studies should consider sampling *Aloe* species from all parts of Kenya where the plant grows. This will be necessary to establish the genetic diversity and population structure of *Aloe* species in the country. .

iii. Extracts of *Aloe* species can be used alongside other methods in managing *F. oxysporum* and *P. ultimum* to reduce over reliance on chemical fungicides.

iv. Further work should include isolation and purification of the active compounds in *Aloe* species and their activity screened against a wide range of economically important plant fungal pathogens.

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APPENDICES

Appendix A: List of maize SRAP primers for genotyping *Aloe* accessions

Forward primer (name: sequence 5'-3')	Reverse primer (name: sequence 5'-3')
Me1:TGAGTCCAAACCGGATA	Em5:GACTGCGTACGAATTAAC
Me2: TGAGTCCAAACCGGAGA	Em7: GACTGCGTACGAATTCAA
Me5: TGAGTCCAAACCGGAAG	Em9: GACTGCGTACGAATTCGA
Me8: TGAGTCCAAACCGGTGC	Em10: GACTGCGTACGAATTCAG
Me11: TGAGTCCAAACCGGTGT	Em11: GACTGCGTACGAATTCCA
Me12: TGAGTCCAAACCGGCAT	Em12: GACTGCGTACGAATTATG
	Em15: GACTGCGTACGAATTTAG
	Em17 GACTGCGTACGAATTGTC

Appendix B: Selective primer-pair combinations screened for *Aloe* species genotyping

	Me 1	Me 2	Me 5	Me 8	Me 11	Me 12
Em 5	Pair#1√	Pair#2√	Pair# 3	Pair# 4	Pair# 5	Pair# 6
Em 7	Pair# 7√	Pair# 8	Pair#9	Pair#10√	Pair# 11	Pair# 12
Em 9	Pair# 13√	Pair# 14	Pair#15	Pair# 16	Pair#17	Pair#18
Em 10	Pair#19	Pair# 20	Pair# 21	Pair# 22	Pair# 23	Pair# 24
Em 11	Pair# 25	Pair#26	Pair# 27	Pair# 28	Pair# 29	Pair# 30
Em 12	Pair# 31	Pair# 32	Pair# 33√	Pair#34	Pair# 35	Pair# 36
Em 15	Pair# 37	Pair# 38	Pair# 39	Pair# 40	Pair# 41	Pair#42
Em 17	Pair# 43	Pair# 44√	Pair# 45	Pair# 46	Pair# 47	Pair# 48

Key: Me - Foward primer, Em – Reverse primer, √ indicates primer combinations that produced polymorphic bands.