GENETIC DIVERSITY OF *POPULUS ILICIFOLIA* POPULATIONS IN KENYA USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) MARKERS

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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

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DEDICATION

To my mother, who kept my hopes alive.

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ABBREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
BTU	British thermal unit
CTAB	Cetyltrimethylammonium Bromide
EDTA	Ethylenediamine Tetra-acetic acid
IUCN	International Union for Conservation of Nature
PCR	Polymerase Chain Reaction
RAPD	Random Amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TBE	Tris Borate EDTA
TE	Tris Ethylenediamine Tetra-acetic acid
TFPGA	Tools for Population Genetics Analysis
UPGMA	Unweighed Pair Group Method with Arithmetic Mean
VNTRs	Variable Number of Tandem Repeats
WCMC	World Conservation Monitoring Center

ABSTRACT

Many species in the genus *Populus* and their hybrids are economically valuable, fast growing, short rotation woody trees for producing wood, fiber, and biofuels, as well as sequestering atmospheric carbon in large plantations and agroforestry settings. Industrial facilities to produce liquid transportation fuels from Poplar feedstock are currently being built in the USA. *Populus ilicifolia* locally known as Tana River Poplar is the only species in the *Populus* genus that falls within the tropics. It occurs in river valleys of Tana, Athi and Ewaso Nyiro in Kenya. *P. ilicifolia* is an endangered species threatened by massive felling by humans, floods and destruction by large herbivores. As such the species populations have become decimated to IUCN vulnerable status. Unlike its relatives in Americas, Europe and Asia, *P. ilicifolia* has received very little attention for utilization and conservation. Incorporating this species into on-going tree improvement and genomics research on temperate *Populus* species is expected to have tremendous economic and scientific benefits to Kenya. Genetic diversity studies using DNA markers have become very crucial in the conservation of tree species that are endangered. The objective of this study was to assess the genetic variation and heterozygosity of four *Populus* populations from Kenya and show the genetic relatedness between Kenyan *P. ilicifolia* and Poplar reference samples from USA by employing Amplified Fragment Length Polymorphism (AFLP) with the aim of providing important information for improvement and *in situ/ex situ* conservation of this species. Leaf samples were collected from three locations along the species natural ranges (i.e. Athi, Ewaso Nyiro and Tana Rivers), Muguga KEFRI (planted) and 10 reference samples from the USA were provided. Six AFLP primer combinations that provided clear and polymorphic bands were used for the analysis. Fragments were resolved using capillary electrophoresis (ABI 3730 DNA analyzer). A sum total 521 bands in the range of 100- 500 base pairs were produced. GenALEx 6.5 was used to reveal the partitioning of variation within and among the populations. Genetic diversity estimates were computed using TFPGA software. The percentage polymorphic loci results were lowest in Ewaso Nyiro population recording 23.80% and Athi population 65.26% which was the highest among the three natural populations. The average Heterozygosity across the populations was between 0.09 and 0.4. According to AMOVA results, there was more genetic variation partitioning within population 60% (P<0.001) than among populations 40% (P<0.001) thus there was a significant variation between populations genetically. UPGMA clustering in form of a dendogram showed two clusters in accordance with their geographical origin where Tana, Athi and Ewaso Nyiro populations clustered together as opposed to the reference samples and KEFRI population. The study showed that Athi population is more diverse than any other Kenyan population and can be used for improvement studies, domestication and conservation of *P. ilicifolia.*

CHAPTER ONE

INTRODUCTION

1.1 Background

Populus ilicifolia (Engl.) Rouleau locally known as Tana River Poplar is a riparian plant endemic to Kenya (Maundu and Tengnas, 2005), occurring along river valleys of River Tana, Athi, and Ewaso Nyiro (Oballa, 1996) in the arid and semi-arid regions. These rivers fall between latitudes 1°N to 3°S and longitudes 37°E to 41°E (Battiscombe, 1926; Dale and Greenway, 1961; Jestaedt, 1986). Thus making *P. ilicifolia* is a unique plant as it is the only member of this genus that falls south of the equator. The species is deciduous, shedding off its leaves during seed maturation period. This occurs mainly in the rainy season a phenomenon called reverse phenology. The *P. ilicifolia* is characterized by opportunistic flowering that is usually triggered by flooding of the rivers. It is also dioecious meaning that it has its two sexes on two different trees (Eckenwalder *et al*, 1996).

P. ilicifolia is widely used by communities living close to its natural habitats as firewood, fodder (from young trees), making straight poles, posts, beehives and dugout canoes since its wood is easy to work on (Battiscombe, 1926). Although its medical potential has not been scientifically evaluated, this species belongs to the same family as willows (Salicaceae) whose bark has salicylic acid an active ingredient in relieving pain and fever. Clusters of this species along the river valley improve the soil structure and fertility as well as create microclimate which ameliorates the soil (Schultz, 2004). Moreover, poplar growing in both temperate and sub-tropical regions is significant in timber production for partitioning and roofing in house construction (Isebrands, 2007). Pulp and paper products are other major resources from poplar (Isebrands, 2007). Plywood from this tree is used for packaging and veneer (Isebrands, 2007). Due to its high biomass yield within a short period, Poplar is also being used in bioenergy production in form of biofuel and bioethanol (U.S.D.O.E., 2006). The species has also been utilized for phytoremediation in wetlands (irrigation channels and sewage lagoons) to remove heavy metals and excess nitrates (Greger and Landberg, 1999).

P. ilicifolia is under threat of extinction (Odera and Kabuye, 1986) due to human activities that have led to massive felling down of this tree species in search of more land for cultivation within the valleys as well as utilizing the tree in producing timber products. Grazing herbivores feed on young *P. ilicifolia* trees which are important in natural regeneration of the species (Oballa, 1996). Moreover, flooding of rivers uproot mature *P. ilicifolia* trees while young saplings are covered by silt or carried away. Therefore, there is a need for conservation measures to be put in place so as to enlarge the *Populus* populations Kenya (Oballa, 1996).

1.2 Problem statement

Populus ilicifolia is among the endangered trees in the Kenya (Odera and Kabuye, 1986). According to International Union for Conservation of Nature (IUCN) this species has also been classified as vulnerable to extinction in the IUCN red list (WCMC, 1998). The major threats to these species survival are habitat degradation by cultivation along river valleys and within the protected areas, heavy floods and large herbivores that uproot mature and young trees along the river systems. Reduced populations translate to loss of genetic diversity of this species which in turn affects adaptation to different environments (Meyers, 2002), reduced resistance to pest and diseases as well as low productivity. Unlike its genus relatives in America, Europe and Asia, *P. ilicifolia* have received very little attention for utilization and conservation. For an efficient conservation strategy, there is need to establish the existing genetic diversity within and among the species population. The genetic study of this species using DNA markers has not yet been carried out before in Kenya. Therefore, this study was carried out at World Agroforestry Center Molecular Laboratories to guide sound conservation strategies and breeding programs of the species.

1.3 Justification

The threat level status of *P. ilicifolia* calls for concerted efforts in the species conservation. Knowledge of the existing genetic diversity within and among the species population will contribute to development of efficient conservation strategies of the species. In addition, the phylogeny analysis will reveal the genetic relatedness of the *P. ilicifolia* to its temperate relatives. This information can be employed in accelerated breeding program of the species. A lot of genome work has been carried out in the temperate species in *Populus* genus with the entire genome of *P. trichocarpa* being sequenced (Tuskan *et al.*, 2006). Borrowing on the knowledge obtained in the improvement of the *Populus* species in America, Europe and Asia can propel *P. ilicifolia* breeding strategies to greater heights. Improvement of *P. ilicifolia* for cultivation as agroforestry tree is therefore important so that quality material that can give good returns in a short time can be availed to farmers. It will also offer a bioenergy investment opportunity in managing the Kenyan River basins (Tana, Athi, and Ewaso Nyiro rivers) and other wetlands as well as a chance to contribute to the Sector Plan for Environment, Water and Sanitation under Vision 2030. By introducing many species in the genus *Populus* and their hybrids is economically valuable since they are fast growing, short rotation woody crops for producing wood, fiber, and biofuel, as well as sequestering atmospheric carbon in large plantations and agroforestry settings.

1.4 Hypotheses

i. There is no significant genetic variation within and among *P. ilicifolia* populations sampled in Athi, Tana and Ewaso Nyiro rivers in Kenya.

ii. There are low levels of heterozygosity among *P. ilicifolia* populations sampled in Athi, Tana and Ewaso Nyiro rivers Kenya.

iii. There are no significant genetic relatedness between Kenyan *P. ilicifolia* and the temperate *Populus* species from USA.

1.5 Objectives of study

1.5.1 General objective

To assess the levels of genetic variations in *P. ilicifolia* populations sampled from Athi, Tana and Ewaso Nyiro rivers using Amplified Fragment Length Polymorphism (AFLP) Markers.

1.5.2 Specific objectives

i. To assess the level of genetic variation within and among *P. ilicifolia* populations from Athi, Tana and Ewaso Nyiro rivers.

ii. To investigate the level of heterozygosity within *P. ilicifolia* populations from Athi, Tana and Ewaso Nyiro rivers.

iii. To establish the genetic relatedness between Kenyan *P. ilicifolia* and other *Populus* genus species members.

CHAPTER TWO

LITERATURE REVIEW

2.1 Taxonomic description of *Populus ilicifolia*

P. ilicifolia belongs to Order Salicales, in family Salicaceae. This family and 29 other families have recently been classified in the Malpighiales using cladistics analysis of angiosperms (Angiosperm Phylogeny Group, 2003). The *Populus* genus consists of Eastern cottonwood (*P. deltoides*), black cottonwood (*P. trichocarpa),* aspens (*P. tremuloides), P. nigra, P. alba and P. fremontii* that have a large natural distribution in the northern hemisphere except for *P. ilicifolia* which occur in the tropics of Africa (Eckenwalder, 1996). *P. ilicifolia* was first classified as a sub-species of *P. euphratica* Oliv in the section *Turanga* (Browicz, 1977). The species has generally had a nomenclature that is confused since its first scientific description by Engler in 1895. This was as a result of the species existing in different morphological forms in separate ecological zones. With time, its taxonomy was revised and *P. ilicifolia* was given a new status under the section *Tsavo* (Browicz, 1977).

2.2 Distribution of *P. ilicifolia* in Kenya

P. ilicifolia occurs in hot dry zones along river valleys of Tana, Athi, and Ewaso Nyiro Rivers (Figure 2.1), 1200 m above sea level (Jestaedt, 1986). Arid and semi-arid areas with annual rainfall of 200-800 mm are suitable for this tree species. The rains have two peaks that occur in March-May and October- December. The mean diurnal temperatures range between17-35°c (Fenner, 1979). When the roots of *P. ilicifolia* are moist, the tree can resist high air temperatures.

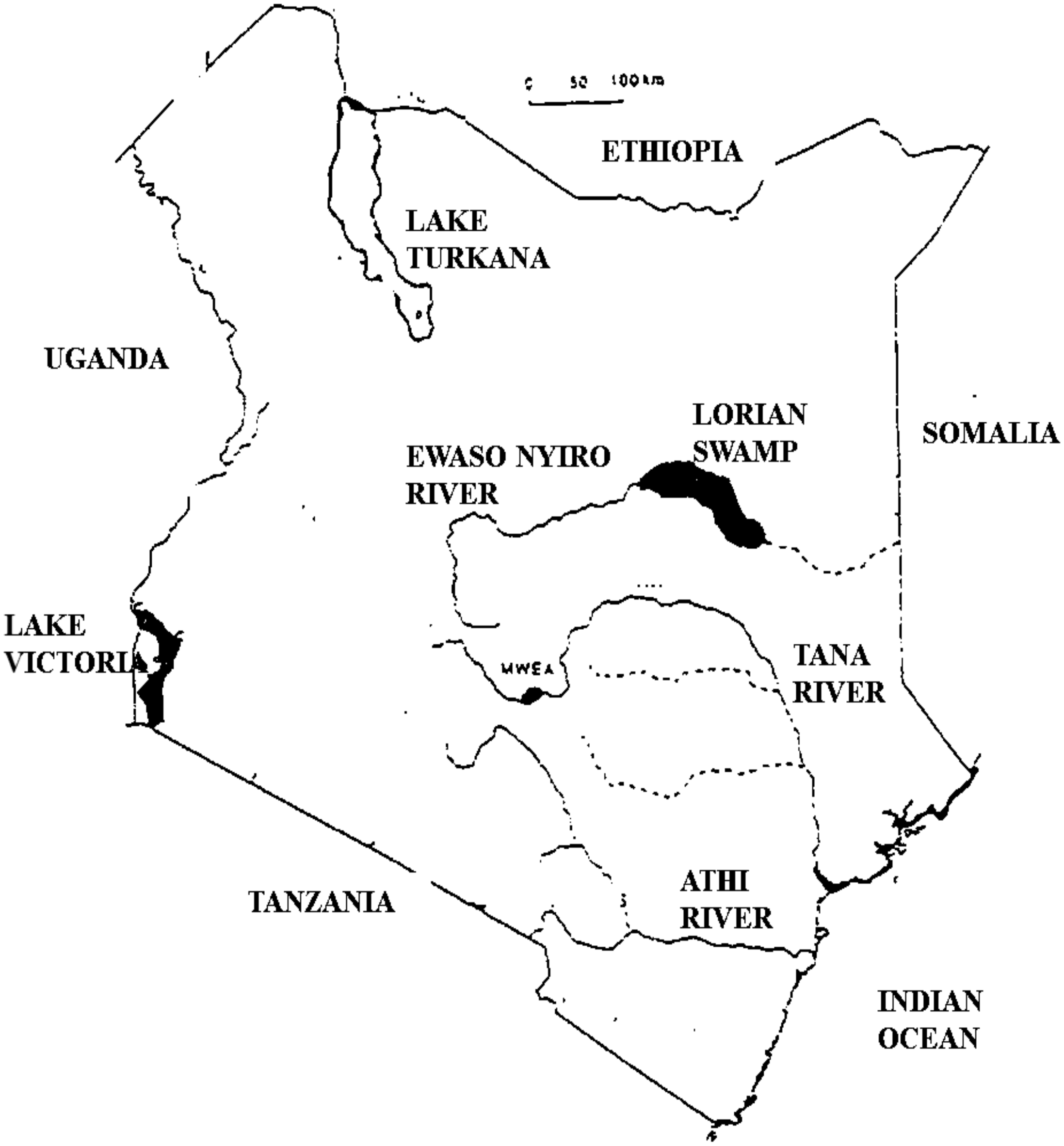


Figure 2.1: Map of Kenya showing the three rivers along which *P. ilicifolia* species is distributed

The riverine forest which is a tropical dry deciduous forest is quite different from the dry shrubland vegetation a few meters from the valleys. This sudden change in environment between these two regions results in attraction of agro-pastoral communities whose activities threaten riparian forest which include *P. ilicifolia* amongst other species. Examples of other tree species occupying the same habitat as *P. ilicifolia* include *Acacia robusta* and *Carissa edulis* (Odera and Kabuye, 1986).

2.3 Reproductive biology of *Populus ilicifolia*

P. ilicifolia reproduce both asexually and sexually which affects the genetic diversity dynamics. Adaptation to changing environments and occupation of new habitats is influenced by these two reproduction mechanisms (Eriksson, 1993). Pollen and seed dispersal of *P. ilicifolia* is important in maintaining genetic variability. In *P. ilicifolia* pollen is dispersed by wind and in 3-6 weeks seeds are formed. Seeds surrounded at the base by silky hair that help in wind and water dispersal are produced in large quantities by the female (Barat-Segretain, 1996). Asexual reproduction on the other hand, is uncommon but occurs through root sucker shoots to maintain the species population during periods of long stable environmental conditions. Thus this results to clonal structures in which one genet may be made up of several trees (Guigou, 2008). In terms of genotype and phenotype, members of the same genet are always identical. In cases of wildfires asexual reproduction is essential for re-establishment of *P. ilicifolia* in the river valleys for conservation purposes (Guigou, 2008).

P. *ilicifolia* can regenerate naturally or artificially. Naturally, this occurs through suckers, coppices and seeds. Suckers develop on roots that are exposed to the surface by water, wind or animals. Seedlings grow on newly formed alluvial soil after seed dispersal but only a few survive to maturity as a result of being washed away by floods or getting damaged by grazing animals (Oballa, 1996). On the other hand, the species can be artificially propagated by seeds, stem, layering and root cuttings but in particular seeds are most appropriate for large scale production (Oballa, 1996).

2.4 Uses of *Populus ilicifolia*

2.4.1 Wood

P. *ilicifolia* is used to provide wood products. In a plantation setting, this species can produce wood which is mostly utilized as pulpwood, veneer, lumber, furniture components, and chopsticks. Pulp is one of the major uses of indigenous *Populus,* in which it makes soft paper, paper board for packaging, tissues, roofing material, hard board ceiling tiles and insulation boards (Isebrands, 2007). Moreover, hybrid *Populus* (cross between two poplar species) can also be used for the same purposes as they have similar properties (Isebrands, 2007). Fast growth in hybrid *Populus* does not affect the fiber length in any way (DeBell *et al*., 1998). Studies which show hybrid *Populus* has higher lignin content and smaller cells (less than 2mm) compared to natural *P*. *tremuloides* have been carried out but this does not affect the quality of paper produced (Labosky, Bowersox, and Blankenhorn, 1983).

Solid wood products have also been produced from poplar. Its wood is utilized in making of lumber products such as furniture components, crates, pallets and boxes. Modern kilns with higher drying capabilities have enhanced poplar lumber opportunities that include paneling, molding, flooring, picture frames and fine furniture (Kretchman, 1999).

As new glues and drying methods are being developed, poplar wood is increasingly being used for composite wood products which are of high demand in the housing industry. Some of these products include fiberboards, oriented strand boards, particleboards, wafer boards, plywood and composite lumber such as laminated strand lumber (LSL), composite I-beams and (LVL) laminated veneer lumber (Isebrands, 2007).

2.4.2 Agroforestry

Agroforestry is a land use system in which herbaceous crops are combined in the same land unit with forest plants or tree crops so as to benefit from the ecological and economical interactions. In Ahero, Bura and Hola, *P. ilicifolia* has been found to perform well as an agroforestry tree. Rice, millet, maize and bananas have been intercropped with this species (Oballa, 1996). The microclimate provided by *P. ilicifolia* plantations favor crops beneath them while mulching from falling leaves reduce soil moisture evaporation (Jaiswal *et al*., 1993; Jain and Singh, 1999). In India, *Populus deltoides* has been utilized to a large extent in agroforestry system due to its ability to grow fast as well as accumulate enough biomass in short rotations of up to 9 years (Lodhiyal *et al.,* 1994). In this system, poplar trees are interspaced together with seasonal crops to improve productivity and generate additional income (Jaiswal *et al.,* 1993; Jain and Singh, 1999). Poplars as an agroforestry tree ensures effective recycling of nutrients since they are deep rooted and reduce soil erosion (Schultz, 2004), nutrient leaching through compacting effect of its roots. Poplar plantations also are involved in creating microclimate such as reduction of evaporation of soil surface temperature through providing shade and mulching. Fertility of soil is also improved through poplar leaf fall and decomposition (Schultz, 2004).

2.4.3 Phytoremediation

Poplars are now being used for phytoremediation in Minnesota. These are some of the emerging opportunities associated with this species. In this case, they are designed to capture and remove water, air and soil contaminants from the environment. *P. ilicifolia* is ideal for phytoremediation due to its ability to grow rapidly and has numerous and deep roots with the capacity to take up large amounts of water (Isebrands, 2007). Nevertheless, the many and deep roots provide a large surface area for proper thriving of fungi and microbes that break down contaminants. Excess fertilizers, pesticides and metals have been remediated by poplar in the past (Licht and Isebrands, 2005). Some of the technologies of phytoremediation using Poplar species include directed plantings on pesticide and petrochemical spills, vegetation filters for disposal of municipal wastewater and stream side buffers between leaking landfill and wetlands or streams (Isebrands, 2007).

2.4.4 Bioenergy production

Sustainable carbon neutral bioenergy source able to reduce the effects of rising atmospheric carbon dioxide and other greenhouse gases can be provided by woody trees in accordance to international Kyoto treaty on climate change (Cannell, 2003; Sims *et al.*, 2006). Moreover, increase in the prices of natural gas and gasoline has renewed interest of bioenergy. *P. ilicifolia* can be grown for its high biomass yield as an energy tree in production of bioethanol which involves cellulosic conversion of cellulose to ethanol (U.S.D.O.E., 2006). Poplar wood, pellets and chips can be directly burnt for energy production or even combined with coal for electricity production. This mixture is cheaper and more environmentally friendly than burning coal alone. Poplar as a bioenergy feedstock is suitable due to its high energy in energy out ratio, it's a short rotating woody tree (Stettler *et al*., 1996; Bradshaw *et al.,* 2000), rapid juvenile growth (Bergez *et al.,* 1989) and high percentage of woody content in the first growing season (Hansen, 1991). In addition, sexual compatibility among many species in the *Populus* genus, allows for generation of hybrids with a combination of characteristics not present in naturally occurring poplar trees.

Poplar wood contains about 7000-8000 British Thermal Units (BTU) per pound which is dependent on the amount of moisture content. A tone of poplar, therefore, contains about 16 million BTU's of energy (Isebrands and Parham, 1974), which is equivalent to above 4 million kilo-calories. This can be estimated to be more than 16000 cubic feet of natural gas or even 133 gallons of gasoline. As an economically viable alternative to other land uses, improvement of biomass yield in hybrid *Populus* for commercial purposes is essential (Updegraff *et al*., 2004). Basal area, tree height and sylleptic branch number are good indicators of final biomass yield in *Populus* (Pontailler *et al.*, 1997). Moreover, besides genotypic effects, water availability, solar radiation and nutrient level also influence biomass yield.

2.5 Threats towards extinction of *P. ilicifolia*

Tree destruction continues to be a menace throughout many countries. Around 60,000 to 100,000 tree species were threatened with extinction by the end of the last millennium (WCMC, 1998). IUCN Red List of tree species that are under threat shows that nearly 1,200 trees and shrubs are critically endangered, 1,700 as endangered while over 3,700 are vulnerable globally. For many forest tree species, the major threats to their existence has been as a result of, climate change, forest fires, insects pests, diseases, natural disasters and human activities such as logging, farming, settlement among others. This has been of great concern to traditional communities that practically depend on these diminishing forests and woodlands. *P. ilicifolia* has over time become an endangered species in Kenya (Odera and Kabuye, 1986) due to various factors. Cultivation along river valleys and within protected areas has completely degraded the species habitat. Moreover, heavy floods and large herbivores uproot young and mature trees along river ecosystems (Figure 2.2, 2.3 and 2.4). Lack of interest by relevant authorities in Kenya to utilize and conserve *P. ilicifolia* in its natural habitat has also contributed to the species being under threat of extinction. Riverine ecosystems have been taken over by irrigation schemes that decrease the species populations to IUCN vulnerable status (WCMC, 1998).

Figure 2.2: Poplars at Samburu Lodge (2005)   Figure 2.3: Remnants of poplars at Samburu Lodge (2012). These pictures were taken during an expedition to Samburu Game Reserve to verify status of *Populus ilicifolia* by Oballa in 2005 and 2012

Figure 2.4 : Elephant damage of Poplar. Picture was taken during an expedition to Tana River to verify status of *Populus ilicifolia* by Oballa in 2012

2.6 Genetic resource conservation

Genetic resource of a plant is the total number of gene information and characteristics present in the species. Forest biodiversity is under serious threat as a result of destruction of forest ecosystem and habitat loss (FAO, 2007). This has in turn led to genetic erosion. Therefore, tree germplasm conservation has become a priority in order to maintain genetic diversity as well as avert extinction (Rao and Ramanatha, 2008). Tree species management and conservation of genetic resources differ from those of food crops. The long generation interval, large number of genera and outbreeding reproductive nature of trees requires a more flexible and complex system for conservation of genetic resources.

There are two fundamental approaches of managing and conserving tree genetic resources; *in situ* conservation that involves maintaining genetic variation of the species at hand in the location where it was encountered that is in the wild or traditional farming systems (Brush, 2000). This approach aims at maintaining the evolutionary processes that shape the species diversity. *Ex situ* conservation, entails the maintenance of genetic resource of a plant in a region away from its original location (Iriondo *et al.*, 2008). *Ex situ* involves planting of seeds or other planting material of a species in a different niche but has similar ecological and climatic conditions as the original species habitat. *In situ* conservation has the advantage over *ex situ* in that it maintains natural genetic interactions between crops, their wild relatives and the local environment. On the contrary, *ex situ* techniques retard adaptive evolutionary development, especially in the case of pest and disease resistance (Hawkes *et al.*, 2000). Adopting either of these strategies or both is paramount in conservation of *Populus* guided by knowledge of its genetic diversity in Kenya.

In Kenya, *in situ* conservation of *Populus ilicifolia* is carried out in some national parks that include Meru, Samburu and Tsavo East (Oballa, 1996). These parks are heavily protected against any animal or human damage. On the other hand, Western part of Kenya (Yala swamp along the Yala river and Ahero irrigation scheme on Nyando river) have been set aside as an ideal regions for *ex situ* plantation development of this species due to adequate accessibility while the natural ranges in the North Eastern lowlands (Tana river, Athi river, Tsavo river) remain for *in situ* conservation.

2.7 Genetic diversity and its importance

Genetic diversity is the measure that quantifies the size of genetic variability within a population (Wright, 1920; Fisher, 1930). It is important in effective conservation of *Populus ilicifolia* in that it enables survival and evolution of the species in different environments through adaptive evolution (Meyers, 2002). Greater yield in different crops is also attributed to large diversity in a species (Smithson and Lenne, 1996). Genetic diversity is also significant in helping the species to resist pest and diseases (Zhu *et al*., 2000).

Genetic diversity in plants can be assessed using heterozygosity and proportion of polymorphic loci. Heterozygosity is the mean proportion of loci that have two different alleles at a single locus within one individual tree (Hughes *et al.*, 2008). The maximum possible heterozygosity is 0.5 accounting for a high genetic diversity. Co dominant molecular markers can be used to estimate observed heterozygosity *(Ho)*. Expected heterozygosity *(He)* can be estimated using both dominant and co dominant molecular markers making assumption on the mode of inheritance, structure and size of population. Proportion of polymorphic loci is the number of loci in genome of an individual that are polymorphic which is represented as a percentage (Hughes *et al.*, 2008). Genetic polymorphism is the presence of multiple alleles at a locus whereby at least two alleles appear at frequencies of more than 1 percent (Satoh, 2007). Alleles that occur at a frequency less than one percent in a population are called rare alleles.

Previous studies based on biochemical markers and Restriction Fragment Length Polymorphisms (RFLP) showed some basic aspects in the population genetics of *Populus species*. Levels of polymorphism (average number of alleles per locus, *A*) and heterozygosity expected under Hardy-Weinberg equilibrium (*He* also known as gene diversity) (Nei, 1973) in *Populus* are close to the mean values for long lived woody species (*A*=1.8, *He* =0.15) and are higher than those for plants in general (*A*=1.5, *He* =0.11) (Hamrick *et al*., 1992). Heterozygosity and levels of polymorphism vary significantly among *Populus* species and also within same species. Exceptionally, in *P. tremuloides*, the number of alleles per locus and genetic diversity appear to be higher than in any other *Populus spp*. This is due to its almost exclusive asexual reproduction and higher frequency of triploid aspens in the Rocky Mountains (Mock *et al.*, 2008).

2.8 Measurement of genetic diversity

Measurement of genetic diversity in tree species is carried out using different markers that include morphological markers, biochemical markers and DNA molecular markers. Of these, DNA markers are the most reliable due to their unlimited numbers and are not affected by developmental or environmental factors. Biochemical markers such as isozymes examine the product of the gene. DNA markers include Amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995), Random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), Restriction fragment length polymorphism (RFLP) (Botstein *et al.,* 1980), Single nucleotide polymorphism (SNP) (Jordan and Humphries, 1994) and Variable number of tandem repeats (VNTR) (Nakamura *et al.,* 1987). For this study AFLPs were used to study the genetic diversity of *Populus ilicifolia.* This is due to their nature as multiloci marker that can simultaneously screen many DNA regions distributed throughout the genome. AFLPs also generate many informative bands within a single reaction. Moreover, they are highly reproducible and reliable in revealing diversity in tree species (Kumar *et al.,* 2009)

2.8.1 Morphological markers

These are the visible traits on a tree or plant species. Just a few Mendelian morphological traits have been discovered in tree species making it difficult to use such markers in forest diversity studies. Many of the identified traits are mutations observed in seedlings such as albino needles, dwarfing and other aberrations (Franklin, 1970; Sorensen, 1973). These markers have limited use because morphological mutants occur rarely.

2.8.2 Isozymes

Isozymes have been used for over 60 years to determine phylogenetic relationships, estimate genetic variability, study population genetics and characterization of plant genetic resources for management purposes (Bretting and Widrlechner, 1995). Isozymes are different forms of an enzyme but with the same catalytic function. They are as a result of amino acid alterations which cause net charge changes or conformation (spatial structural) changes which results to a shift in their electrophoretic morbidity. Addition of a specific enzyme stain can reveal isozyme profile of individual samples (Soltis and Soltis, 1989). Isozymes are advantageous since no DNA extraction, primers, probes or sequence information is required. However, they have low level of polymorphism, can be affected by environmental factors and are limited in number. Despite these major short comings, isozymes have been used in outcrossing rate measurements of crops and to assess their population divergence (Kumar *et al*., 2009).

2.8.3 Restriction fragment length polymorphism (RFLP)

These were the first DNA markers to be used Botstein *et al.*, 1980). RFLP technique is a hybridization based molecular marker which was first used for plant genome analysis in 1986 (Helentjaris *et al.,* 1985; Weber and Helentjaris, 1989). Polymorphisms in plant genome are identified by restriction enzymes that reveal different patterns between DNA fragments based on their sizes. Insertions, deletions, translocation, mutations and inversions may cause changes in the DNA sequences at sites of restrictions by enzymes such as *EcoR1*. Such effects may result to no cutting at the appropriate nucleotide sequence thus a longer DNA fragment will be formed. On the contrary these changes may result in new recognition sites for the different restriction enzymes thus giving shorter DNA fragments (Botstein *et al.,* 1980).

RFLPs have the advantage of showing co-dominant alleles with high genomic abundance. They are relatively polymorphic and highly reproducible. They can detect coupling stage of molecules of DNA as fragments of homologous chromosomes are detected. The demerits of this technique however, lie in the large amounts of pure and high molecular weight DNA (1-10µg) required for the digest and southern blotting. It also requires radioactive isotopes which are hazardous and expensive in the analysis process (Kumar *et al.,* 2009). RFLPs have been widely used in mapping studies since they are abundant, randomly distributed throughout the genome and a large number of restriction enzymes exist. RFLPs have been used to reveal genetic diversity in cultivated plants and their wild relatives (Weising *et al.,* 2005). They have also been used to show the genetic differentiation in some *Populus* species that is *P. euphratica*, and *P. deltoides* (Vanden *et al.,* 2006; Slavov *et al.,* 2009).

2.8.4 Random amplified polymorphic DNA (RAPD)

RAPD is a PCR based technology in which random DNA sequences are amplified (Williams *et al.,* 1990). It is a dominant marker that uses single short primers (8–10 base pairs) under low temperature conditions which facilitates binding at many sites spread throughout the plant genome. Amplicons generated from the DNA of different populations are then separated on agarose gel. This technique has the advantage in that no prior knowledge of the DNA sequence of the species being studied is required same as for AFLP. The method is also cheap in that the primer used has a random chance of generating a PCR product (amplicons). Despite these advantages it is highly ineffective as the data it generates is limited by poor repeatability of results preventing accurate assays of results on DNA concentration (Black, 1993). RAPD has been used to analyze the genetic diversity of *Prunus Africana* which is a threatened African species (Muchugi *et al.,* 2006). *Vitex fischeri* which is a hard wood tree of importance in central Kenya has been studied genetically using RAPD (Lengkeek, 2006). Recently, RAPDs have been used to reveal variation in *Commiphora wightii*, a medicinal tree (Suthar *et al.,* 2008).

2.8.5 Variable number of tandem repeats (VNTRs)

There are highly variable regions scattered all over in the genome of a plant species which contain DNA sequence known as variable Number of Tandem Repeat (Nakamura *et al.,* 1987). Multiple copies of base pair sequence arranged in head tail fashion together are known as tandem repeats. When the repeating base pair unit is less than four the VNTR is called microsatellite but when more than four units are repeating they are called minisatellite (Armour *et al.,* 1999). Microsatellites (Litt and Luty, 1989) are also known as simple sequence repeats (SSRs); Tautz *et al.,* 1986) or simple sequence length polymorphisms (SSLPs); McDonald and Potts, 1997). Over representation of different forms of simple repetitive DNA sequence motifs gives rise to microsatellites (Tautz *et al.,* 1986). Slipped strand mispairing has been established as the mechanism of mutation that results to microsatellites (Levinson and Gutman, 1987). The differences in microsatellite alleles arise from the variable number of repeat units within its structure. If nucleotide sequences flanking the microsatellite repeats are known, then specific primers (20-25bp) can be designed to amplify the microsatellite region (Kumar *et al.,* 2009).

Unlike RAPD, PCR amplification of SSRs is carried out in presence of both reverse and forward primers which anneal at 3´ and 5´ end of the DNA strand template respectively.

SSRs have been used in the study of interspecific relationships of Sesbania (Kumar *et al.*, 2010). In Bukina faso these markers have been used in the study of genetic polymorphism of *Adansonia digitata* species (Larsen *et al.*, 2009) and in Malawi in the study of genetic differentiation and diversity of *Adansonia digitata L* (Baobab) (Munthali, 2012). SSRs are advantageous in that they are highly polymorphic even with closely related species, reproducible and are co-dominant markers. On the down side, SSR markers require prior knowledge of DNA sequence of the species to be studied, require high development cost and effort to obtain working primers for a given study (Semagn *et al.*, 2006).

2.8.6 Amplified fragment length polymorphism (AFLP)

AFLP markers combine both the RFLP and PCR-based technologies in which it ligates primer recognition sites to the restricted DNA (Lynch and Walsh, 1998). AFLP analysis involves first, genomic DNA restriction digestion using both rare cutter (*EcoRI*) and frequent cutter (*MseI*) endonucleases. Oligonucleotide adaptors that are double stranded are then designed such that the first restriction site is not restored soon after ligation. These adaptors are ligated on both sides of the DNA fragments so as to create DNA sequence for amplification that is known (Blears *et al.,* 1998; Mueller and Wolfenbarger, 1999; Ridout and Donini, 1999). PCR amplification occurs when the designed primers have annealed to those DNA fragments that possess the adaptor sequence (Vos *et al.*, 1995). Two subsequent PCR amplicons are subjected to an aliquot under strict conditions with primer that are complimentary to the adaptors containing 3 prime selective nucleotides (Vos *et al.,* 1995).

AFLPs were first used in trees to genetically map a disease resistance in *Populus species* (Cervera *et al.,* 1996). Genetic linkage maps have been constructed for *Eucalyptus globulus* based on AFLPs (Marques *et al.,* 1998). Since then AFLPs have been used widely in population genetics studies. AFLPs have successfully been used to assess the genetic diversity of tree species such as *Adansonia digitata* (Assogbadjo *et al*, 2006), *Allanblackia* (Russell *et al.,* 2009), Shorea species (Dipterocarpaceae) in Indonesia (Ping *et al*., 2009), *Astragalus ampullarioides* (Breinholt *et al.,* 2009) and Kenyan *Warburgia ugandensis* (Muchugi *et al*., 2012).

2.8.6.1 Merits and demerits of AFLP

AFLPs have high genomic abundance, considerable reproducibility and they generate many informative bands per reaction as compared to RFLPs, RAPDs and SSRs (Kumar *et al*., 2009). They also do not require sequence data for primer construction as well as can easily be analyzed by automated sequencers. The fact that AFLPs reveal a high number of polymorphic markers in a single reaction has made this method the main technology in tree species genetic studies (Vos *et al.,* 1995). AFLPs have many applications in genetic identity, parentage and phylogenetic studies in closely related species due to their highly informative fingerprinting profiles associated with them.

The major disadvantage of AFLP markers is that they are dominant markers thus they cannot differentiate dominant homozygotes from heterozygotes (Kumar *et al*., 2009). The technology requires high molecular DNA which is highly purified and free from contaminants that may interfere with restriction process. Complete digestion is paramount in AFLP technique as incompletely digested fragments may be recorded as different bands which may lead to misinterpretation of false polymorphism (Vos *et al.*, 1995). Moreover, this technique is labor intensive as it entails additional digestion and ligation steps which are not found when dealing with SSRs.

To compensate for the dominant nature of AFLP markers, Hardy Weinberg equilibrium was assumed. From the hardy Weinberg equation $p^2 + 2pq + q^2 = 1$, we were able to estimate the genotype frequencies that is p^2- frequency for dominant homozygotes, 2pq frequency for heterozygotes and q^2 frequency of recessive homozygotes. The allele frequencies were estimated based on the square root of the recessive genotype (Miller, 1997). For dominant markers, presence of a band is assumed to represent a single locus. Dominant markers produce binary data in form of 1s (presence) and 0s (absence) where presence of a band is assumed to represent the dominant genotype while 0 or blank represents the homozygous recessive genotype (Miller, 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant material

About 10 leaf samples were collected randomly per tree in the available tree clusters along rivers Athi, Tana and Ewaso Nyiro in Kenya. The 10 leaves per tree were pooled together and regarded as one sample. Where the tree density was high, the trees were sampled maintaining a distance of about 100m to avoid sampling of genetically related individuals. Four samples of *Populus* planted at Muguga KEFRI were also sampled, Table 3.1. Ten reference samples from the genus *Populus* were provided by Virginia Polytechnic Institute and State University (USA) that is *P. tremuloides, P. deltoides, P. fremontii, P. tremula and P. balsamifera.* A large number of sample collections were not possible for this study due to inaccessibility of the species in its endemic habitat as well as its limited distribution. The molecular work was carried out at World Agroforestry Center Molecular Laboratories in Nairobi, Kenya. The leaves collected were placed in a snap top bag containing silica gel. Once dry, the leaves were stored at -20°C.

Table 3.1: Details of leaf samples collected and DNA samples from USA

Lab Code	River	Tree location	Tree number
1.	Athi	Tsavo East Sala Gate	T5
2.		Tsavo East Sala Gate	T4
3.		Tsavo East Sala Gate	T7
4.		Tsavo East Sala Gate	T2
5.		Tsavo East Sala Gate	T1
6.		Tsavo East-Epiya Chipeya camp	T3
7.		Tsavo East – Epiya Chipeya Camp	T1
8.		Tsavo-Athi junction near Lugard’s Falls	T3
9.		Tsavo Athi junction near Lugard’s Falls	T4
10.		Tsavo Athi junction near Lugard’s Falls	T2
11.	Uwaso Ngiro	Samburu Lodge	T1
12.		Samburu Lodge	T2
13.		Samburu Lodge	T3
14.		Samburu Lodge	T4
15.		Samburu Lodge	T5
16.		Samburu Lodge	T6
17.	Tana	Rajowero River Trib	T1
18.		Rajowero River Trib	T2
19.		Rajowero River Trib	T3
20.		Tana river	T1
21.		Tana river	T3
22.		Tana river	T4
23.		Tana river	T6
24.		Tana river	T8
25.		Tana river	T9

26.		Tana river	T10
27.	KEFRI (Planted)	KEFRI compound	T1
28.		KEFRI compound	T2
29.		KEFRI compound	T3
30.		KEFRI Gate	T4
31.	USA reference samples	Ready to use extracted DNA	0.1tremuloides edmonton
32.		Ready to use extracted DNA	229M tremula
33.		Ready to use extracted DNA	deltoids ufo214
34.		Ready to use extracted DNA	edmonton 13
35.		Ready to use extracted DNA	fremontii RmT4C1
36.		Ready to use extracted DNA	deltoids ufo190
37.		Ready to use extracted DNA	tremula327.9 M
38.		Ready to use extracted DNA	fremontii RmT4C2
39.		Ready to use extracted DNA	balsamifera GAL2
40.		Ready to use extracted DNA	balsamifera GAL13

3.2 DNA extraction and purification

Genomic DNA was extracted from approximately 50mg of each of the dried leaf samples using the CTAB method according to Doyle and Doyle (1987). The leaf tissues were washed in 70% ethanol and rinsed with distilled water. The sterilized leaves were ground into powder by the use of a tissuelyser (QIAGEN) and the DNA was extracted with 550 µl of CTAB extraction buffer (2% CTAB), 100 mM Tris-HCL (PH 7.5), 1.4 M NaCI, 20 mM EDTA (PH 8.0) 0.2% mercaptoethanol and 0.8g of polyvinyl pyrrolidone (PVP) preheated at 65°C. The grinded tissue mixed with the buffer was then incubated for 30 minutes at 65°C with intermittent shaking. 550 µl of chloroform-isoamyalcohol (24:1) was then added to the tubes with the mixture in a fume hood and shaken gently.

The mixture was then centrifuged at 14000 rpm for 10 minutes at room temperature. The supernatant (top layer) containing DNA was pipetted out into a 1.5ml tube to which DNA was precipitated with 0.7 volume of ice cold isopropanol which was allowed to take place overnight. The samples were centrifuged for 10 minutes at 13000 rpm at 4°C so as to obtain the nucleic acid pellet. The solution was then poured out carefully so as not to dislodge the pellet. 500 µl of 70% ethanol was added to the pellet making sure the pellet was suspended fully. Centrifugation was then done for 10 minutes at 13000 rpm at 4°C and the ethanol poured out. This cleaning step was repeated again including centrifugation and ethanol carefully poured out so as not to dislodge the pellet. The samples were then left to air dry for one hour on a tissue in such a way that the tubes were left open and in an inverted position. After this, each of the samples was suspended in a cocktail of TE buffer with RNase (A and T_1) and incubated at 37°C for 30 minutes. The dissolved DNA was then stored at 4°C.

3.3 Analysis of DNA quantity and quality

DNA quantity and purity for each sample solution was evaluated using a spectrophotometer which employed the Thermo Scientific Nano drop 2000 system (Wilmington, USA). The absorbance ratio at 260 and 280 ($A_{260/280}$) was used to assess the purity of the DNA. In essence, $A_{260/280}$ of 1.8-2.0 represents pure DNA.

This was followed by agarose gel (0.8%) in 100ml TBE electrophoresis. The gel was first pre-stained using gel red fluorescent dye. 2 µl of each sample mixed with 1µl of loading dye (bromophenol blue, xylene cyanol and sucrose) was loaded into each well and electrophoresis carried out at 100V power for 1 hour. Band intensities from the UV electrograph were compared with that of a 100 ng/µl molecular weight marker (Lambda DNA, New England Biolabs, Ipswich, USA). Gels were viewed under UV illumination and photographed using Uvitec gel documentation system (Cambridge, UK).

3.4 AFLP analysis

Analysis of amplified fragment length polymorphism (AFLP) was done according to Vos *et al.*, (1995) with modification as in the AFLP^{TM} Plant Mapping Protocol of Applied Bio systems.

3.4.1 DNA template preparation

Before AFLP analysis was carried out, the ability of the restriction enzymes to cleave DNA was verified. The genomic DNA from a few samples were digested for 3 hours at 37°C temperature using frequent cutter *MseI* and rare cutter *EcoRI* from New England Biolabs. This was carried out using one restriction enzyme at a time and then a combination of both afterwards. 1 µl of 20 U/µl EcoRI, 1 µl of 4 U/µl MseI, 2 µl of 5X T4 DNA ligase buffer (10 mM Tris-HCL (PH 7.8), 10 mM $MgCI_2$, 10 mM dithiothreitol, 1mM ATP, 25µg/ml bovine serum albumin) and 11 µl of sterile triple distilled water. 10 µl of the digested DNA was mixed with 2 µl of loading dye and was run on 1.5% gel red stained agarose gel to check for complete digestion against 100 bp DNA ladder.

Restriction and ligation were then carried out together in which restriction ligation master mix was prepared. The master mix consisted of 0.2 µl 5X T4 DNA ligase buffer with ATP, 0.1 µl 1mg/ml NaCI, 0.05 µl 1mg/ml BSA, 1unit of *MseI*, 5 units of *EcoRI* and 1 unit of T4 DNA ligase and sterile water for topping up. The adaptor pairs (Table 3.2) were first annealed by heating at 95°C for 5 minutes and then allowed to cool down for 10 minutes. The restriction ligation reaction was then prepared by mixing 2.0 µl 5X T4 DNA ligase buffer, 0.1 µl 0.5M NaCI, 0.5 µl 1mg/ml BSA, 1 µl *MseI* adaptor, 1 µl of *EcoRI* adaptor and 1 µl enzyme mater mix. About 5.5 µl of genomic DNA was then added and mixed gently and the mixture was incubated at 37°C for 3 hours. The restricted ligated products were then diluted 20 fold using sterile water to obtain the precise template concentration for subsequent PCR. The diluted products were then store at 4°C.

Table 3.2: *EcoRI* and *MseI* adaptors used for AFLP analysis

Name	**Sequence**
EcoRI adaptor	5'CTCGTAGACTGCGTACC 3' 3'CTGACGCATGGTTAA 5'
MseI adaptor	5'GACGATGAGTCCTGAG 3' 3'TACTCAGGACTCAT 5'

3.4.2 Pre-selective amplification

To minimize artifacts, two amplification processes are involved that is preselective and selective amplification. A primer extension of one, two or three bases minimizes the number of amplified fragments by factors of 4, 16 and 64, respectively. This results in an adequate number of products in form of bands, not very many bands to result to smears or high levels of band comigration during electrophoresis, but enough to provide adequate information (Mueller and Wolfenbarger, 1999).

PCR pre-selective amplification was performed on the samples in which the threshold of selection was low since only a single nucleotide was used. Thus, a large number of fragments that form the final templates for selective amplification were generated. The PCR amplification reaction were carried out with a Gene Amp 9700 PCR system (PE Applied Bio systems, foster city, CA) in a 20 µl reaction containing 4 µl of the restriction / ligated DNA and 16µl of a mixture containing 1µl of *EcoRI* and *MseI* AFLP pre-selective primers (Table 3.3) and 15µl of AFLP core mix (Applied Biosystems). The PCR amplification protocol consisted of 94°C for 2 min followed by 20 cycles of the following profile; 94°C for 30 sec, 56°C for 30 sec and 72°C for 2 min with a final hold of 60°C for 30 min.

The success of the preselective amplification was confirmed by running 10 µl of the preselective product mixed with 2 µl of 6X loading dye on 2% agarose gel prestained with gel red fluorescent dye in 1X TBE buffer for 2 hours at 100 v. The gel was then viewed on a UV trans-illuminator. Successful amplification was shown by smears in the range of 100-1500bp. The amplified products were then diluted 20 fold using triple distilled sterile water.

Table 3.3: Preselective amplification primers

Primer name	**Sequence**
EcoRI +1-A	5’GACTGCGTAACCAATTC+A-3’
MseI+1-C	5’GATGAGTCCTGAGTAA+C-3’

3.4.3 Selective amplification

For selective amplification, AFLP primers sets were tested and the 6 most suitable *EcoRI* and *MseI* sets were used. The reaction mixture for each selective amplification contained 15µl AFLP core mix, 1 µl AFLP *EcoRI* dye labeled primer with 3 additional user selected nucleotides and 1µl AFLP *MseI* unlabeled primer with 3 user selected nucleotides. The concentration of the primers was 2µM. The labels used to label *EcoRI* primers were FAM, NED and JOE. The PCR profile consisted of an initial heat up at 94°C for 2 min then one cycle of 94°C for 20 sec, 66°C for 30 sec, and 72°C for 2 min, followed by 10 cycles of each with 1°C lowering of annealing temperature and finally 25 cycles of 94°C for 20 sec, 56°C for 30 sec and 72°C for 2 min with a final hold of 60°C for 30 minutes.

A sum total of 16 primer set combination adapted from previous work by Muchugi *et al*. (2008) were used for trials (Table 3.4). Only 6 combination sets showed polymorphism across all the 5 populations (Table 3.5).

Table 3.4: Primer set combinations tested for AFLP analysis

	MseI	CTT	CTC	CAT	CTT	CAA	CAC	CTA
EcoRI								
ACT				X	X	X		
ACA			X	X			X	
ACC				X				
AAG					X			X
ACG		X		X				
AGC							X	
AGG				X	X			
AAC		X			X			

Table 3.5: Six primer sets that showed polymorphism and used in AFLP analysis

Name	Sequence
EcoRI +3-ACA MseI+3-CTC	5’GACTGCGTAACCAATTC+ACA 3’ 5’GATGAGTCCTGAGTAA+ CTC 3’
EcoRI +3-ACT MseI+3-CAA	5’GACTGCGTAACCAATTC+ACT 3’ 5’GATGAGTCCTGAGTAA+CAA 3’
EcoRI +3-ACT MseI+3-CAT	5’GACTGCGTAACCAATTC+ACT 3’ 5’GATGAGTCCTGAGTAA+CAT 3’
EcoRI +3-AAG MseI+3-CTT	5’GACTGCGTAACCAATTC+AAG 3’ 5’GATGAGTCCTGAGTAA+CTT 3’
EcoRI +3-AGC MseI+3-CAC	5’GACTGCGTAACCAATTC+AGC 3’ 5’GATGAGTCCTGAGTAA+CAC 3’
EcoRI +3-AGG MseI+3-CAT	5’GACTGCGTAACCAATTC+AGG 3’ 5’GATGAGTCCTGAGTAA+CAT 3’

3.5 Product resolution

The selective amplicons were then resolved on 2% gel red stained Agarose gel and buffered in 1× TBE to check for successful amplification. The gels were viewed on a trans-illuminator and photographed. Following successful amplification viewed on the gel, the products were resolved using automated capillary array system (ABI prism 3730 DNA analyser). In preparation for this resolution, 8 µl of a loading buffer mixture containing 12 µl of gene scan 500 LIZ internal size standard and 800 µl deionized formamide was added to 1.5 µl of the selective amplification products samples in a micro-Amp 96 PCR plate.

Following the addition of this LIZ standard mixture, the AFLP products were denatured by heating at 95°C for 5 minutes and then cooled in ice before loading in the capillary electrophoresis system. The samples were loaded and the capillary and electrode were placed on the samples and electric current applied. The DNA fragments together with the LIZ size standards were separated as they moved through the polymer filled capillary electrodes with illumination of capillaries at the detection cell. The ABI Genetic Analyzer recorded the fluorescence intensity as a function of time and wavelength from the regions on a CCD camera that correspond to different detection wavelength ranges. These signals were transmitted to a computer that has data collection Software version 4.1. The data was then further analyzed using GeneMapper software to display the fragment sizes as electrophoregrams and binary data.

3.6 Data analysis

From the automated capillary electrophoresis, the fragment data was scored by GeneMapper^{TM} 3.7 software which generated binary data coded by presence (1) or absence (0) and AFLP electrophoregrams of DNA fragments produced by the most suitable primer sets.

To determine genetic diversity of the populations, the unbiased average Heterozygosity and the percentage of polymorphic loci at 99% confidence interval were computed using (TFPGA) Tools For Population Genetics Analysis software (Miller, 1997). The allele frequencies were estimated based on the square root of the null genotype and Hardy Weinberg equilibrium assumed.

GenALEx 6.5 software (Peakall and smouse, 2009) was used to show analysis of molecular variance (Excoffier *et al*., 1992) by revealing the partitioning of the variation between and among the populations. The percentage variation shown by the population partitioning showed the degree of differentiation across the populations.

Principle coordinate analysis is a method that visualizes similar and dissimilar data. It assigns similar or dissimilar matrix a location in a three dimensional space. This was conducted using GenALEx 6.5 software (Peakall and smouse, 2009) to show visually the relationship between the sample populations using genetic distances. It was chosen to complement the UPGMA cluster analysis.

The genetic relationship among populations was shown based on Nei's genetic distances (Nei, 1973) produced by TFPGA 1.3 software (Miller, 1997). Based on these genetic distances, cluster analysis was conducted by using unweighed pair group method with arithmetic mean, UPGMA (Sneath and Sokal, 1973). This was represented in a dendogram showing the relationship among and between the populations. A dendogram to show relationship between individuals in Kenyan *P. ilicifolia* populations was also drawn. To verify this cluster analysis bootstrapping was carried 1000 times using TFPGA.

CHAPTER FOUR

RESULTS

4.1 Analysis of quality and quantity of extracted DNA

Clean, high molecular weight DNA is an essential requirement for successful AFLP technique. Such Poplar DNA extracted using CTAB method was run on 0.8% agarose gel which had been pre-stained using gel red dye. Lambda DNA (100ng/µl) was also used as a standard and photo taken (Figure 4.1). The DNA yield was between 100 ng/µl to 1000 ng/µl. The numbers on the gel (Figure 4.1) represent the lab codes assigned to each sample as indicated on Table 3.1. Sample code numbers 1 to 10 represent Athi River population, code numbers 11 to 16 represents Ewaso Nyiro River population, code numbers 17 to 26 represents Tana River population while sample code numbers 27 to 30 represent KEFRI samples. Sample code number 7 and 8 from Athi River population had the lowest concentration of DNA of 283 ng/µl and 376 ng/µl respectively. KEFRI samples, code number 28 and 30 had the highest yield of DNA of 1673 ng/µl and 1576 ng/µl respectively.

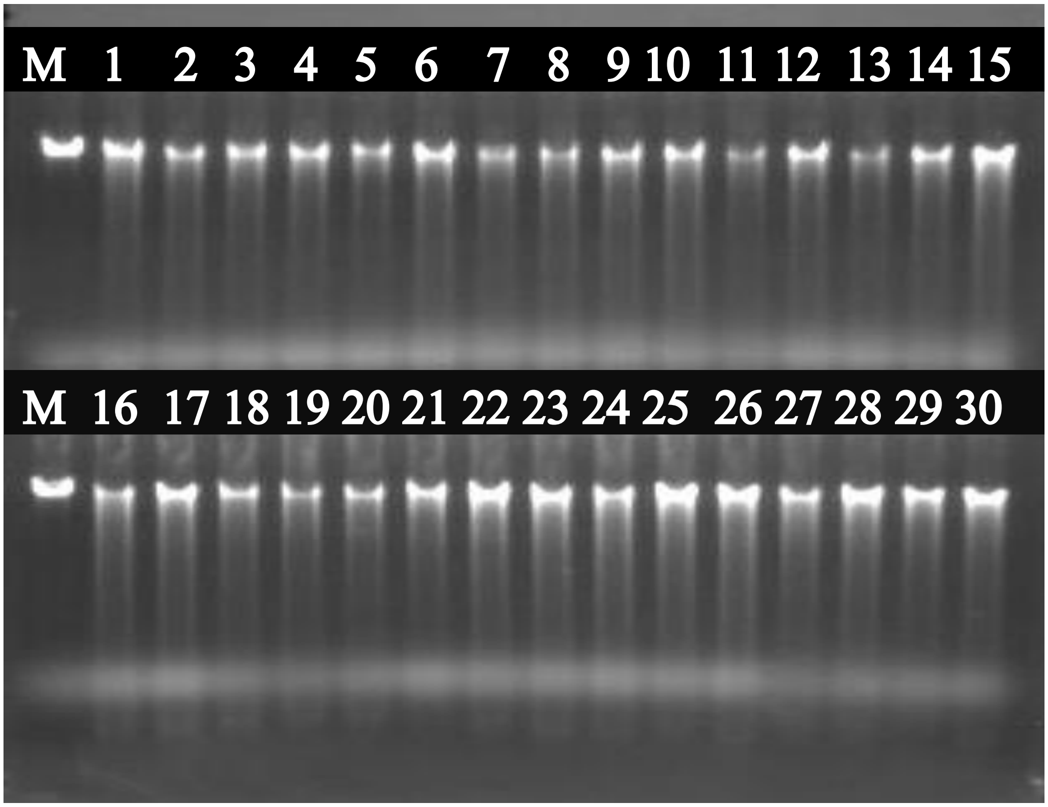
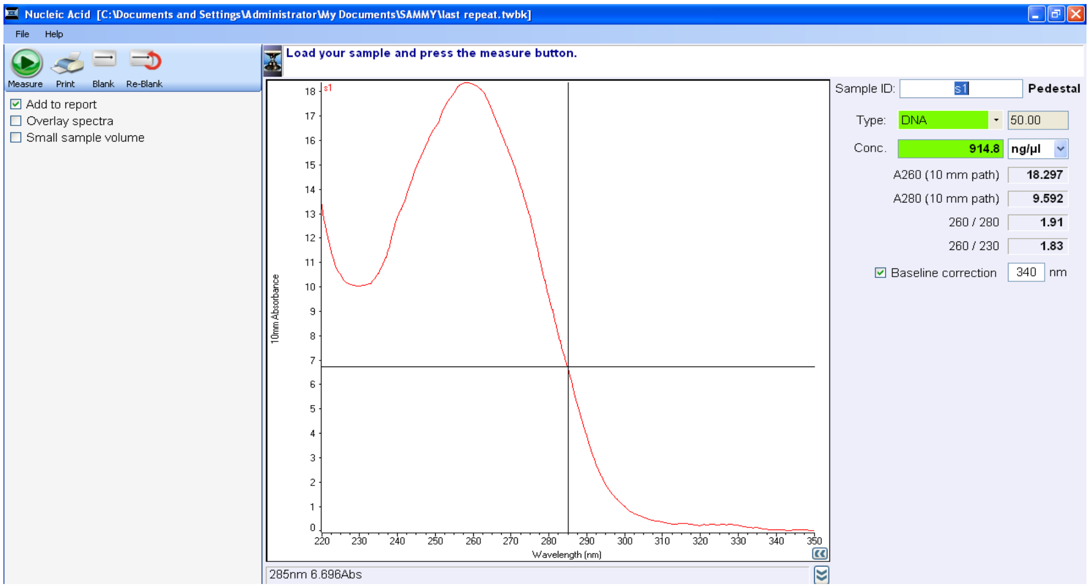


Figure 4.1: Gel image showing thirty *Populus* DNA samples extracted using CTAB method. Code numbers 1 to 10 represent Athi River population, code numbers 11 to 16 represents Ewaso Nyiro River population, code numbers 17 to 26 represents Tana River population while sample code numbers 27 to 30 represent KEFRI samples. M is the lambda DNA size marker of 100 ng/µl (New England Biolabs, Ipswich, USA)

The DNA purity and concentration was determined by a Nano drop spectrophotometer (Figure 4.2). DNA has absorbance maxima at 260 nm, proteins at 280 nm and other contaminants e.g. carbohydrates have absorbance at 230 nm. DNA contamination by proteins was evaluated at 260:280 absorbance ratios while that of other organic contaminants was evaluated at 260:230. A range of ratios between 1.8-2.0 was considered as pure DNA for both absorbance ratios. For instance, the DNA yield was 914.8 ng/µl with 260:280 ratios at 1.9 and 260:230 ratios at 1.83 for sample T1 from Athi East Salagate (Figure 4.2). Wavelength of the trough in the sample spectrum was at ~230 nm. Absorbance by a contaminant at a low wavelength would have shifted the wavelength of the trough. Wavelength of the peak in the sample spectrum was at 260 nm. Absorbance by a contaminant would have shifted the peak absorbance wavelength. A low 260:230 ratio would have indicated contaminant absorbing at 230 nm or less. A low 260:280 ratio would have indicated contaminant absorbing at 280 nm or less.



Load your sample and press the measure button.

☑ Add to report
☐ Overlay spectra
☐ Small sample volume

Sample ID: s1 Pedestal
Type: DNA 50.00
Conc. 914.8 ng/µl
A260 (10 mm path) 18.297
A280 (10 mm path) 9.592
260 / 280 1.91
260 / 230 1.83
☑ Baseline correction 340 nm

285nm 6.696Abs

Figure 4.2: Spectrophotometer curve showing absorbance against wavelength of a pure 914.8 ng/µl DNA sample from Athi East Salagate T1

DNA concentration of all the samples that is, from Athi River, Tana River, Ewaso Nyiro River, KEFRI and USA samples was determined (Table 4.1). The lab codes represented the number assigned to each sample. The table also showed the DNA contamination by proteins which was evaluated at 260:280 absorbance ratios while that of other organic contaminants was evaluated at 260:230. A ratio of 18-2.0 was accepted as pure DNA for both absorbance ratios.

Table 4.1: DNA concentration and purity of the 40 samples as recorded by the spectrophotometer

Lab code	Nucleic Acid Conc.	Unit	260/280	260/230	Sample Type
1	1006.3	ng/µl	1.93	1.81	DNA
2	1029.5	ng/µl	1.92	1.76	DNA
3	928.9	ng/µl	1.8	0.96	DNA
4	850	ng/µl	1.87	1.98	DNA
5	914.8	ng/µl	1.91	1.83	DNA
6	1185.3	ng/µl	1.87	1.76	DNA
7	283.7	ng/µl	1.99	1.86	DNA
8	376.4	ng/µl	1.79	1.87	DNA
9	785.2	ng/µl	1.41	1.9	DNA
10	1163.3	ng/µl	1.98	1.97	DNA
11	654.2	ng/µl	1.77	1.86	DNA
12	1059.4	ng/µl	1.9	1.87	DNA
13	1061	ng/µl	1.9	1.98	DNA
14	733.4	ng/µl	2.06	1.84	DNA
15	1214.1	ng/µl	1.82	1.87	DNA
16	1335.7	ng/µl	1.76	1.9	DNA
17	1166	ng/µl	1.92	1.97	DNA
18	822.9	ng/µl	1.92	1.82	DNA
19	803	ng/µl	1.84	1.89	DNA
20	1456.1	ng/µl	1.88	1.85	DNA
21	768.8	ng/µl	1.88	1.96	DNA
22	1243.9	ng/µl	2.01	1.79	DNA
23	1295.9	ng/µl	1.88	1.98	DNA
24	1094.3	ng/µl	1.98	1.99	DNA
25	1140.5	ng/µl	1.88	2.01	DNA
26	1132	ng/µl	1.98	1.84	DNA
27	1306.5	ng/µl	1.85	1.8	DNA
28	1673	ng/µl	1.82	1.9	DNA
29	1235.7	ng/µl	1.88	1.8	DNA
30	1576.5	ng/µl	1.92	1.9	DNA
31	61.9	ng/µl	1.99	1.95	DNA
32	56.9	ng/µl	1.88	1.87	DNA
33	65.3	ng/µl	1.89	1.86	DNA
34	79.1	ng/µl	1.76	2.01	DNA
35	67.2	ng/µl	1.87	2.02	DNA
36	56.7	ng/µl	1.97	2.01	DNA
37	57.3	ng/µl	2.01	1.87	DNA
38	89.1	ng/µl	1.86	1.98	DNA
39	87.8	ng/µl	1.97	1.92	DNA
40	56.9	ng/µl	1.88	1.9	DNA

4.2 Amplified Fragment Length Polymorphism (AFLP) analysis

AFLP technique involves restriction and ligation, and in this case *MseI* and *EcoRI* enzymes and adaptors were used. They generated visible smears that were in the range of 100-500bp (Figure 4.3). Presence of smears indicated that digestion of the genomic DNA using *MseI* and *EcoRI* enzymes was successful as well as the ligation of the *MseI* and *EcoRI* adaptors at the restriction sites on the DNA fragments. The restriction produces a large number of small size DNA fragments that results in the visible smears.

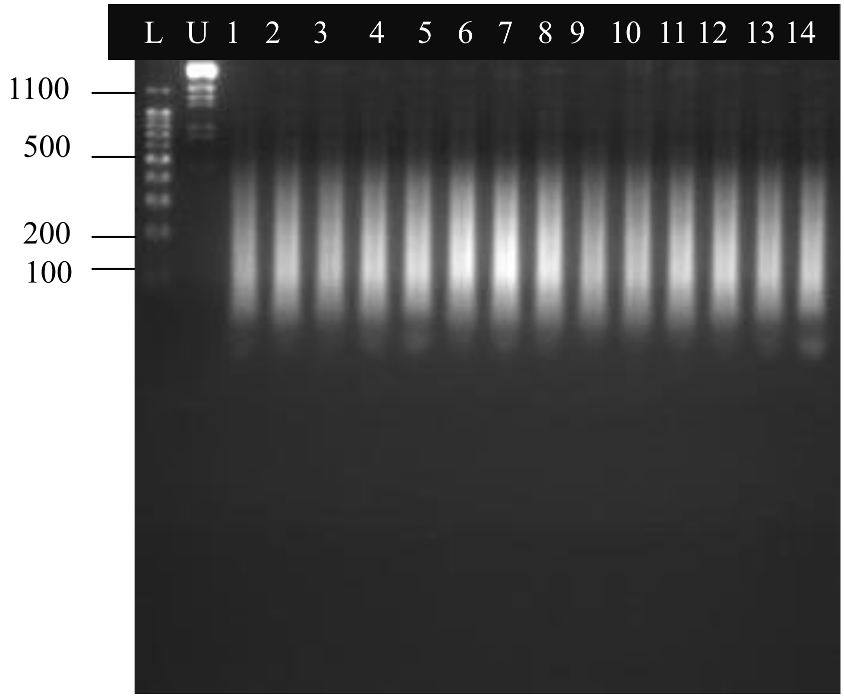


Figure 4.3: Gel electrophoresis image showing restriction/ligation products of fourteen samples from, Athi River and Ewaso Nyiro River populations. Lane 1 to 10 represents Athi River population while lane 11-14 represent Ewaso Nyiro population. The numbers represent the lab codes assigned to each sample. L depicts 100 bp molecular ladder while U is undigested Tana River sample DNA

Preselective amplification generated visible smears on each sample. The smears were in the range of 100- 700 bp when run on 2% (w/v) agarose gel (Figure 4.4). During preselective amplification only a part of the restricted fragments are amplified. This results to homogenous DNA smears in the range of 100- 700 bp with L as the DNA size marker.

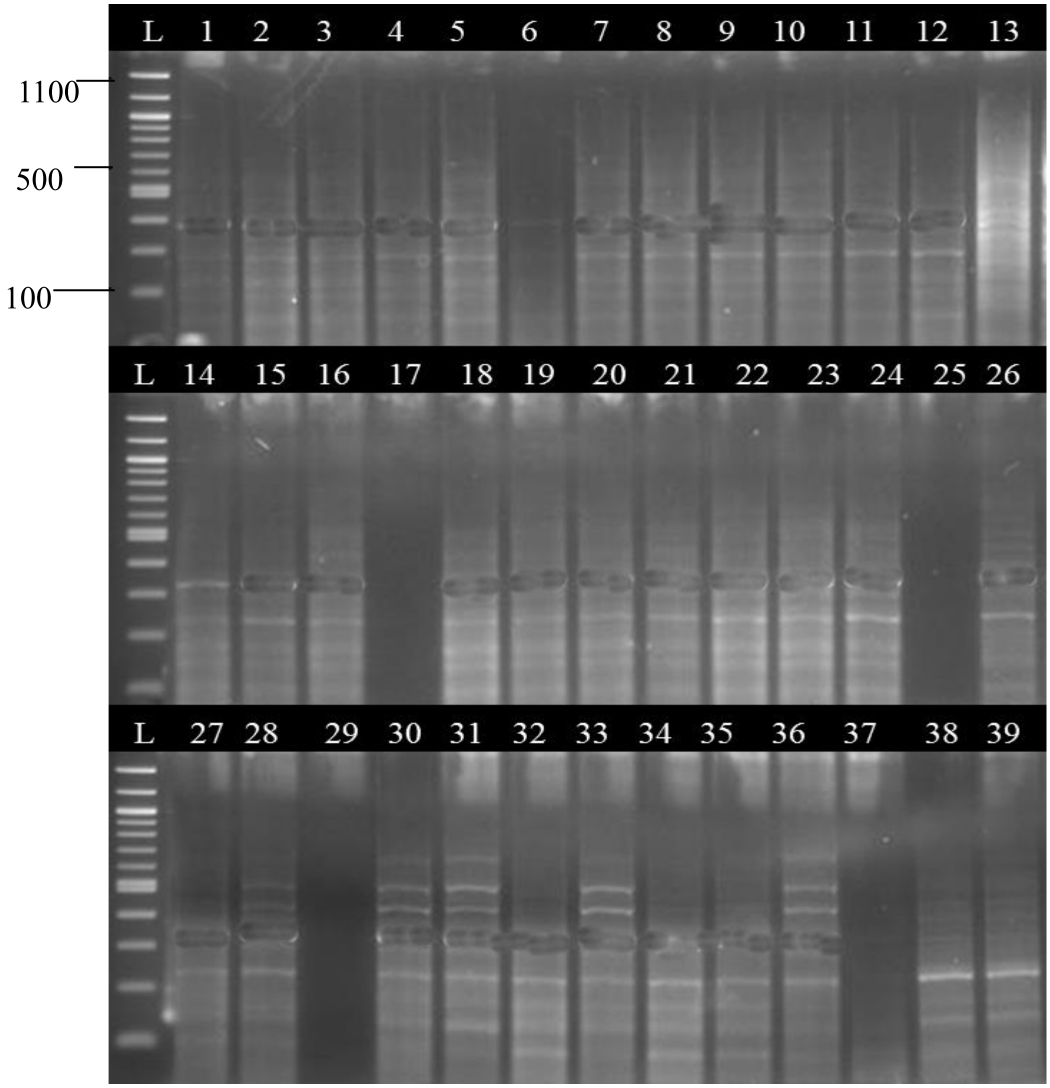


Figure 4.4: Agarose gel image showing preselective amplification of thirty nine *Populus* samples. Lane 1-10 represents Athi River samples, Ewaso Nyiro River samples (Lane 11-16), Tana River samples (Lane 17-26), KEFRI samples (Lane 26-30) and USA samples (Lane 31-39). L denotes the 100 bp ladder and C denotes the control.

Successful selective amplification was verified by running the amplicons on 2%w/v agarose gel (Figure 4.5). For the selective amplification, the *EcoR1* primers were labeled with a fluorescent dye to enable visualization of DNA amplicons. FAM, JOE and NED were the dyes used. Three more nucleotides were added at the 3' end of the primer sequence used for the preselective amplification. This resulted to more selectivity and minimized the number of the restricted fragments amplified. The result of this was smears that were less prominent as compared to the preselective amplification. Figure 4.5 shows the selective amplification smears.

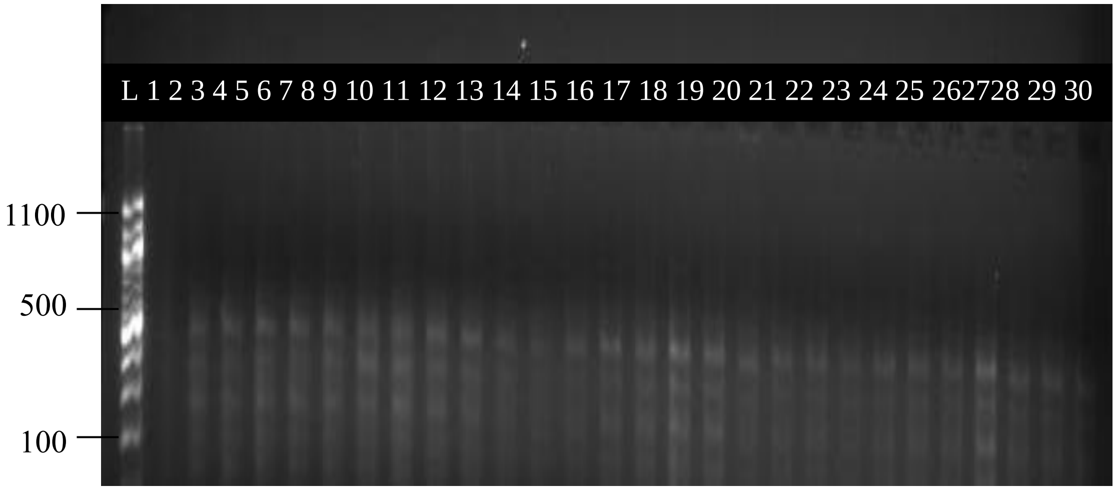


Figure 4.5: Agarose gel image showing selective amplification of thirty *Populus* samples. Lane 1-10 represent Athi River samples, Ewaso Nyiro River samples lane 11-16, Tana River samples lane 17-26 and KEFRI samples lane 27-30. The primer combination used was E-ACA/M-CTC labeled with FAM fluorescent dye. L denotes the 100 bp ladder

The PCR products were resolved in a capillary electrophoresis where the fragment analysis resolved the DNA fragments differing with even a single base pair. The fragments were separated with regard to length with smaller fragments running first. As the fragment passed through the laser, a dye attached to the primer was excited to produce a fluorescent signal that was collected on a computer. The results of the fluorescence were then visualized as peaks called electrophoregrams as shown by GeneMapper software (Figure 4.6). Each peak represented a band on a normal acrylamide gel. The amplified fragments range from 50 to 500 base pairs.

Figure 4.6: AFLP electrophoregrams for three *P. ilicifolia* samples using E-ACA/M-CTC primer combination. Each peak corresponds to a DNA restricted fragment of a particular length. For the three sample electrophoregrams, the peaks start at 50 base pairs and end at 354 base pairs

4.3 Genetic diversity estimates

Based on 521 unambiguous AFLP loci analyzed across the three Kenyan natural populations using unbiased Nei's genetic diversity, the least average heterozygosity (H) was from Ewaso Nyiro River population (H=0.0905) while Athi River population (H=0.2189) showed the highest heterozygosity (Table 4.2). The percentage polymorphic loci for the Kenyan populations reflected similar results with Ewaso Nyiro River population recording 23.8% which was the least, while Athi River population 65.26% which was the highest percentage polymorphic loci. Thus heterozygosity (H) and percentage polymorphic loci values concurred.

Table 4.2: Genetic diversity estimates in five *Populus* populations

Population	**Number of individuals**	**% polymorphic loci**	**Mean heterozygosity (H)**
ATHI	10	65.26	0.2189
TANA	10	59.69	0.2049
EWASO NYIRO	6	23.80	0.0905
MUGUGA KEFRI	4	52.98	0.2396
REFERENCE SAMPLE FROM USA	10	71.98	0.3170

Nei's genetic distances (Table 4.3) computed by Tools for Population Genetics Analysis software were used to generate the Principle Coordinate Analysis and the phylogenetic analysis in form of Dendograms. Genetic distances showed the extent of genetic differences within and among populations, thus giving the genetic relationship between and among the populations. The genetic distances were measured as a function of gene frequencies (Nei, 1972). The shortest genetic distance was between Athi River and Tana River populations (0.0341) an indication of close relatedness between the two populations. The largest genetic distance was between Ewaso Nyiro River population and the KEFRI samples (0.06987). This was an indication that the two populations were distantly related.

Table 4.3: Genetic distance matrix of 5 *Populus* populations based on 521 AFLP loci

POPULATIONS	ATHI	EWASO NYIRO	TANA	MUGUGA KEFRI	USA
ATHI	0				
EWASO	0.0467	0			
TANA	0.0341	0.0416	0		
MUGUGA KEFRI	0.5869	0.6987	0.6048	0	
USA	0.5228	0.6424	0.5315	0.0751	0

4.4 Principle coordinates analysis (PCoA)

The principle coordinate analysis was derived from the genetic distances (Table 4.3) and the first two principle axes accounting for 45.56% and 11.48% respectively and showed two groups (Figure 4.7). One group was made of reference samples from USA and the KEFRI samples. The reference samples consisted of different species in the genus *Populus* that is, *P. tremuloides, P. deltoides, P. fremontii, P. tremula and P. balsamifera.* The second cluster was composed of Tana River, Athi River and Ewaso Nyiro River populations which were Kenyan natural populations. The PCoA geometrically arranged the individuals of each population such that the distance between them on the graph represented their ecological distances. Individuals that are close together in the graph (Figure 4.7) were interpreted to have similar characteristics (closely related) while those apart interpreted to be different or distantly related

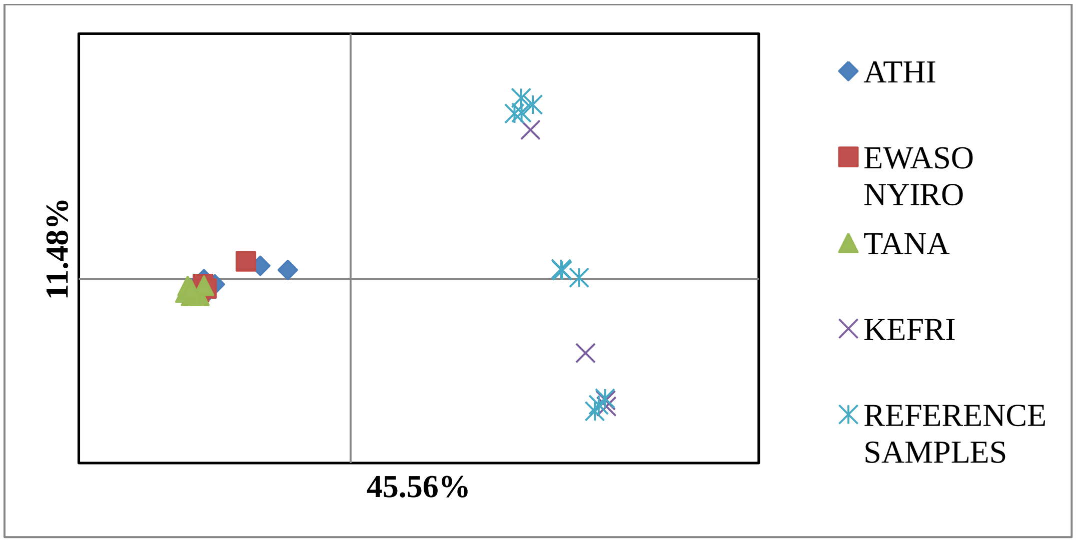


Figure 4.7: Principle coordinate analysis of the 5 *Populus* populations based on 521 AFLP loci

4.6 Phylogenetic analysis

To show genetic differences between the populations and individuals, Nei's unbiased genetic distances were used again (Nei, 1978) taking into consideration unambiguous 521 AFLP loci were scrutinized (Table 7). The smallest genetic distance was between Athi River and Tana River populations (0.0341) while the greatest distance was between (0.6987) Ewaso Nyiro River and KEFRI sample populations (Table 7). Two main population clusters were formed according to the dendogram constructed from these genetic distances (Figure 4.8). One cluster consisted of Tana River, Athi River and Ewaso Nyiro River populations while the other was composed of the reference samples from USA and KEFRI sample populations which had a small genetic distance between them (0.0751). Another dendogram showing relationships between individuals in the Kenyan *P. ilicifolia* populations was also constructed (Figure 4.9).

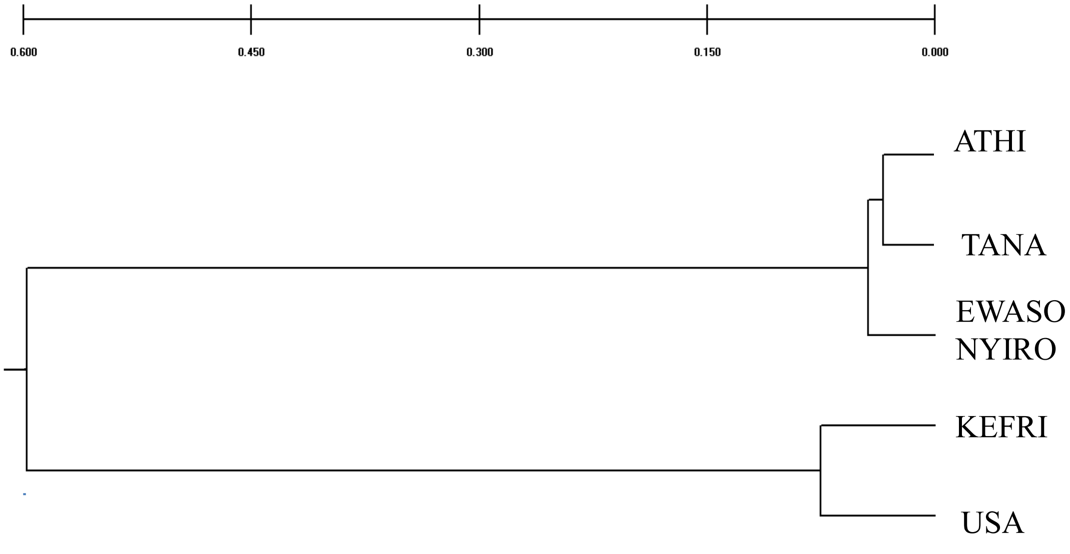


Figure 4.8: UPGMA dendogram showing relationship among the five Poplar populations based on 521 AFLP loci

Considering only the Kenyan natural populations, three distinct clusters were formed from the individual tree samples each cluster corresponding to each of the rivers that is Tana River, Ewaso Nyiro River denoted by Samburu and Athi River denoted by Tsavo (Figure 4.9). Samples Tsavo Athi T2, Tsavo East T1 and Tsavo East T6 appeared to cluster on their own an indication of distant relatedness from the rest of the clusters.

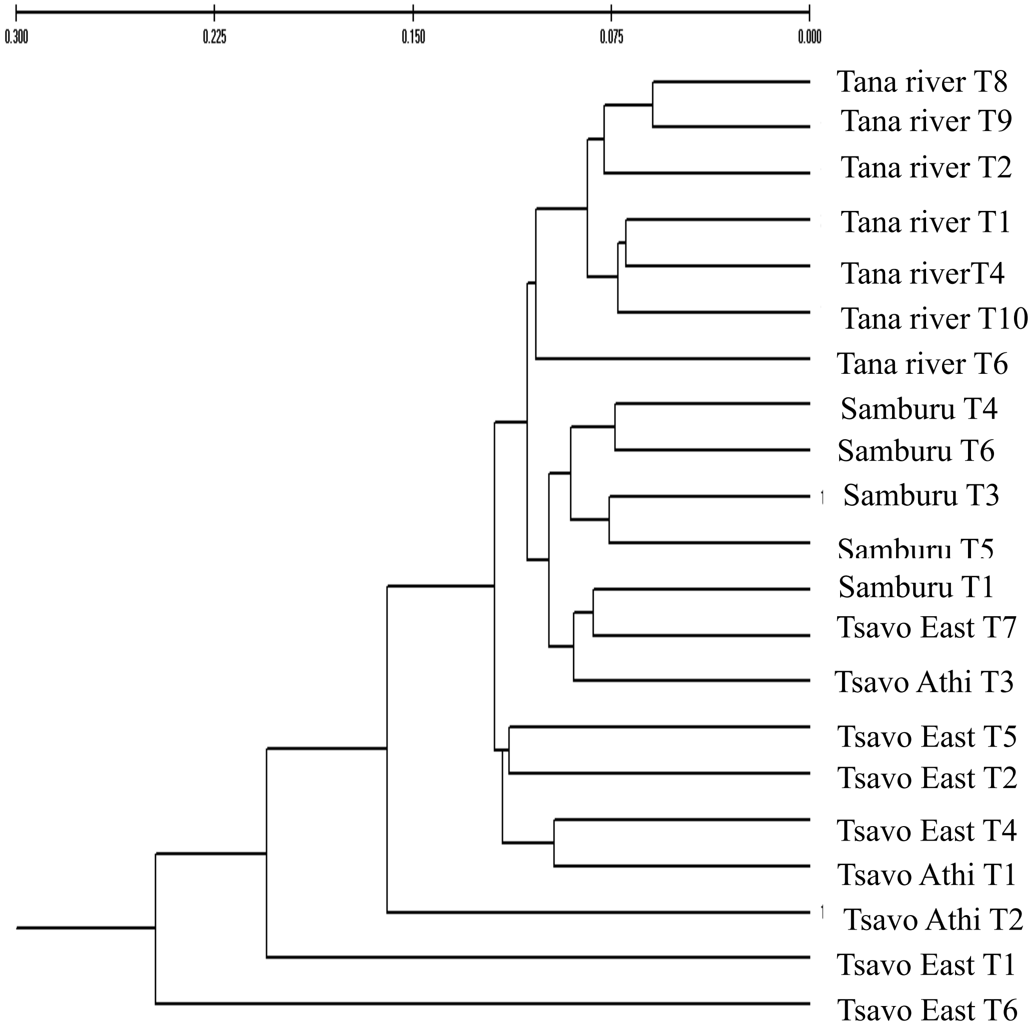


Figure 4.9: UPGMA dendogram showing relationship among individuals in the three Kenyan populations based on 521 AFLP loci

4.5 Analysis of molecular variance (AMOVA)

Analysis of molecular variance showed significant genetic variation within all the five populations 60% (P<0.001) and among populations 40% (P<0.001), (Table 4.4). When nested analysis were carried out considering only the Kenyan natural Poplar populations, that is, Athi River, Tana River and Ewaso Nyiro River, low but significant genetic variation among populations was evident. 13% (P<0.010) of genetic variation partitioned among populations and 87% (P<0.010) partitioned within the populations (Table 4.4).

Table 4.4: Summary of analysis of molecular variance (AMOVA). Degrees of freedom (df), sum of squares (SS), mean of square deviation (MSD), % variation and P-values are shown

Source of variation	df	SS	MSD	% variation	P-value
P. ilicifolia only					
Among populations	2	131.1	65.6	13%	
Within populations	23	1113.8	48.4	87%	<0.01
All samples					
Among populations	4	1465.2	366.3	40%	
Within populations	35	2079.8	59.4	60%	<0.001

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

Although a large sample size is important for genetic analysis (Bashalkhanov *et al.*, 2009), use of small sample size in genetic diversity is common (Breinholt *et al.*, 2009). To compensate for this, analysis of average heterozygosity and genetic distance of a given species can use a small sample size so long as a large number of loci are studied (Nei, 1978). In this study 521 loci were used for the genetic studies.

Genetic estimates produced by Tools for Population Genetics Analysis (TFPGA) software, based on six AFLP primer combinations showed significant genetic diversity for most of the populations. For the Kenyan Poplar populations, they all had a lower mean heterozygosity (H) values than a similar study in Kenya of a tropical tree (*Vitex fischeri*) that used RAPD markers (Lengkeek *et al.*, 2005). The highest diversity estimate for *P. ilicifolia* was from Athi River population (H= 0.2189) which was relatively lower than (H=0.278) observed by Lengkeek *et al.* (2005). Similar results of between H=0.22 to H= 0.35 were obtain by Kyndt *et al.* (2009). Percentage polymorphic loci values greater than 50% depict high genetic variation (Changadeya, 2009). Therefore, Athi River population is the most diverse according to these diversity estimates. This suggests that it could be the center of origin for the Kenyan *P. ilicifolia* populations thus spreading through wind dispersal to Tana River and Ewaso Nyiro rivers. This was in accordance with Oballa (1996) findings. The lowest mean heterozygosity was recorded from Ewaso Nyiro River population (H=0.0905). Low population densities of tropical tree species (due to flooding, anthropogenic factors) result to a decrease in gene diversity according to Dusan (1992). This was true for *P. ilicifolia* species in Ewaso Nyiro River, Kenya. Ewaso Nyiro River population had low genetic diversity (H=0.0905) which was in line with studies carried out on the same species by Oballa (1996) using isozyme markers. This could be attributed to reduced population size thus the remaining trees maybe closely related. Decreased population size (genetic bottle neck) facilitates high chances of inbreeding within a population brought about by random genetic drift (Luan, 2006). As a result the effective population size (*Ne*) is reduced translating to low heterozygosity (Hamrick and Godt, 1996). Additionally, founder effects could have caused the low gene diversity (Muchugi *et al.,* 2012) in Ewaso Nyiro River population where a small proportion of the species that does not represent the total genetic diversity of the species fragments from the main population and starts a new population. This isolation may have led to subsequent loss of unexploited genetic potential (Kotzé and Muller, 1994). This could have resulted in erosion of genetic diversity in this population which eventually leads to detrimental health effects for this species.

With reference to temperate poplar species that is USA reference samples, larger diversity estimates than tropical Kenyan poplar populations were recorded. For the USA, H = 0.32, % = 71.98 which was highly variable as compared to any of the Kenyan Poplar populations. These reference samples had high genetic diversity values that agreed with other studies on temperate *Populu*s species (Slavov *et al.,* 2009). This could be attributed to long-term natural selection for adaptation of these populations. Furthermore, the reference samples from the USA were composed of different species in the *Populus* genus. They include *P. tremuloides, P. deltoides, P. fremontii, P. tremula* *and P. balsamifera.* This mixed nature could also have resulted in high diversity values. USA population of poplar could be deduced to be highly adaptive to changing ecological niches as opposed to Kenyan populations. Similarly high diversity values were recorded for Muguga KEFRI samples that is H = 0.24, % = 52.98. The KEFRI samples (planted) composed of *P. deltoides*, *P. alba* and a hybrid of *P. deltoides* X *P. trichocarpa* (*P. beaupre*). The hybrid is a better performer in terms of growth compared to the other two species. These samples had been brought in from the Mississippi, USA in 2005 and so no much evolution had taken place hence a large diversity estimates like their USA relatives. Nevertheless, different conservation and management strategies are therefore required for protection of Kenya *P. ilicifolia* populations. The possibility of hybridization of the reference samples with the Kenyan local *P. ilicifolia* for genetic improvement should be explored especially those that appear to be suffering from bottleneck effect like Ewaso Nyiro River population.

The breeding system in trees is one factor that influences the genetic variation within and among populations (Loveless and Hamrick, 1984). Forest trees have more genetic differentiation within populations than among populations. Outcrossing plants, of which many tropical trees are, exhibit higher levels of genetic differentiation within populations than selfing plants (Hamrick and Godt, 1996). *Populus* being a genus of outcrossing trees (Ridley, 1930; Nilsson *et al.*, 1991) exhibited similar partitioning for this study as there was 60% variation within populations as compared to 40% among populations. Considering only the Kenyan Poplar populations the same was true as 87% partitioned within population while 13% among populations. Chase *et al.* (1995) showed that outcrossing plants species tend to have 10-20% of genetic variation among population while species that self-pollinate have a 50% variation among populations. This was in agreement with other studies carried out on other tropical woody perennial trees that showed most of the genetic variation is maintained within populations (Hamrick *et al.*, 1992). Kyndt *et al.* (2009) found differentiation among populations of baobab ranging from 2% to 28% in West Africa. *Warburgia ugandensis* a tropical tree in Eastern Africa showed similar genetic variation of 59% within populations (Muchugi *et al.*, 2012). Mbatudde *et al.* (2013) has also shown the same genetic structuring in *P. Africana* recently.

The low genetic variation (13%) among populations and high variation (87%) within populations could imply that many alleles are common among the populations with few rare alleles present due to environmental adaptation. High similarity of alleles may deduce sharing of common ancestral alleles and the low frequency allele subsequent as a result of evolution (Esselman *et al.*, 2000). The weak but significant differentiation among populations was also in agreement with direct studies of gene flow, the results of which suggested that long-distance pollination can be extensive in *Populus* (Tabbener and Cottrell, 2003; Pospíšková and Šálková, 2006; Vanden Broeck *et al.*, 2006; Slavov *et al.*, 2009). Therefore, for conservation of *P. ilicifolia* in Kenya, any single population can be targeted in the conservation strategies. Studies of genetic differentiation are therefore important for conservation of *P. ilicifolia* especially in determination of which population should be conserved and how genetic mixing between populations should be carried out in introduction programs.

Using the UPGMA cluster analysis method based on unbiased Nei's genetic distances, two outstanding groups were formed. This was in agreement with the principle coordinate analysis (Peakall and smouse, 2009) that showed two groups which represent distance matrix relationship between groups. The PCoA was selected to complement the cluster analysis which shows relationship between closely related individuals. The two clusters from UPGMA analysis were due to the difference in geographical location between Kenyan and U.S.A populations which may have resulted to the wide variation and separate clustering. This meant distant relatedness between the Kenyan and U.S.A populations. The resultant dendogram from UPGMA cluster analysis grouped Kenyan *P. ilicifolia* populations in the same cluster in accordance with their geographical locations.

The other cluster was composed of USA reference samples and KEFRI samples though they are geographically located miles apart. This clustering could be attributed to the fact that the KEFRI samples were composed of temperate poplar species introduced in Kenya (KEFRI) recently in 2005 and so no much evolution had taken place hence the small genetic distance between the USA population and KEFRI samples. According to the stepping stone model increase in geographical distance results in decreased genetic similarities (Cox and Derrett, 2002) assuming mating movements are limited to short distances. Comparisons of USA reference samples and Kenyan populations (from the three riverine habitats), the two were distantly related as shown by the different clustering and the genetic distances. This is as a result of divergent evolutionary process of isolation by distance (Wright, 1943) and adaptation to different environments. The two are located in two different continents thus natural selection has played a big part in the wide diversity.

For the Kenyan populations, Athi River and Tana River had the smallest genetic distance (0.0341) between them an indication of recent divergence. To some extent this confirms some degree of regional similarity. Both populations belong to the Somalia-Masai center of endemism associated with unique ecological conditions and vegetation cover (White, 1983). The two riverine habitats (Athi River and Tana River) are also geographically closer to each other when compared to Ewaso Nyiro River. Ewaso Nyiro River had a relatively larger genetic distance between both Tana River and Athi River (0.0416 and 0.0467 respectively) than between Athi River and Tana River an indication of early divergence. Isolation by relatively large geographical distance between the Ewaso Nyiro River population and both Athi River and Tana River populations suggests some degree of isolation and limited gene flow thus explaining the relatively large genetic distance. Additionally Ewaso Nyiro River drainage is slightly far in Northern Kenya where it drains into Lorian swamp. Tana River and Athi River populations are relatively closer to each other geographically that is southern part of Kenya and draining into Indian ocean, thus explaining the small genetic distance due to possible gene exchange.

The greatest distance (0.6987) was between Ewaso Nyiro River and KEFRI population, an indication of no genetic exchange between the two, a case of isolation by distance, regardless of the fact that the two are in the same country, Kenya. This was an indication that interspecies hybridization had not taken place yet. Though long distance pollination for *Populus* species has been documented, natural and man-made barriers between the two populations could have hampered any gene exchange between these populations.

This study validated Imbert and Lefèvre (2003) genetic studies on Poplar species in the temperate regions by show that *P. ilicifolia* typically display high levels of genetic diversity within populations at molecular loci (Yeh *et al.,* 1995; Stevens *et al.*, 1999) an important factor in conservation and breeding (Munthali *et al.*, 2012).

Amplified Fragment Length Polymorphism markers were sufficient in determination of genetic diversity of *Populus ilicifolia* in Kenya. Their ability to screen many loci distributed throughout the genome of this species made it possible to show the genetic structure within and among populations as well as evaluate the level heterozygosity in population at the gene level.

5.2 CONCLUSION

Kenyan *P. ilicifolia* populations had a high genetic diversity as shown by heterozygosity and percentage of polymorphic loci values with the exception of Ewaso Nyiro River population. Conservation strategies should therefore prioritize Tana River and Athi River populations. On the other hand, low genetic diversity in Ewaso Nyiro River population should be of major concerned as its not healthy for outbreeding species faced with environmental and climatic changes. Further decrease in genetic diversity in Ewaso Nyiro River population should be curbed through establishment of conservation stands on farms and in plantation set up. Low genetic variation among Kenyan natural populations (Athi River, Tana River and Ewaso Nyiro River) showed that any single population can be targeted in the conservation strategies. Furthermore, there was significant genetic difference between Kenyan and USA populations even for populations introduced 10 years ago (KEFRI samples). This led to the clustering in different groups. Improvement programs of *P. ilicifolia* should incorporate temperate poplar species through hybridization so as to economically attract farmers to grow them.

5.3 RECOMMENDATION

The recommendations made based on this study include;

i. Urgent measures should be taken to conserve the species as the survey established decreasing populations across the species ecology.
ii. Seed collection from many randomly sampled trees is encouraged in order to capture the diversity in conservation and use of the species.
iii. Further studies on *P. ilicifolia* biology to be undertaken to understand how the species survival in its natural ecology can be enhanced.
iv. Assessment of possibility of hybridization with other temperate poplar species that is geared towards faster bioenergy tree production.

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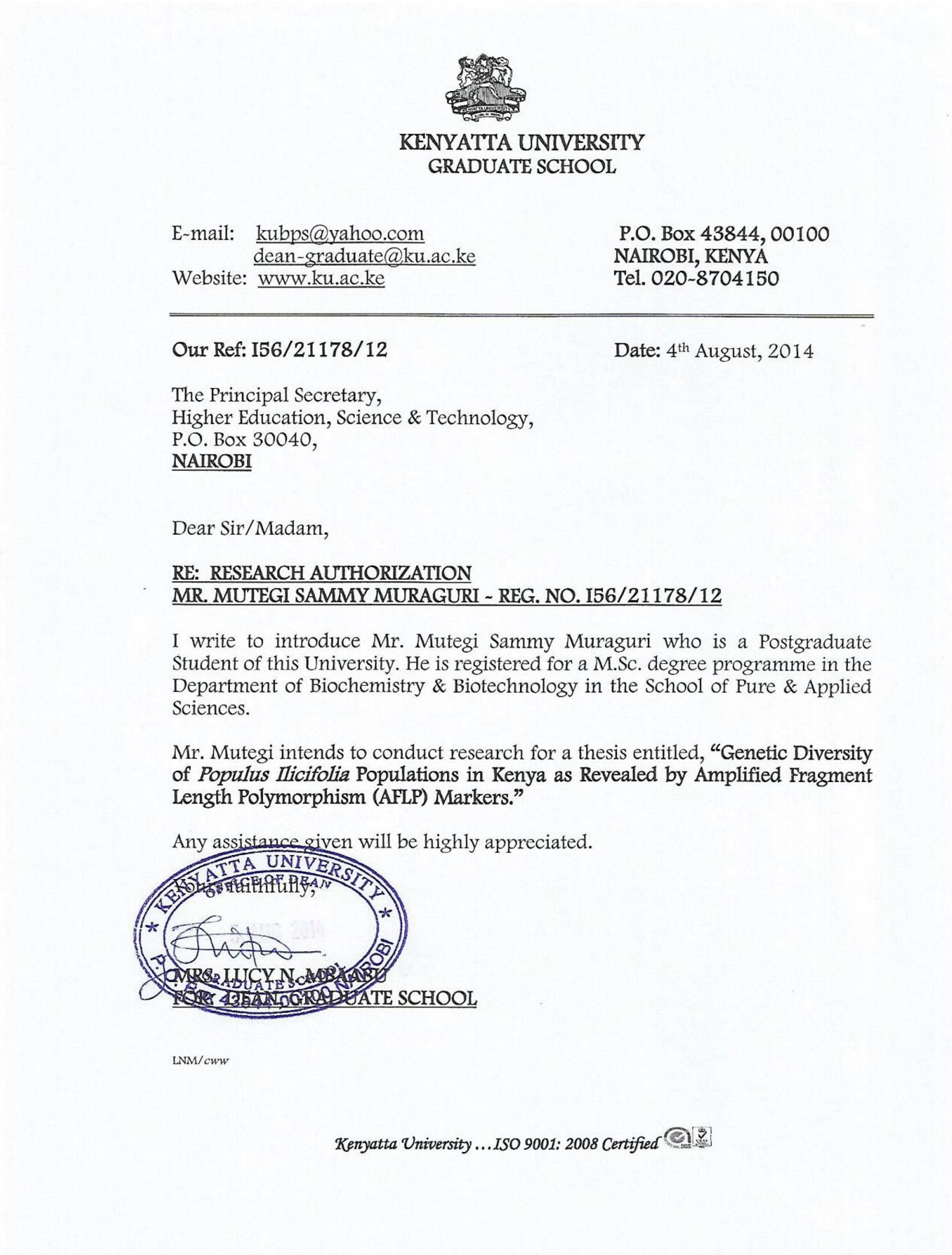
APPENDIX



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Our Ref: I56/21178/12

Date: 4th August, 2014

The Principal Secretary,
Higher Education, Science & Technology,
P.O. Box 30040,
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION
MR. MUTEGI SAMMY MURAGURI - REG. NO. I56/21178/12

I write to introduce Mr. Mutegi Sammy Muraguri who is a Postgraduate Student of this University. He is registered for a M.Sc. degree programme in the Department of Biochemistry & Biotechnology in the School of Pure & Applied Sciences.

Mr. Mutegi intends to conduct research for a thesis entitled, **"Genetic Diversity of *Populus Ilicifolia* Populations in Kenya as Revealed by Amplified Fragment Length Polymorphism (AFLP) Markers."**

Any assistance given will be highly appreciated.

Yours faithfully,

5 AUG 2014

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

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