IMPACT OF DOMESTICATION ON GENETIC DIVERSITY OF WARBURGIA UGANDENSIS SPRAGUE WITHIN LAKE VICTORIA REGION

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A thesis submitted in partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology in the School of Pure and Applied Sciences of Kenyatta University

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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 award

Nkatha Gacheri Muriira

Signature ______________________ Date 02/02/2012

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

To my late dad Lawrence Muriira, your inspiration keeps me abreast.
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### ABBREVIATIONS AND ACRONYMS

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<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism.</td>
</tr>
<tr>
<td>AMOVA</td>
<td>Analysis of molecular variance</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic acid</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>TE</td>
<td>Tris Ethylenediamine Tetra-acetic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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<td>TFPGA</td>
<td>Tools for Population Genetic Analysis</td>
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ABSTRACT

*Warburgia ugandensis* Sprague (Canellaceae) occur in East and Central Africa, and is of great medicinal importance. The demand for *W. ugandensis* bark in herbal medicinal industry has led to unsustainable harvesting in the natural forests thereby threatening the species survival. The species natural habitat is also under threat of clearance for farming. Growing of this species on-farm will ensure sustainable medicinal source and its conservation. The material planted on-farm should be of wide genetic base hence protect subsequent generations of founder effects. However, genetic diversity of material currently on-farm is unknown. The genetic diversity of three on-farm *W. ugandensis* populations and their proximate natural populations from Lake Victoria region were analysed using the amplified fragment length polymorphism (AFLP) markers. Three reference populations each from Kenya, Uganda and Tanzania were also included in the study. Twenty primer pairs were initially used in the screening for polymorphisms, of which four primer pairs were selected for the final analysis. Genetic diversity estimate, genetic distances and phenogram was developed using TFPGA 1.3 statistical software. Analysis of molecular variance (AMOVA) and principal coordinate analysis (PCA) were generated using GenAlEx 6.3 molecular statistical software. Four primer combinations (EcoRI+ACC/MseI+CAA, EcoRI+ACT/MseI+CAT, EcoRI+ACT/MseI+CAG, and EcoRI+AGC/MseI+CAG) produced 223 polymorphic bands. Both the natural and on-farm populations had high genetic diversity ranging from $H = 0.2892$ to $H = 0.1278$. Principal co-ordinate analysis and phenogram showed the ten populations falling into two major groupings of Kenya and Tanzania, with Uganda populations splitting into the two groupings; this is probably because Uganda is believed to be the centre of diversity for *Warburgia ugandensis*. The two cluster analyses revealed close genetic relatedness between the on-farm and their proximate natural population. The AMOVA showed 54% of the variation to be within populations and 46% among populations ($P<0.01$). These study findings are in agreement with earlier suggestions that most of the trees on-farm are remnants of forest in cultivated forest lands. These revealed close genetic relationship between the on-farm and their proximate natural population. The high genetic diversity of *W. ugandensis* populations currently on-farm suggest that the material can be used for future seed sources and conservation of the species.
CHAPTER ONE
INTRODUCTION

1.1 Background

*Warburgia ugandensis* Sprague (Canellaceae) is a multi-purpose tree found in the lowland rainforest and upland dry evergreen forest of eastern and central Africa. It is ranked second among the top ten traditional medicinal plant species according to utility value and sustainable use (Kariuki and Simiyu, 2005). The World Health Organization estimates that about 80% (WHO, 2002) of the local communities in sub Saharan Africa rely on traditional medicines while global trend in medicinal qualities has also been on rise (Lange, 2002). Due to the high demand for the medicinal extract from its bark, roots and leaves, *W. ugandensis* are overexploited, as shown in Figure 1.

The natural forests that this species inhabit are currently under threat of destruction by clearing for farming and unsustainable exploitation of the species for medicinal purposes (Maundu and Tengnas, 2005). As such, the species is reported as becoming rare in areas where they were once plentiful (Beentje, 1994). Due to the increasing pressure for bark demands for herbal preparations, there is need to look into alternative supply rather than natural forest.

The plant is propagated through seeds and stem cuttings; mass propagation, though tissue culture is also possible. The species is fast growing and easy to grow. It has been incorporated in agroforestry systems as shown in Figure 2, as well as being grown in urban areas for aesthetic value because of its ever green nature. There is need for an intensive cultivation program for *W. ugandensis* to ensure sustainable supply of the
herbal extracts and the species conservation. Cultivation of the medicinal species in plantations and on-farm has been advocated to ensure sustainable supply of the herbal products and conservation of the species in the world (Simons et al., 2000; On et al., 2001). However, most farmers and nursery managers often collect germplasm from a relatively narrow range of maternal parents during propagation in agroforestry systems (Lengkeek et al., 2005a) suggesting that on-farm stands may be at risk of genetic erosion. A wide genetic base provides the ability to withstand potential inbreeding depression through future generations of farmer propagation (Boshier, 2000).

However, little is known about the influence of cultivation on genetic diversity within the species and whether on-farm stands are suitable sources of material for further planting. Molecular genetic studies provide valuable data on diversity through its ability to detect variation at DNA level (Karp and Edwards, 1996). Studies on structuring of Warburgia genus using AFLP markers showed that genetic variation among *W. ugandensis* populations was high, hence intensive sampling of a few populations of the species would capture most of genetic variations (Muchugi et al., 2008). The knowledge of the species genetic diversity ensures that planting material of wider genetic base is incorporated in planting program hence cushions subsequent generations of founder effects (Simons et al., 1993).

Molecular marker techniques have provided valuable information about genetic diversity of natural plant population (Xiao-Ru and Alfred, 2001). Among polymerase chain reaction (PCR) based methods, the amplified fragment length polymorphism (Vos et al., 1995) technique is considered a highly informative fingerprinting tool (Schnell et al., 2001) and has been used successfully to analyse the genetic structure of the population of
tropical tree species (Muluvi et al., 1999; Russell et al., 1999) and investigated genetic variation in a wide variety of micro-organisms, plants and animals (Sawkins et al., 2001; Maguire et al., 2002).

Figure 1: Severely debarked *W. ugandensis* at Tree in Geita forest reserve in Kagera district, Tanzania.

Figure 2: Coppicing *W. ugandensis* tree stumps Cherangani, Kenya.

1.2 Statement of the Problem

*Warburgia ugandensis* is an important multipurpose tree species which has been over-exploited due to unsustainable harvesting through ring barking for medicinal extracts, indiscriminate felling for timber, firewood and agricultural expansion thus, threatening its genetic diversity. Domestication of the species is currently taking place on-farm which could provide important reservoirs of the tree diversity. However, most farmers and nursery managers are known to collect germplasm from a relatively narrow range of maternal parents for propagation; this may result into a narrow genetic base of the on-farm trees.
A narrow genetic base within a population can lead to inbreeding depression in future generations of on-farm trees which would lead to accumulation of deleterious alleles, making the species unable to cope with changing biotic and abiotic stresses.

1.3 Justification

Domestication results in either gain or loss of genes, thereby altering gene and genotype frequencies. Knowledge of natural species and population level genetic diversity is essential for monitoring the effects of in situ or ex situ conservation. Information on the biological characteristics of the species therefore provides a sound basis in the conservation strategies planting programs, especially if the established stands would in future be of use in germplasm collection.

Knowledge of the species genetic diversity ensures that planting material of wide genetic base is incorporated in the planting program hence protects subsequent generations of founder effects. Knowledge of phenotypic and genetic variation is a prerequisite in the domestication. For species with high population differentiation, more populations need to be preserved or sampled to ensure that allelic and genotypic diversity can be retained on existing on-farm trees. Possible losses in genetic diversity in on-farm stands of *W. ugandensis* were estimated by comparing with proximate natural forest material. As *W. ugandensis* has gained popularity as an agroforestry species, the knowledge generated would guide future germplasm sampling for plant establishment and conservation strategy. Molecular characterization of the variability may help to manage and preserve genetic resource for long term survival of species and for further applications such as domestication and use in breeding programmes.
1.4 Research questions

i. Has domestication changed the genetic diversity of *W. ugandensis* in the Lake Victoria region?

ii. How is the genetic structuring of *W. ugandensis* populations in the Lake Victoria region?

1.5 Hypothesis

The on-farm population of *W. ugandensis* is of narrow genetic base when compared to their proximate natural populations in the Lake Victoria region.

1.6 Objectives

1.6.1 General objective

To determine genetic diversity of populations of *W. ugandensis* within the Lake Victoria region of Kenya, Uganda and Tanzania.

1.6.2 Specific objectives

i. To evaluate genetic diversity of on-farm *W. ugandensis* populations and their proximate natural populations in the Lake Victoria region in Kenya, Uganda and Tanzania using AFLP technique.

ii. To evaluate genetic variation partitioning among *W. ugandensis* populations in the Lake Victoria region in Kenya, Uganda and Tanzania.
2.1 Taxonomy and botanical description of *Warburgia ugandensis*

The genus is named after Dr. Otto Warburg (1859-1938), born in Hamburg, lecturer in botany at the University of Berlin and author of numerous botanical paper (Beentje, 1994). *Warburgia ugandensis* Sprague (synonym *W. breyeri* pott) belongs to family Canellaceae within genus *Warburgia* the others being *W. salutaris*, *W. stuhlmannii* and *W. elongata* (Beentje, 1994). It is a spreading evergreen tree 4.5-30 m tall, 70 cm in diameter, bark smooth or scaly; pale green or brown, slash pink: bole short and clear of branches for about 3m: crown rounded. The leaves are alternate, simple, dotted with glands, stipules absent; petiole 1-5 mm long; blade oblong-lanceolate, elliptic or oblong-elliptic, 3-15 x 1.4-5 cm, apex and base tapering, margins entire, glossy dark green above, paler green and dull below, midrib frequently slightly off-centre (Najma, 2002).

Flowers are solitary or in small 3-4 flowered cymes, auxiliary, regular bisexual bracts ovate to kidney shaped thick covering only the young buds, sepals are green ovate, petals in whorls white or greenish yellow obovate dotted with glands, fruit is a berry at first green and ellipsoidal and later sub spherical and turning purplish, skin leathery, glandular seeds have oily endosperm and are cordate (Najma, 2002). *Warburgia ugandensis* is widely distributed in lowland rainforest and drier highland forest areas as shown in Figure 3 at altitude between 1000 m to 2000 m above sea level (Maundu and Tengnas, 2005). The tree requires mean annual rainfall of between 1000-1500 mm and it withstands swamp forest soils (Maundu and Tengnas, 2005).
Figure 3: Distribution of *Warburgia ugandensis* in Africa (Dots refer to documented voucher specimen not the species population density).
2.2 Reproductive biology

*Warburgia ugandensis* is hermaphroditic, flowering at the beginning of the rainy season with fruiting following later in the rainy season, fruits may remain on the tree for a long time (Orwa *et al.*, 2009). *W. ugandensis* is insect-pollinated species, though its flowers do not have a strong scent, dish-shaped flowers with brightly coloured inner petals which suit small bees (e.g. *Trigona*) and other insects.

The estimate of out-crossing rates of *W. ugandensis* shows a mixed mating system, which is predominantly out-crossing (Muchugi *et al.*, 2008). Out-crossing is predominant in many tropical tree species, and the average out-crossing rate is more than 80% (Nason and Hamrick, 1997). Out-crossing rates may be strongly influenced by flowering-tree density (Murawski *et al.*, 1994) and the types and behaviors of pollinators governing the pollen movement (Ghazoul and Moray, 1998).

The low selfing rates contrasts the expectations of species with bisexual flowers where majority of the seed are thought to result from self-fertilization (Murawski *et al.*, 1994). This may be as a result of presence of active self incompatibility factors (Muchugi *et al.*, 2008).

2.3 Uses of *Warburgia ugandensis*

*Warburgia ugandensis* is one of the highly valued medicinal species within the traditional health systems of the communities where it naturally occurs (Beentje, 1994; Ssegawa and Kasenene, 2007). The curative efficacy of the species extracts is ascribed to its antibacterial and antifungal medicinal properties which have been scientifically proven in
different *in vivo* and *in vitro* trials of the isolated major bioactive ingredients (Rabe and Staden, 2000; Olila *et al.*, 2001).

This tree species has a high pharmaceutical value both for humans and livestock, exhibiting a broad spectrum antimicrobial activity (Olila *et al.*, 2001) and antiparasitic activity (Irungu *et al.*, 2007). The species are rich in terpene compounds specifically drimane and coloratane skeletons (Mashimbye *et al.*, 1999). Active sesquiterpenes isolated include; warburganal (Haraguchi, 1998), muzigadial, polygodial (Taniguchi and Kubo, 1993), ugandensidal (Haraguchi, 1998) and cinnamolide (Rugutt *et al.*, 2009).

Although conventional medicine has become widespread in developing countries, many rural communities are still heavily dependent on plant-based therapies for their primary healthcare (Taniguchi and Kubo, 1993; Zschockes *et al.*, 2000). According to the WHO approximately 80% of the world’s population relies on traditional medicine to fulfil their daily health needs (Marshall, 1998).

Many people in East Africa prefer traditional medicine to modern medicines; this is attributed to their accessibility, affordability, local knowledge and expertise among local Communities (Kariuki and Simiyu, 2005). Traditional healers use *W. ugandensis* to treat malaria, chest pains, toothache and manufacture of some skin creams. *W. ugandensis* has a great potential for commercial market, development.

Medicinal products from *W. ugandensis* are currently available in Kenya and Uganda but the processing is at small scale as shown in Figure 4. Extracts from *W. salutaris* a species in the same genus, are already being processed commercially in South Africa as shown in Figure 5 and marketed internationally at highly competitive prices (Botha *et al.*, 2004).
Warburgia ugandensis other uses include; Food, the fruits are edible with all parts having a hot peppery taste (Orwa et al., 2009). The leaves and seeds are sometimes used to add flavor to curries. It is also used as fodder: Leaves, pods and seeds are fed to livestock (Orwa et al., 2009). The wood has high oil content and burns well with an incense-like smell hence useful as fuel. It saws timber for building and furniture. The resin from the tree is used locally as glue to fix tool handles. The old leaves have also been used as mulch for soil conservation. It can also be a source of ornamental shade (Maundu and Tengnas, 2005).

Figure 4: Warburgia ugandensis products (Kenya and Uganda).

Figure 5: Warburgia salutaris products (South Africa).
2.4 Poverty, diseases and environmental degradation in Lake Victoria region

Lake Victoria is the world’s second largest freshwater lake and the largest in Africa, with a total catchment of 250,000 square kilometres, of which 68,000 km² is the actual lake surface. Located in the upper reaches of the Nile River Basin, the lake waters are shared by the three East African Countries of Kenya (6%), Uganda (43%) and Tanzania (51%). Rwanda and Burundi are a part of the upper watershed that drains into Lake Victoria through the Kagera River and between them, occupy about 18% of the lake catchment (Orodho et al., 2011).

The lake is a major trans-boundary natural resource that is heavily utilized by its bordering countries for fisheries, transportation, tourism, water supply and waste disposal. The problems of human poverty and unemployment are widespread. They are compounded by the rapid increase in population, the ongoing public health challenges posed by the high incidence of HIV/AIDS and malaria, unplanned urbanization and environmental degradation (Awange and Ong‘anga, 2006).

Persistent poverty remains a main cause and consequence of environmental degradation and resource depletion. These practices include cultivating marginal and fragile areas such as wet lands and clearing vital forests to open up new arable lands or to get access to fuel wood culminating intensified soil erosion (Awange and Ong‘anga, 2006).

Indigenous knowledge on the use of medicinal plants offers a wide range of subsistence and cultural benefits. It provides affordable means of primary health care especially in impoverished rural areas. Before the patient is clinically diagnosed for active respiratory disease in the region, they are attended-to by traditional health practitioners (THPs)
whose medicines are plant based. This has been due to easy availability and sometimes only source of fast available health care (Adhikari, et al., 2010). Some plants are being used in greater quantities depending on number of diseases and average number of patients per month such as *Warburgia ugandensis*, *Cordia africana* and *Entada abyssinica*. Despite their multiple medicinal applications, they are among rare plant species in the area.

Medicinal plants with market value should be treated as important resources for sustainable development through commercial cultivation. *Rubia cordifolia* and *W. ugandensis* are proposed for commercial cultivation (Otieno et al., 2011). *W. ugandensis* is among the second of the six most used plant species to treat TB in Lake Victoria basin (Orodho et al., 2011). Domestication of medicinal wild varieties is constrained by number of factors including misconceptions, attitudes and unawareness on the specific propagation conditions. For example, some assume that domestication lessen medicinal potency of wild plants (Otieno et al., 2011). This has resulted in reduced number of many important trees.

2.5 Genetic diversity in tropical trees

Tropical rainforests are the most biologically diverse terrestrial ecosystems, but much of this diversity is under threat from extensive forest loss and fragmentation (Myres, 1986). The threat lies not only in the outright loss of species but also in the potential loss of genetic diversity and fitness of remnant populations (Myres, 1986). The maintenance of genetic variation is considered essential for the long-term survival of a species since genetic diversity provides a species evolutionary potential (Frankel and Soule, 1981). A reduction in diversity through the loss of alleles reduces a population’s ability to respond
to biotic challenges such as pathogens and to changes in the abiotic environment such as
temperature and rainfall. A reduction in the number of reproductive individuals in a
population represents a decrease in the number of pollen donors and in the quantity of
pollen deposited and may be accompanied by a decrease in the abundance of pollinators
(Cunningham, 2000).

Forest fragmentation also affects seed predation, seed dispersal and herbivory levels of
remnant plant populations (Dirzo and Miranda, 1991). Edge effects associated with forest
fragmentation, decrease seed and seedling establishment of tropical trees (Bruna, 1999).
Due to their demographic and reproductive characteristics, tropical trees are particularly
vulnerable to the effects of forest fragmentation.

Many tropical trees occur at low densities, are pollinated by animals, have high out-
crossing rates, and have breeding systems that involve complex mechanisms of self-
incompatibility. The process of forest fragmentation may also lead to changes in the
 genetic structure of the remnant tree populations (Loveless and Hamrick, 1984; Hamrick
et al., 1992; Nason and Hamrick, 1997).

The number of available genotypes decreases with respect to the original tree populations
because of a loss of reproductive individuals and a higher probability of mating with
related individuals. In the long term, this is likely to cause a loss of genetic variability in
the remnant tree populations due to factors associated with inbreeding or genetic drift
(Ellstrand and Elam, 1993).

Forest loss and fragmentation also alters the composition configuration and connectivity
of the landscape (Taylor et al., 1993). These changes can affect genetic diversity in
several ways: through the direct loss of genotypes as habitants and its residents are extinguished, through process related to reduced population sizes and through the isolation of remnant populations which can modify and limit gene flow (White et al., 2002).

Such impacts can lead to genetic bottlenecks, increased random genetic drift and inbreeding depression which can ultimately result in loss of genetic variation and increased genetic differentiation between remnant populations (Young et al., 1996, Simons et al., 2000). Young et al., 1996, predicted a situation where human population in many areas will increase to the extent that many natural resources of important tree products are exhausted. This would be followed by a lag phase before farmers compensate by increasing cultivation of trees for these products.

In some tropical areas where farmers have an active tree planting culture, farmer cultivation already provides important reservoirs of tree biodiversity (Lengkeek et al., 2005a). Bringing more inter-specific and intra-specific diversity into efficient usage on-farm is a survival mechanism used by farmers (SGRP, 2000; Tapia and De la Torre, 1998) and an important approach for conducting tree domestication activities (Kindt and Lengkeek, 1999; Weber, 2001; Lengkeek et al., 2005a). At an intra-specific level the proportion and structure of variation maintained on-farm in tree species during the development of tropical agro-forestry systems is largely uncharacterized.

Genetic diversity protects a species against extinction by providing multiple phenotypes. These phenotypic differences may provide diseases resistance adaptability to changing
climate, or some other traits necessary for the species to survive in the ever changing habitats (Josh and Vrieling, 2005).

The long term viability of individual tree species in farm landscapes depends upon a wide genetic base providing the capacity to adapt to environmental fluctuations or changing farmer requirements such as a change in species use or planting niche. However, since most tree species are-out-breeding, a wide genetic base provides the ability to withstand potential inbreeding depression through future generations of farmer propagation (Boshier, 2000).

A number of authors have however, indicated that farmers and nursery managers often collect germplasm from a relatively narrow range of maternal parents during propagation (Lengkeek et al., 2005a; Lengkeek et al., 2005b), suggesting that on-farm stands may be at risk of genetic erosion. The long term survival of a species depends on the ability of populations to respond to environmental changes and is related to the amount of genetic diversity present in the species (Beardmore, 1983).

2.6 Factors affecting genetic diversity in tropical tree species

Plant mating pattern vary with reproductive biology and spatial structure of a species and these two factors influence the levels and dynamics of genetic diversity (Loveless, 1992). It has been shown that the mating system has a marked effect on the level and distribution of genetic variation of plant species (Hamrick and Murawski, 1983). The mating system has an important influence on the amount and distribution of genetic diversity within and among populations of plant species (Godt and Hamrick, 1989).
Reproductive isolation and mating of closely related individuals would increase frequency of inbreeding (Templeton et al., 1990; Young et al., 1996) hence, affect the species genetic diversity. Information on the biological characteristics of the species provides a sound basis in the conservation strategies planting programs, especially if the established stands will in future be of use in germplasm collection (Muchugi et al., 2008). However, domestication of *W. ugandensis* is taking place in many areas neighbouring the remnant populations and there is the likelihood of genetic exchange between the planted populations and the remnant populations.

Random change in allele frequency are related to population sizes; the smaller the population the more likely chance events are to change allele frequencies (Lowe et al., 2005). This random process of allele frequency change is called genetic drift. Extreme drift can lead to the extinction of alleles and the losses of polymorphism such that locus becomes fixed for a single allele. Gene flow involves movement of gametes such as pollen or zygote for example seeds, such patterns of allele movement have profound impacts on the structure of genetic diversity and without gene flow populations diverge and differentiate (Lowe et al., 2005).

2.7 Threats to genetic diversity

Humans have changed forests in size, composition and in dependence on climatic conditions. Utilisations of forest genetic resources for fuel, timber, and clearing forests for agricultural crops and domestic animals and human dwellings have altered the gene pool of many forest tree species (Chew, 2001). As human activity on natural landscape increase, habitats become fragmented and the interactions between wild and domesticated populations become complex.
Wild populations that were large and had connected gene flow become fragmented and the population size is diminished (Frankham *et al.*, 2009). Genetic impact of deforestation depends on natural population structure. If natural genetic pattern is unknown and losses through deforestation cover a range of different climatic conditions, genetic impact is certainly severe.

- Deforestation has often led to fragmented forests, leading to reproductively isolated populations (Young and Boyle, 2000). For those populations that are small, inbreeding and stochastic effects may finally extirpate local population. Clearing forest, habitat modification, unsustainable land management and impact of exotic species and genetic pollution of native forests are all potential threats to maintenance of genetic resources (Geburek and Turok, 2005).

### 2.8 Approaches of measuring genetic diversity

Genetic diversity can be measured using genetic markers. Genetic markers can be divided into three classes; morphological, biochemical and DNA based markers.

#### 2.8.1 Morphological markers

Morphological markers displaying Mendelian inheritance have been in use for long to characterise genetic variation. They are difficult to use in forest genetics for the following reasons: they are often recessive in nature therefore heterozygotes are not identifiable, they are generally mutations and often confer a deleterious phenotype to the organism, therefore decreasing its fitness, they may also exhibit epistatic effects (interaction between genes) or there expression may be affected by environmental conditions and
may confer a phenotype that is only apparent at one stage of an organism (Geburek and Turok, 2005).

### 2.8.2 Biochemical markers

Biochemical markers can either be at protein level such as isozymes or at organic chemical levels such as terpenes and they examine the products of genes (Ferguson and Grebe, 1986). Protein markers were among the first group of biochemical markers exploited for genetic diversity assessment and genetic linkage map development (Hash and Bramel-Cox, 2000).

They have been used to produce the distribution of genetic diversity in European tree species such as Norway spruce (*Picea abies*) (Morgante *et al*., 1991), beech (*Fagus sylvatica* L.) (Comps *et al*., 2001). Such variation has, however, remained restricted to a few numbers of loci. Many diversity statistics have been developed specifically with isozymes (Slatkin and Barton, 1989).

They are typically codominant and are of known genomic origin thus, suitable for estimating diversity. However, they are unordered and only low levels of polymorphism may be found limiting their applications in genetic distance estimation (Lowe *et al*., 2005) and analysis may be complicated by effects of gene expression, epistasis (which is interactions between genes) and redundancy of the genetic code (Glaubitz and Moran, 2005). Enzyme activity may be tissue and age dependent and requirement of fresh or appropriately frozen material to maintain enzyme activity may be a drawback (Dawson *et al*., 1995). This is especially true for tropical species where field work is often difficult.
2.8.3 DNA based markers

Molecular markers are neutral and detect variation in non-coding regions of the genomes which are fast evolving (Powell et al., 1996). These are therefore able to detect high levels of genetic variation such as single nucleotide mutation as well as insertions and deletions (Powell et al., 1996). Among these molecular markers, hybridization based DNA markers such as restriction fragment length polymorphism (RFLP) and PCR based DNA markers such as random amplified polymorphic DNAs (RAPDs), microsatellites and amplified fragment length polymorphisms (AFLP), have been used in genetic diversity studies.

2.8.3.1 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) refers to restriction fragments of different sizes produced by restriction enzymes from the same stretch of genomic DNA of different genotypes. Restriction fragment length polymorphisms were the first DNA markers to come into wide spread use (Botstein et al., 1980).

Among the various kinds of DNA-based markers characterized so far, restriction fragment length polymorphisms (RFLPs) were the first to provide the means to directly detect variations present at the DNA level (Diers and Osborn, 1994). RFLPs have been used to document genetic diversity in cultivated plants and their wild relative (Zschockes et al., 2000; Weising et al., 2005). Variations in fragment length between individuals or species can arise either when mutations alter restriction sites, or result in insertions/deletions between them (Burr et al., 1983).
The experimental procedure of RFLP consists of extraction and purification, of genomic DNA is digestion with restriction enzymes and separation according to the size of the DNA fragments on an agarose gel (Karp and Edwards, 1996). After electrophoresis, the DNA fragments are denatured and blotted on to a nitrocellulose or nylon membrane. Specific DNA fragments are identified by hybridization with a labeled probe (Weising et al., 2005).

All DNA fragments on the membrane which have homology to the probe are visualized. Because heterozygous are distinguishable, RFLPs are co-dominant markers. The complexity in performing RFLP analysis, coupled with the widespread use of short-lived radioisotopes, has led to its limitation for routine application in large-scale crop improvement programmes (Yamamoto et al., 1999). Although highly specific, performing RFLPs is quite tedious and expensive since it requires large amounts of pure quality DNA and expertise in handling radioactivity.

2.8.3.2 Random amplified polymorphic DNA (RAPD)

The development of polymerase chain reaction (PCR) for amplifying DNA led to a revolution in the applicability of molecular methods and a range of new technologies were developed which could overcome many of the technical limitations of RFLPs. The most common version is RAPD (Random amplified polymorphic DNA) analysis, in which the amplification products are separated on an agarose gels in the presence of ethidium bromide and visualized under ultraviolet light (Williams et al., 1990).

It is cost-effective and an easy to perform approach which gives satisfactory results even with crude DNA preparations. The enormous attractions of these arbitrary priming
techniques are; there is no requirement for DNA probes or sequence information for the design of specific primers, since the procedure involves no blotting or hybridizing steps, it is quick, simple and automatable and very small amounts of DNA (10 ng per reaction) are required.

It is absolutely critical, however, to maintain strictly constant PCR reaction in order to achieve reproducible profiles (Karp and Edwards, 1996). RAPDs have therefore been extensively used in assessing genetic relationships amongst various accessions of different plant species (Wachira et al., 1997). RAPDs have been widely employed to assess genetic variation in a range of African tree species (Bouvet et al., 2004; Jamnadass et al., 2005). However, RAPD technique has limitations associated with dominance and the potential non-homology of apparently similar bands states and its lack of specificity and reproducibility. It has been observed that RAPD profiles are highly sensitive to variations in the concentrations of template DNA (Davin-Regli et al., 1995), magnesium ions, Taq and thermal cycler used. Thus, the results obtained through RAPDs can be arbitrary.

2.8.3.3 Microsatellite DNA markers

Microsatellites are DNA sequences composed of a tandem repetition of a simple short sequence, occurring in the genome of many higher organisms (Rafalski, 1993). Provided the sequence of the DNA surrounding a microsatellite is known and suitable PCR primers can be designed, the segment of DNA incorporating the microsatellite can be amplified and its length determined by electrophoresis. Multiple allelic length variants can be identified at most microsatellite loci (Rafalski, 1993). Microsatellite markers are highly polymorphic, co-dominant and are abundant in genomes of species most.
However, the high number of alleles per locus causes some bias in diversity estimates due to increased heterozygosity levels. The high mutation rates also means that microsatellites suffer from homoplasy problems (Schlotterer et al., 1998) and may also increase within-population component of variation. Since each marker has advantages and disadvantages, the choice of a specific marker depends on project objectives, time required for analysis, availability of equipments and skilled personnel as well as financial resources (Glaubitz and Moran, 2005).

2.9 Amplified fragment length polymorphism

Amplified fragment length polymorphisms, a PCR based assay for plant DNA fingerprinting, combines the specificity of restriction analysis with PCR amplification (Zabeau and Vos, 1993; Vos et al., 1995). AFLP markers have been successfully applied to address a range of genetic question in forest tree species such as in genetic diversity analysis in \textit{Vitex fischeri} (Lengkeek et al., 2005b), genetic molecular analysis of \textit{Populus nigra} (Arenas et al., 1998), genetic variation analysis in \textit{Calycophllum spruceanum} (Russell et al., 1999) and genetic variation analysis studies in \textit{Moringa oleifera} (Muluvi, 1999).

2.9.1 Principle of the AFLP method

AFLP involves digestion of genomic DNA with restriction endonucleases, a rare cutter and a frequent cutter, followed by ligation of terminal adapter sequences to generate template DNA for amplification. Selective PCR primers are modified by adding two or three selective nucleotides (Vos et al., 1995; McGregor et al., 2000) in the selective amplification stage to allow for fragment detection in the sequencer.
Site-specific adapters are then ligated to the resulting DNA fragments to generate template DNA for PCR as shown in Figure 6. Template DNA is amplified using primers complementary to the adaptor sequences. Addition of extra nucleotides at the 3' end of the PCR primers allows the selective amplification of only those restriction fragments starting with nucleotides homologous to those of the primers (Vos et al., 1995).

Consequently, a virtually unlimited number of markers can be generated. Resolution of the resulting DNA fragments on standard sequencing gels allow for the detection of the amplified fragment length polymorphism. Previously radio-labeled primers (with Y-[\textsuperscript{33}P]) were used, however, to minimize the danger of exposure to radioactive materials fluorescent-labeled primers are now used (Palacios et al., 1999).

According to Vos et al. (1995), the reason for using two different enzymes are, that the frequent cutter generates small DNA fragments, which amplifies well and are in the optimal size range for separation on denaturing gels, the number of fragments to be amplified is reduced by using the rare cutter, since only the rare cutter fragments are amplified. This limits the number of selective nucleotides needed for selective amplification. Additionally, the use of two restriction enzymes makes it possible to label one strand of the double strand PCR products, which prevents the occurrence of 'doublets' on the gels due to unequal mobility of the two strands of the amplified fragments. Using two different restriction enzymes gives the greatest flexibility in 'tuning' the number of fragments to be amplified hence large numbers of different fingerprints can be generated by the various combinations of a low number of primers (Vos et al., 1995).
The AFLP procedure is insensitive to the template DNA concentration, although it is affected by DNA quality. The protocol is optimized such that the amplification reaction ceases when the labeled primer is exhausted (Vos et al., 1995). This ensures that fingerprints of equal intensity are produced despite variation in template concentration. However, at very high template dilutions (picogram quantities), the nucleotide sequences flanking the restriction site are no longer random for a small pool of restriction fragments and variations in the banding patterns may be observed (Blears et al., 1998).

Figure 6: Schematic flow of the AFLP analysis (Muller and Wolfenbarger, 1999).
2.9.2 Advantages of AFLP

AFLP technique has several advantages over other finger printing methods. It is capable of detecting small sequence variations using only small quantities of genomic DNA (0.05-0.5 µg). The numerous bands on a gel are analyzed simultaneously making AFLP an extremely efficient technique. For example AFLP has allowed detecting highest number of polymorphisms in a single assay than other marker types in a study of relationship among early European maize inbreeds (Lubberstedt et al., 2000). AFLP technique is valuable for studying species where there is no prior sequence information and has been widely employed in taxa identification, determination of phylogenetic relationships as well as assessment of intra specific diversity at molecular genetics level (Russell et al., 1999; Muluvi et al., 1999).

The AFLP technology is extremely robust and proficient at revealing intra population diversity estimating genetic distance between individuals and populations (Winfield et al., 1998). Thus, AFLPs provide a cost effective procedure to monitor the extent and distribution of diversity in agroforestry species (Muluvi, 1999). AFLP analysis is able to detect high levels of polymorphism and has high repeatability and speed of analysis thus, AFLP finger prints can be tailored to produce patterns of desired complexity (Karp and Edwards, 1996). AFLP can be applied to any DNA samples including human, animal, plant and microbial DNAs, giving it the potential to become a universal DNA fingerprinting method (Muller and Wolfenbarger, 1999).

2.9.3 Limitations of AFLP

The technique demands genomic DNA of high purity to ensure complete digestion by the restriction endonuclease. Incomplete restriction of DNA generates partial fragments,
predominantly of high molecular weight. Amplification of fragments that are not fully digested generates an altered banding pattern, and may be misinterpreted as false polymorphisms (Vos et al., 1995). Other studies have indicated poor reproducibility of AFLP fragments as a result of incomplete digestion of genomic DNA (Arnau et al., 2002; Goulao et al., 2001). They share many limitations with RAPDs with respect to band homologies and identities (Karp and Edwards, 1996).

AFLP markers usually detect variation in anonymous nuclear sequences and show predominantly dominant Mendelian inheritance, which limits their usefulness for population analysis (Paglia et al., 1998; Lerceteau and Sznidt, 1999; Nikaido et al., 1999).

AFLP is a dominant marker technique hence not possible to distinguish between homozygotes and heterozygotes however, genetic diversity by scoring bands as present or absent can be estimated by assuming hardy Weinberg equilibrium (Kremer et al., 2005). Densitometric software has been developed that discriminate between heterozygotes and homozygotes based on allelic density (Geburek and Turok, 2005). Fragment analysis software for automated sequencers have been developed (Genemapper version 3.7) which makes it possible to generate enough polymorphism.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental site

DNA extraction for primer testing was carried out at Kenyatta University molecular laboratory while AFLP analysis of all individuals was done at the ICRAF molecular laboratory after which fragments produced were analysed at BECA laboratory at ILRI using ABI PRISM 3730.

3.2 Experimental design

Two sites, one from natural forest and the other on-farm as shown in Table 1 the Lake Victoria region in Kenya, Uganda and Tanzania were studied. From each site between twelve and twenty two trees were sampled. The selection of these sites was done in line with the project funding, the Lake Victoria Initiative (VicRes), that aims at addressing several challenges facing communities in the Lake Victoria region, among them environmental degradation and improving of rural livelihoods (www.vicres.net).

Reference populations outside the lake region were also sampled in each country to place the results in regional context. These include; Rumuruti (Kenya), Lushoto (Tanzania) and Kibale (Uganda). Natural trees for sampling were chosen at random separated by a minimum distance of 100 m. However, in some areas this distance could not be maintained since trees had died. Strategy for sampling farm trees involved the collection from single individuals from a series of separate small farms and did not have a minimum distance requirement.
3.3 Sample collection

Three young clean leaves were picked from each tree which were then cleaned with 70% ethanol and put in snap-top bags containing silica gel to dry. After drying, the leaves were stored at -20 °C. This method allows adequate preservation for several months without affecting the yield and DNA quality of the DNA extracted (Milligan, 1992). Details of the sampled populations for this study are shown in table 1 and Figure 7.

3.4 Genomic DNA extraction

Genomic DNA extraction was carried out using CTAB method as described by (Doyle and Doyle, 1987). Approximately 1 cm² dry leaf material was placed in a sterile microfuge tube and the tube gently submerged in liquid nitrogen. A small amount of polyvinyl pyrrolidone (PVP) was added and then frozen leaf material ground to a fine powder using epperndorf grinders. Five hundred microlitres of extraction buffer (2% CTAB, 100 mM Tris-HCL (PH 7.5), 1.4 M NaCl; 20 mM EDTA (PH. 8.0), 0.05% 2-mercaptoethanol), preheated to 65 °C was added to the ground leaf material. The contents of the microcentrifuge tubes were then incubated in a water bath at 65 °C for 30 minutes.
Figure 7: Collection sites for *W. ugandensis* samples used on AFLP study.
Table 1: Details of the populations sampled.

<table>
<thead>
<tr>
<th>Country</th>
<th>Population</th>
<th>Mean annual precipitation</th>
<th>Mean annual Temperature</th>
<th>GPS reading</th>
<th>Altitude (M)</th>
<th>Population type</th>
<th>Number of samples</th>
<th>Population designation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kenya</strong></td>
<td>Cherangani</td>
<td>1200 mm</td>
<td>22 °C</td>
<td>N 01° 00' 01'</td>
<td>2189</td>
<td>On-farm</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Rumuruti</td>
<td>700 mm</td>
<td>21 °C</td>
<td>N 00° 07' 01'</td>
<td>2100</td>
<td>On-farm</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Kitale</td>
<td>1269 mm</td>
<td>20 °C</td>
<td>N 01° 00' 01'</td>
<td>2189</td>
<td>Natural</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Rumuruti</td>
<td>700 mm</td>
<td>21 °C</td>
<td>N 00° 07' 01'</td>
<td>2100</td>
<td>Natural</td>
<td>15</td>
<td>4</td>
</tr>
<tr>
<td><strong>Uganda</strong></td>
<td>Mabira</td>
<td>1400 mm</td>
<td>17 °C</td>
<td>N00° 22.798' 04.804'</td>
<td>1215</td>
<td>Natural</td>
<td>22</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Mabira</td>
<td>1400 mm</td>
<td>17 °C</td>
<td>N00° 22.798' 04.804'</td>
<td>1215</td>
<td>On-farm</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Kibale</td>
<td>1300 mm</td>
<td>24 °C</td>
<td>N00°13' 01'</td>
<td>1300</td>
<td>Natural</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td><strong>Tanzania</strong></td>
<td>Lushoto</td>
<td>1100 mm</td>
<td>18 °C</td>
<td>S 04° 35' 05'</td>
<td>1780</td>
<td>Natural</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Kagera</td>
<td>1750 mm</td>
<td>21 °C</td>
<td>S 01° 05' 07'</td>
<td>1167</td>
<td>Natural</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Lushoto</td>
<td>1100 mm</td>
<td>18 °C</td>
<td>S 04° 38' 54'</td>
<td>1950</td>
<td>On-farm</td>
<td>17</td>
<td>10</td>
</tr>
</tbody>
</table>
Seven hundred microlitres of chloroform /isoamylalcohol (24:1) was then added and the mixture placed on a rotator for 15 minutes at room temperature (RT). The mixture was then centrifuged at 14000 rpm for 5 minutes at RT and the aqueous layer (450 μl) transferred to a fresh microfuge tube. An equal volume of ice-cold isopropanol (450 μl) was then added, mixed and incubated at -20 °C overnight.

The microfuge tube contents was then centrifuged at 14000 rpm for 5 minutes at RT to pellet DNA and the pellet was then washed with 450 μl of 70% ethanol by centrifuging at 14000 rpm for 5 minutes. The excess alcohol was poured off and the microfuge tubes inverted to air dry the pellet. The pellet was then resuspended in 100 μl TE (10 mm Tris-HCL, 1 mm EDTA (PH 7.5). The isolated genomic DNA was stored at 4 °C.

**3.5 Determination of DNA quantity**

Ten microlitres of each DNA sample was mixed with 5 μl of 1 X gel loading buffer III (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol) and run in 1 % agarose gels containing ethidium bromide (0.5 μg/ml) buffered in 1 X TBE (89 mM Tris-HCL [PH 8], 89 mM Boric acid and Mm EDTA) in a horizontal electrophoresis apparatus. Standards of 50 ng and 100 ng of uncut lambda DNA (Promega, USA) was loaded and gels run at 100 V for 1.30 hr.

A gel was then viewed under UV illumination (312 nm) and photographed using an Uvitec Gel Doc camera (UVItec Ltd Cambridge, UK). DNA concentration was estimated against the standards, where the DNA concentration was low, re-extraction was done for that sample then the two were freeze dried and resuspended in core volume.
3.6 Amplified fragment length polymorphism analysis

The AFLP method was carried out following the standard procedure described by (Vos et al., 1995) and adapted in the AFLP™ Plant mapping protocol of the Applied Biosystems (ABI), USA.

3.6.1 Template preparation and adapter ligation

Before carrying out the AFLP analysis, the suitability of the restriction enzyme chosen to cut genomic DNA was first tested. This was carried out by digesting the genomic DNA with *Mse*I (frequent 4-base cutter) and *EcoRI* rare (6-base cutter) restriction enzymes separately and then in combination of both.

Three micrograms of the genomic DNA was incubated overnight at 37°C in a 10 μl reaction mix consisting of 5 U of *EcoRI*, 5 U of *MseI*, 8 μl of 5 X restriction-ligation buffer (100 mM Tris-HCL (PH 7.8) 10 mM MgCl₂, 10 mM dithiothreitol, 25 mg/ml bovine serum albumin, 0.25 μl of sterile distilled water) 5 μl each of the digested products consisting of 1 μl of 10 X dye and 4 μl of water was run on 1.5 % agarose gel to check for complete digestion with size markers (100 bp) DNA and *EcoRI/ MseI* Restricted DNA).

In the ABI AFLP™ protocol the restriction and ligation steps was carried out together. A restriction-ligation enzyme master mix was first prepared by combining 1 X T₄ DNA ligase buffer with ATP 5 μm NaCl, 5 μg/μl BSA, 1 unit of *MseI*, 5 units of *EcoRI* and 1 Weiss units of T DNA ligase and topped up with sterile triple distilled water to a total volume of 10 μl for each sample. The adaptor pairs was first heated at 95 °C for 5 minutes to denature them and the tubes allowed to cool to room temperature over a 10
minutes period for the adaptor pairs to denature completely. The restriction ligation mix was prepared as follows: 1.0 μl of 10 X ligase buffer with ATP 50μm Msel adaptor 1.0 μl EcoRI adaptor and 5μm enzyme master mix.

Genomic DNA (0.5 μg) in 5.5 μl sterile triple distilled water was then added, mixed thoroughly and spun down for 10 seconds. This reaction mixture was incubated at room temperature overnight. The restriction-ligation products were diluted by adding 189 μl of 1 X TE to obtain the appropriate concentration for subsequent PCR. The diluted products were stored at 4 °C for use within one month or at -20 °C in case of longer storage periods.

3.6.2 Pre-selective amplification

PCR amplification of the adaptor ligated restriction fragments was performed to generate large quantities of the sequences on the final PCR products. The PCR reactions was performed in a 20 μl volume consisting of 4.0 μl diluted restrictions-ligation DNA products, 5 μm AFLP pre-selective primer pairs and 15 μl AFLP core mix.

Pre-amplification was carried out at initial hold time of two minutes at 72 °C followed by 20 cycles of 20 seconds at 94 °C, 30 seconds at 56 °C and at 72 °C for two minutes and a further hold time of 30 minutes at 60 °C. To check the success of amplification reaction 10 μl of preselective amplification products were mixed with 2 μl of 10 X loading dye and run on 1.5 % agarose gel in 1X TBE buffer at 4 V/cm for 3-4 hours.

The gel was stained with ethidium bromide and viewed on a UV trans-illuminator. After successful amplification which was shown by presence of smear products from 100-1500
bp ten microlitres pre-selective amplification products was then diluted with 190 µl 1 X TE and stored at 4 °C.

3.6.3 Selective amplification reaction

Selective amplification was conducted using various combinations of two AFLP primers specific for *MseI* and *EcoRI* primer adaptors on a test panel of representative samples. These primers had three additional 3’ nucleotides. The *EcoRI* primers were fluorescent labelled. The primer combination showing the best results were then used in the final analysis.

The selective PCR was performed in 20 µl volumes consisting of 3 µl of the diluted preselective amplification products, 1 µl fluorescent labelled *EcoRI* primer, 1 µl of *MseI* primer and 15 µl of AFLP core mix. The selective PCR Amplification was programmed for an initial 2 minutes at 94 °C followed by one cycle of 94 °C for 20 second, 66 °C for 30 seconds and 72 °C for 2 minutes. This cycle was repeated 12 times with a lowering of the temperature of 1 °C per cycle. This was followed by 20 cycles of 94 °C for 20 seconds, 56 °C for 30 seconds and 72 °C for 2 minutes and a further hold time of 30 minutes at 60 °C.

A total of 20 primer combinations (ABI, USA) which were adopted from previous work by Muchugi *et al* 2008 as shown in Table 2 were used to run the trials. Six combinations that showed good amplifications and polymorphisms across all populations were selected for use on full set of individuals as shown in Table 3. The final analysis was done using four primer combinations. The AFLP analysis using four primer combinations
EcoRI+ACC/MseI+CAA, EcoRI+ACT/MseI+CAT, EcoRI+ACT/MseI+CAG and EcoRI+AGC/MseI+CAG, gave a total of 223 polymorphic markers.

Table 2: Primer combination tested for AFLP analysis.

<table>
<thead>
<tr>
<th>Primer Combination</th>
<th>CAA</th>
<th>CAC</th>
<th>Mse 1</th>
<th>CAT</th>
<th>CTA</th>
<th>CTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-AAC</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>-AAG</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>-ACA</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>-ACC</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ACG</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-ACT</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>-AGC</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>-AGG</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: Ticks represent the primer combination tested.

Table 3: Primers selected for all population analysis.

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Primer Combination</th>
<th>Primer Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E-ACT/M-CAT</td>
<td>Blue(FAM)</td>
</tr>
<tr>
<td>2</td>
<td>E-AGC/M-CAT</td>
<td>Yellow(NED)</td>
</tr>
<tr>
<td>3</td>
<td>E-ACT/M-CAG</td>
<td>Blue(FAM)</td>
</tr>
<tr>
<td>4</td>
<td>E-ACC/M-CAA</td>
<td>Yellow(NED)</td>
</tr>
<tr>
<td>5</td>
<td>E-AGG/M-CAC</td>
<td>Green(JOE)</td>
</tr>
<tr>
<td>6</td>
<td>E-AGC/M-CAG</td>
<td>Yellow(NED)</td>
</tr>
</tbody>
</table>

3.6.4 Selective amplification product resolution

The AFLP product was prepared for analysis on ABI prism 3730 DNA analyzer by making a 1:3 dilution. A loading buffer mix was prepared by adding 10 μl of gene scan 500 LIZ internal size standard ABI to 990 μl deionised formamide. Nine microlitres of the size standard mix was added to 1 μl of the diluted selective amplification products in a micro-Amp PCR plate.
The amplification products were first denatured by heating at 95 °C then cooling in ice before loading into the genetic analyzer. After loading the samples, the first step involved electro kinetic injection. The capillary and electrode was placed into the sample and voltage applied. The DNA fragments were then separated by capillary electrodes with the illumination of capillaries at the detection cell. The florescence emitted was separated by wavelength and collected on CCD camera and the signals were relayed into a computer that has data collection software.

The separation parameters include; the length of the capillary, separation medium, separation voltage, separation temperature and electro kinetic injection. From the ABI PRISM 3730, the sample data was directed to the GeneMapper™ software to analyze and display the sizing results as electrograms and tabular data.

3.7 Data analysis

The resulting fragments from the ABI Prism 3730 automated sequencer (Applied Biosystems) were described and screened for quality using Gene Mapper 3.7 software. The GeneMapper generated allele frequencies data of all individuals as products presence (1) and product absence (0) which were converted into Microsoft excel spread sheet.

3.7.1 Principal coordinate analysis (PCA)

Principal coordinate analysis (PCA) via distance matrix of AFLP was performed using GenAlEx 6.3 (Peakall and Smouse, 2009) software to provide visual representation of genetic diversity.
3.7.2 Analysis of molecular variance (AMOVA)

The Analysis of molecular variance (Excoffierer et al., 1992) based on phiPT statistic useful for dominant marker analysis was run to calculate variance components within and among populations by GenAlEx 6.3 using 99 permutations to assign significance values to estimates. Within population diversity values were calculated using Nei’s unbiased diversity statistic (Nei, 1978), averaging over individual AFLP products.

3.7.3 Nei’s unbiased diversity

Population allele frequency diversity values were calculated and analysed with Popgene program version 1.31 (Yeh et al., 1997) assuming Hardy-Weinberg equilibrium within populations and using Nei’s unbiased statistic (Nei, 1978).

\[ H = n\left(1 - \sum [p_i^2] / (n - 1)\right) \]

Where \( n \) = number of individuals analysed and \( p_i \) is the frequency of the \( i \)th allele.

Diversity values were averaged across loci.

3.7.4 Nei’s genetic distance and cluster analysis

Genetic distance (D) between population frequency data sets was generated with TFPGA 1.3 (Miller, 1997) from the equations of Nei (1972):

\[ D = \ln \left[ J_{xy} / \left( J_x J_y \right)^{1/2} \right] \]

Where, \( J_x, J_y \) and \( J_{xy} \) are the arithmetic means of individual loci identities \( \Sigma x_i^2, \Sigma y_i^2 \) and \( \Sigma x_i y_i \) over all the loci respectively. Cluster analysis based on Nei’s genetic distances was undertaken using an unweighted pair-group method with arithmetic averaging (UPGMA) (Sneath and Sokal, 1973) to generate a phenogram showing relationship among
populations. This was done by bootstrapping at 1000 times using TFPGA. This allows confidence to be assigned to the overall pattern of relationship.
CHAPTER FOUR

RESULTS

4.1 DNA extraction and AFLP

Clean and high molecular weight DNA is prerequisite for AFLP. DNA extracted based on CTAB procedure was run on 1% [w/v] agarose gel against standards and gel photo taken as shown in Figure 8.

Figure 8: Ethidium bromide-stained agarose gel (1% [w/v]) showing CTAB DNA extraction of thirty two *W. ugandensis* samples from Cherangani and Rumuruti, Kenya. The first lane is $\lambda$ DNA size markers of 100ng/ml.
Complete restriction digestion and ligation of adaptors is required for successful amplification. Products of restriction ligation run on 2% [w/v] agarose gel gave smears as shown in Figure 9.

![Figure 9: Ethidium bromide-stained agarose gel (2% [w/v]) showing Restriction ligation smears of thirty two *W. ugandensis* samples (Cherangani-on-farm and Rumuruti on-farm). M is the marker.](image)

Pre-selective amplification products were run on 2% [w/v] agarose gel which gave homogeneous DNA smears as shown in Figure 10.

![Figure 10: Ethidium bromide-stained agarose gel (2% [w/v]) showing Preselective amplification smears of thirty two *W. ugandensis* samples (Cherangani-on-farm, Rumuruti on-farm). M is 1kb ladder marker.](image)

Before resolving the selective amplification products on the ABI prism 3730 DNA analyzer, the products were run on 2% [w/v]) agarose gel to check amplification as shown in Figure 11.
Figure 11: Ethidium bromide-stained agarose gel (2% [w/v]) showing selective amplification of thirty two *W. ugandensis* samples (Cherangani-on-farm and Rumuruti on-farm) Primer combination E-ACC/M-CAA.

The GeneMapper™ software analyzed and displayed the sizing results as electrograms and tabular data as shown in Figure 12 and Table 4.

Figure 12: Automated AFLP peaks for selected *W. ugandensis* Samples for primer combination E-ACCXM-CAA.
Table 4: Binary data for automated AFLP peaks in Figure 12.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Allele no. 100</th>
<th>Allele no. 120</th>
<th>Allele no. 140</th>
<th>Allele no. 160</th>
<th>Allele no. 180</th>
<th>Allele no. 200</th>
<th>Allele no. 220</th>
<th>Allele no. 240</th>
<th>Allele no. 260</th>
<th>Allele no. 280</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

4.2 Measurement of Nei’s Unbiased Genetic Diversity

The mean Nei’s genetic diversity for all the 223 loci in the 10 populations ranged from $H = 0.1278$ (Rumuruti on-farm) to $H = 0.2892$ (Kibale) as shown in Table 5. The percentage polymorphic loci corresponded to the diversity estimate, as population with higher diversity estimate also showed higher percentage polymorphic loci.

Comparing individual on-farm populations with their proximate natural populations genetic diversity estimate showed that, Cherangani on-farm ($H = 0.2119$) was higher than the proximate Kitale natural population ($H = 0.1464$), same results were shown for Lushoto on-farm ($H = 0.2467$) and Lushoto natural ($H = 0.2210$). Mabira on-farm ($H = 0.1963$) was slightly higher than proximate Mabira natural ($H = 0.1946$) while Rumuruti natural ($H = 0.1787$) was higher than proximate Rumuruti on-farm ($H = 0.1278$).

Among all the natural population Kitale had unusually low genetic diversity estimate ($H = 0.1464$) with Kibale natural having highest ($H = 0.2892$). The level of genetic diversity as shown by Shannon’s diversity index was average for most of the populations varying from 0.1920 (Rumuruti on-farm) to 0.4000 (Kibale natural) as shown in Table 5. Based on the mean polymorphism of the loci within population, the diversity ranged from 37.22% (Rumuruti on-farm) to 88.79% (Kibale natural) as shown in Table 5.
Random sampling for analysis to check the consistence of values obtained new genetic diversity. The highest genetic diversity was Kibale with $H = 0.2539$, Shannon index = 0.3844, percentage polymorphism of 80.27% with the lowest being Rumuruti on-farm with $H = 0.1281$, Shannon index = 0.1920 and percentage polymorphic loci = 37.22).
Table 5: Mean diversity estimates ($H$) for 10 populations of *W. ugandensis* generated from 223 AFLP marker

<table>
<thead>
<tr>
<th>Country</th>
<th>Provenance</th>
<th>N</th>
<th>$H$</th>
<th>%Polymorphic loci</th>
<th>Shannon index</th>
<th>Ns</th>
<th>$H$</th>
<th>%Polymorphic loci</th>
<th>Shannon index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kenya</td>
<td>Rumuruti on-farm</td>
<td>12</td>
<td>0.1278</td>
<td>37.22</td>
<td>0.1920</td>
<td>12</td>
<td>0.1278</td>
<td>37.22</td>
<td>0.1920</td>
</tr>
<tr>
<td></td>
<td>Rumuruti natural</td>
<td>15</td>
<td>0.1787</td>
<td>59.64</td>
<td>0.2725</td>
<td>12</td>
<td>0.1666</td>
<td>53.14</td>
<td>0.2525</td>
</tr>
<tr>
<td></td>
<td>Cherangani on-farm</td>
<td>15</td>
<td>0.2119</td>
<td>69.96</td>
<td>0.3158</td>
<td>12</td>
<td>0.1979</td>
<td>66.59</td>
<td>0.3049</td>
</tr>
<tr>
<td></td>
<td>Kitale natural</td>
<td>15</td>
<td>0.1464</td>
<td>50.22</td>
<td>0.2273</td>
<td>12</td>
<td>0.1359</td>
<td>45.29</td>
<td>0.2078</td>
</tr>
<tr>
<td>Uganda</td>
<td>Mabira natural</td>
<td>22</td>
<td>0.1946</td>
<td>63.23</td>
<td>0.2973</td>
<td>12</td>
<td>0.1920</td>
<td>61.44</td>
<td>0.2924</td>
</tr>
<tr>
<td></td>
<td>Mabira on-farm</td>
<td>12</td>
<td>0.1963</td>
<td>57.85</td>
<td>0.2735</td>
<td>12</td>
<td>0.1963</td>
<td>57.85</td>
<td>0.2735</td>
</tr>
<tr>
<td></td>
<td>Kibale natural</td>
<td>20</td>
<td>0.2892</td>
<td>88.79</td>
<td>0.4000</td>
<td>12</td>
<td>0.2539</td>
<td>80.27</td>
<td>0.3844</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Lushoto natural</td>
<td>17</td>
<td>0.2210</td>
<td>63.68</td>
<td>0.3097</td>
<td>12</td>
<td>0.2138</td>
<td>58.74</td>
<td>0.3245</td>
</tr>
<tr>
<td></td>
<td>Lushoto on-farm</td>
<td>16</td>
<td>0.2431</td>
<td>74.89</td>
<td>0.3673</td>
<td>12</td>
<td>0.2424</td>
<td>73.09</td>
<td>0.3666</td>
</tr>
<tr>
<td></td>
<td>Kagera natural</td>
<td>15</td>
<td>0.2195</td>
<td>61.88</td>
<td>0.3673</td>
<td>12</td>
<td>0.1943</td>
<td>63.92</td>
<td>0.2988</td>
</tr>
</tbody>
</table>

Key: N=Total number of individuals in a population.
Ns= Resampled individuals for reanalysis.
4.3 Analysis of molecular variance (AMOVA)

Partitioning of genetic variability by analysis of molecular variance (AMOVA) based on all surveyed 10 populations shown in Table 6 revealed that most of the AFLP variation was found within population (54% $P<0.01$) while the remaining split among population (46% $P<0.01$) as in Table 6.

Table 6: Analysis of molecular variance (AMOVA) for AFLP among *W. ugandensis* sampled from Lake Victoria region in Kenya, Uganda and Tanzania.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Df</th>
<th>SS</th>
<th>MSD</th>
<th>% of total variance</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>9</td>
<td>2882.06</td>
<td>320.22</td>
<td>46</td>
<td>0.01</td>
</tr>
<tr>
<td>Within populations</td>
<td>131</td>
<td>3277.38</td>
<td>25.01</td>
<td>54</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>140</td>
<td>6159.39</td>
<td>345.24</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

Key: Analysis based on all stands. Df= degrees of freedom, SS= Sums of squares, MSD= Mean square deviations and P= Significance of variance. Significant values were based on the random permutations 99 times.

4.4 Principal coordinate analysis

Principal co-ordinate analysis gave two general groupings. One group consisted of Lushoto (natural) Lushoto (on-farm), Kagera (natural), and some individuals from Kibale (natural) population. The other group consisted of Mabira (on-farm), Mabira (natural), Rumuruti (on-farm), Rumuruti (natural), Cherangani (on-farm), Kitale (natural), and some individuals from Kibale (natural) as shown in Figure 13.
4.5 Measurement of Nei’s genetic distance and cluster analysis

Phenogram generated using Nei’s genetic distance in Table 6 showed two major clusters as shown in Figure 14. The first cluster consisted of Kibale natural, Kagera natural, Lushoto natural and Lushoto on-farm while the other comprised Cherangani on-farm, Kitale natural, Mabira natural, Mabira on-farm, Rumuruti on-farm and Rumuruti natural. However each of the two major clusters has two distinct subgroups.

The first cluster has Lushoto natural and Lushoto on-farm separated from Kagera and Kibale natural while the second has Rumuruti on-farm separating from Kitale natural, Cherangani on-farm, Mabira natural and Mabira on-farm. Most on-farm populations also clustered with the proximate natural populations. These results correlate with the Principal coordinate analysis (PCA) results in Figure 13.
The shortest genetic distance was observed between Lushoto natural and Lushoto on-farm (0.0246), while most distant population were Lushoto natural and Kitale Natural (0.7587) as shown in Table 7.
Table 7: Matrix of unbiased genetic distance according to Nei (1978) among 10 *W. ugandensis* populations of based on 223 AFLP markers.

<table>
<thead>
<tr>
<th>Population</th>
<th>Cherangani-on-farm</th>
<th>Rumuruti-on-farm</th>
<th>Kitale-Natural</th>
<th>Rumuruti-Natural</th>
<th>Mabira-Natural</th>
<th>Mabira-on-farm</th>
<th>Kibale-Natural</th>
<th>Lushoto-Natural</th>
<th>Kagera-Natural</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cherangani-on-farm</td>
<td>0.1622</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rumuruti-on-farm</td>
<td>0.0485</td>
<td>0.1472</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kitale-Natural</td>
<td>0.1498</td>
<td>0.0531</td>
<td>0.1399</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rumuruti-Natural</td>
<td>0.0612</td>
<td>0.1428</td>
<td>0.0425</td>
<td>0.1014</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mabira-Natural</td>
<td>0.0929</td>
<td>0.1813</td>
<td>0.1182</td>
<td>0.1215</td>
<td>0.0463</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mabira-on-farm</td>
<td>0.4367</td>
<td>0.5231</td>
<td>0.5342</td>
<td>0.4979</td>
<td>0.4959</td>
<td>0.4823</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kibale-Natural</td>
<td>0.6269</td>
<td>0.6721</td>
<td>0.7587</td>
<td>0.6424</td>
<td>0.7059</td>
<td>0.6651</td>
<td>0.0642</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lushoto-Natural</td>
<td>0.6042</td>
<td>0.6621</td>
<td>0.7412</td>
<td>0.6282</td>
<td>0.6866</td>
<td>0.6442</td>
<td>0.0445</td>
<td>0.0496</td>
<td></td>
</tr>
<tr>
<td>Kagera-Natural</td>
<td>0.5796</td>
<td>0.6565</td>
<td>0.7185</td>
<td>0.6221</td>
<td>0.6718</td>
<td>0.6333</td>
<td>0.0566</td>
<td>0.0246</td>
<td>0.0365</td>
</tr>
</tbody>
</table>
Figure 14: Phenogram based on Nei (1978) genetic distance for 10 populations of *Warburgia ugandensis* from Kenya, Uganda and Tanzania generated using 223 AFLP markers.
CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Results on genetic diversity revealed interesting results when comparing on-farm populations with their proximate natural populations. The genetic diversity estimate of Cherangani on-farm ($H = 0.2119$) showed higher genetic diversity than its proximate population Kitale natural ($H = 0.1446$). This may be as a result of the planting materials used for the on-farm population having been introduced from different sources. However, if the sampled trees are remnants of the natural forest, which is most likely the case scenario, this difference shows the effect of forest fragmentation.

This was once a continuous natural forest stretching all the way from Kitale but farming and human settlement must have restricted gene flow between the two populations hence the difference in their genetic diversity. This is in agreement with Epperson, (1992) who found that isolated populations evolve separately as they adapt to new ecological habitat leading to changes in allele frequencies hence the genetic differentiation.

There was no significant difference in genetic diversity of Lushoto on-farm ($H = 0.2431$) when compared to Lushoto natural ($H = 0.2210$), also Mabira natural ($H = 0.1946$) compared to Mabira on-farm ($H = 0.1963$). This could be an indication that some planting materials were sourced from wide range of maternal plants from nearby forest while others could have been from a different source, others could also be remnants of the forest trees in the now cultivated land. The close genetic diversity could also imply
that there is unrestricted gene flow between the proximate populations. The transfer of pollen by insects between different individuals may increase the possibility of sexual recombination and subsequently increase within population genetic diversity.

In the Kenyan reference populations, significance difference was observed in genetic diversity between Rumuruti on-farm \((H = 0.1278)\) and Rumuruti natural \((H = 0.1787)\) populations. This may be an indication that the planting materials were sourced from this natural population, and then a few mother plants were involved. However, during the collection, most farmers clearly stated that they never planted the trees and that the trees on-farm had regenerated from remnants of natural forest trees.

In this case the loss in genetic diversity in on-farm can be linked to selection pressure (e.g. logging for timber, charcoal and firewood of bigger trees) in the now cultivated land resulting to significant loss of alleles (Ratnam and Boyle, 2000). In addition, the issue of restricted gene flow could have contributed to the difference as the barrier between the two populations increase with increased farming and settlement. Resampling ten times by randomly picking a sample size of 12 individuals per population captured new genetic diversity which maintained the same pattern as the results analysed using varying number of sample size.

A number of authors have compared the genetic diversity of both natural populations of tropical trees and cultivated materials. Studies directly comparing natural forest and proximate farm stands of the timber tree Meru oak \((Vitex fischeri, \text{ synonym } Vitex keniensis)\) in Central Kenya using RAPDS (Lengkeek et al., 2005b) and studies employing SSRs geographically matched natural and agroforests tree stand, of sheanut
(Vitellaria paradoxa) (Kelly et al., 2004) found little difference in diversity levels between stand categories, with no evidence of genetic bottlenecks events in agroforestry (Hollingsworth et al., 2005).

In both cases however, the majority of trees tested from agroforests stand were expected to represent remnants or dispersed natural regenerants thus, providing an appropriate comparison with the present study.

Hollingsworth et al. (2005) using Inga edulis examined matched natural and planted stands at five locations in Peruvian Amazon, finding limited but significant bottlenecks in on-farm material. The differences between the Vitex fischeri and Vitellaria paradoxa and I. edulis results may be explained by the different origins of agroforestry material and the intensity of human management in each case (Hollingsworth et al., 2005).

Natural populations are expected to have high genetic diversity as shown by Kagera and Kibale populations however, Kitale showed a relatively low genetic diversity. The same population showed lowest genetic diversity among the populations studied by Muchugi et al. (2008). The low genetic diversity in Kitale population could probably be as a result of stochastic loss of alleles probably as a result of strong selective pressures due to deforestation for settlement and cutting down of big trees for timber. Low genetic diversity in Kitale could also be attributed to extensive exploitation of the tree species by local communities, changes in landscape caused by deforestation, habitat destruction altering population density, diversity and abundance of pollinator communities thereby impinging on the mating systems (Lowe et al., 2005).
Most of the on-farm populations tested remains relatively diverse; they do not appear to have experienced genetic bottlenecks which result from sampling. Therefore concerns about loss of adaptability and potential inbreeding caused by genetic (Boshier, 2000; Lengkeek et al., 2005b) do not appear serious at this time.

Plant species, especially the perennials like trees, rely on the available genetic diversity for stability and survival under the ever-changing environments. Populations with high level of genetic variation are valuable since they offer a diverse gene pool from which gene conservation and improvement programs can be made (Machua et al., 2007). The decrease in the genetic diversity limits a species ability to keep pace with the changing selection pressure and their roles in the ecological and evolutionary development of the biosphere (Machua et al., 2007; Runo et al., 2004).

Consequently, maintenance of genetic diversity is important as the diversity carries forward both ecological adaptation and microevolution. The overexploitation of W. ugandensis thus, threatens their genetic diversity and hence might limit their ecological and evolutionary development of the remaining populations. Therefore, knowledge of the levels and distribution of genetic diversity is important for designing conservation strategies for such a threatened and endangered species (Francisco-Ortega et al., 2000).

Low levels of genetic diversity are detrimental to populations when they lead to inbreeding depression but can be of special scientific interest and may indicate ongoing evolution and speciation (Machua et al., 2007; Francisco-Ortega et al., 2000) The results signify the importance of focusing on population with more variability for conservation purposes. Studies on genetic variations in Melia volkensii using RAPDs have found that
on-farm conservation strategies should be emphasized (Runo et al., 2004). These findings are in agreement with Muchugi et al (2008) study that also revealed high genetic diversity among the sampled *W. ugandensis* populations. There is considerable genetic variation depicted by the Ugandan populations which will be of importance in laying the conservation strategies. Kibale should be considered more distinct for the purposes of seed collection and management.

The mean diversity estimate indicates that considerable within genetic diversity exists in *W. ugandensis* which is in agreement with its life history traits and geographical distribution. *W. ugandensis* is a long lived species with a mixed mating system which is predominantly out-crossing (Muchugi et al., 2008) and spreads in lowland rainforest and upland dry evergreen forest of Eastern and Southern Africa. This combination is expected to result in a high level of genetic diversity (Godt and Hamrick, 1989).

The high genetic diversity observed indicates that these populations would have capacity to adapt to environmental changes. High genetic diversity observed in the populations studied is in agreement with the trend shown by species whose seeds are ingested by animals (Hamrick et al., 1992). Wild animals (monkeys and elephants) eat the *W. ugandensis* fruits and disperse the seeds. Factors such as population size, isolation and gene flow, all of which directly affect effective population size may have a major influence on levels of genetic diversity observed within a population (Godt and Hamrick, 1989).

The partitioning of genetic variation of populations of *W. ugandensis*, obtained by AFLP markers showed that within population component was higher (54%) than the among
population component (46%). This shows strong within population variation with small
differentiation among the 10 population. The high levels of variation found within
populations suggest that sampling from a few localities for either breeding or
conservation could capture a large proportion of the variation within the species (Chase et al., 1995).

High levels of genetic variation are important in safe guarding against co-evolving biotic factors such as pest and diseases (Machua et al., 2007). The results agree with the general observation that woody, perennial out-breeding species maintain most of their variations within populations (Hamrick et al., 1992). This has been previously demonstrated for woody, widely distributed, predominantly out-crossed, long lived perennial species using different markers (Russell et al., 1999).

These values are also similar to those obtained by other workers, for example a study utilizing isoenzyme in Cordia alliodora a neotropical tree species indicated that the variance within population from Central America accounted for 88% of total diversity recorded (Chase et al., 1995). Harris et al., (1994) found that the variation within populations of Pentaclethra macroba, a tree species restricted to Atlantic region within Costa Rica, accounted for 78.1% of the total variation observed within this species while Melia volkensii had 75.5% of the variation within population (Runo et al., 2004).

The phenogram and principal coordinate analysis showed the on-farm populations clustering together with their proximate natural populations implying their genetic relatedness. The high percentage of the variation (73.93%) explained by the first two
components in the PCA could be attributed to high extent of genetic variability between individuals of each population than the low variation among the populations.

When considered separately, the natural populations grouping did not necessarily follow the geographical distance trend. This also does not agree with the predicted vegetation classification. Mabira and Kagera fall in the Lake Victoria Mosaic while the Kibale and Kitale fall in the Guineo-Congolian phytochoria (White, 1983). The Kibale and Kitale samples displayed this correspondence in the study by Muchugi et al., (2008).

The clustering of geographically distant populations of Lushoto, Kibale and Kagera suggests a different migration theory from the Muchugi et al. (2008) study. A probable explanation is the effect of Lake Victoria as a gene flow barrier, especially in the case of Mabira and Kagera populations. Interestingly the genetic disjunction revealed among Kenyan populations in Muchugi et al., (2008) is also revealed here with the Rumuruti populations clustering away from the Kitale population despite their geographical closeness; in this case the Rift valley which is between the two populations could have acted as a significant barrier hence the two evolving separately. Kitale population also belongs to Guineo-Congolian vegetation block and Rumuruti falls outside this vegetation.

This study further confirmed the implied theories of African floral evolution (White, 1983), which considers western Kenya as the most eastern remnant of the Guineo-Congolian phytochoria while eastern Kenya populations fall within the Somalia-Maasai centre of endemism. Such genetic differentiation reflects the different evolutionary history of the species in the different ecological niches combined with different gene dispersal mechanisms (White, 1983).
This revelation calls for more genetic analysis in understanding the species migration and colonization from its centre of diversity which is suggested to be Uganda.

Although plant species are being rescued by *ex situ* methods, and reintroductions, the single most important way to conserve a plant species is through the protection of the habitat in which it lives (Li *et al.*, 2002). Therefore, the strategy preferred for preserving genetic variation of *W. ugandensis* in the region should be to protect its habitat. On the other hand, the seed and germplasm collections in botanical gardens should also be of practical value for the conservation of genetic variation in *W. ugandensis*, in such cases the study show sampling intensively from a few populations captures most of the genetic species genetic diversity. However, conservation of existing natural forest is still crucial and considering the high population genetic structuring revealed in the study, there is need to put this into country based conservation strategies.

5.2 Conclusion

Based on the findings of the study the following conclusions were made;

i. Despite concerns that *W. ugandensis* trees currently on-farm may be of low genetic base, results show on-farm populations have high genetic diversity comparable to their proximate natural populations.

ii. Close genetic relatedness exist between on-farm population and their proximate natural population.

iii. High genetic structuring was evident among the natural populations although most genetic variation was within populations.
5.3 Recommendations

The following recommendations were made based on findings and conclusion;

i. Materials currently on-farm can be used from seed sources and conservation of the species. However, there is need to look into the density and pollinator activity of on-farm populations as these factors influence mating and gene flow which impacts on the overall genetic diversity of germplasm collected on-farm.

ii. Individual country need to implement strict *in situ* conservation strategies, as discriminate felling and debarking of *W. ugandensis* trees was evident in the natural sites visited which are supposed to be protected forest reserves.
REFERENCES


