MOLECULAR ANALYSIS OF GENETIC VARIATION IN AN INDIGENOUS DRYLANDS TREE SPECIES, *MELIA VOLKENSII* (Gurke.) AND IMPLICATIONS FOR ITS CONSERVATION

BY

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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

This thesis is dedicated to my parents, Samuel and Wanjiru Runo.

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ABSTRACT

Melia volkensii (Gurke.) is a popular tree species in the East Africa's arid and semi arid lands (ASALs) valued because of its high quality termite resistant timber. It is fast growing thus a good choice for commercial forestry establishment. Goats eat its fruits and small-scale farmers appreciate the tree as an agroforestry species. The benefits from the tree and the risks of depletion looming over its natural distribution have made it a priority species for conservation and improvement programmes. However, information on its existing genepool is lacking. Studies were therefore carried out on the species genetic diversity in order to propose appropriate conservation measures and improve breeding strategy.

Eight RAPD primers generated 38 scorable bands. Genetic distances were computed between all populations and used to construct neighbour-joining phenograms. An Analysis of Molecular Variance (AMOVA) was also used to partition genetic variation components between regions, between populations, and between individuals within populations.

The phenograms grouped the nine populations into two groups, one consisting of populations from eastern Kenya, and the other one consisting of coastal populations. AMOVA results indicated significant genetic differentiation between populations in the eastern and the coastal regions with 21.1 %, ($P < 0.0002$) of the total variation attributed to a difference between these areas. Little differentiation was recorded between populations (3.5 %, $P < 0.0002$) and 75.4 %, ($P < 0.0002$) of the total variation was attributed to variation within populations.

The difference between populations in the eastern and coastal regions could be due to ecogeographical association with genetic variation and therefore they should both be conserved to retain the full breadth of genetic variation of the species.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Conservation of biodiversity

Species conservation is a subject that has recently generated a lot of interest. This is manifested by the high political profile of the topic and in the number of activities related to conservation strategies such as organization of conferences and meetings, the involvement of many national and international institutions and the increased level of scientific research and documentation. However despite all these efforts, only a small fraction of biodiversity so far is under proper conservation especially in the tropics (National Research Council, 1991). Mismanagement, adverse environment as well as socio-economic changes threaten many ecosystems.

Currently, forests are among the most threatened ecosystems in the world (National Research Council, 1991; Williams, 1991). The majority of the highly exploited tropical forests are not under any conservation programmes. This trend of over exploitation posses a threat to the highly demanded forest tree species. Population genetic theory predicts that the decrease in the genetic diversity limits a species ability to keep pace with the changing selection pressure (Young and Merriam, 1992). Plant species, especially the perennials such as trees, rely on the available genetic

diversity for stability and survival under the ever-changing environments (National Research Council, 1991). This variability forms the base on which natural selection may act and has been exploited ever since the first domestication by man for breeding purposes. The benefits of forests and trees both in ecological terms and as a source of forest products cannot be over emphasized (Bawa and Krugman, 1991), thus the questions currently being raised do not any longer concern the rationale for forest conservation and improvement programmes but rather how they can be effected.

As natural forest resources decline, there is an increasing need to resort to plantation and on-farm forestry. The main obstacle to this development is lack of knowledge on the biology of most of the tropical tree species and the ecosystems (National Research Council, 1991). Understanding species population structure and potential genetic resources is essential for their conservation, planning and sustainable management (Sun *et al.*, 1998).

Traditionally, provenance and progeny tests coupled to biometrical analysis of phenotypic traits have been the standard methods for describing and quantifying genetic variation in forest tree species (National Research Council, 1991). This approach however, besides taking years to complete, requires the establishment of expensive field trials and frequently the measurement of traits under strong environmental effects. It is clearly a

slow approach when the goal is to rapidly estimate the patterns and distribution of genetic variation for making decisions in conservation and monitoring of genetic resources.

1.2 Molecular markers in forest conservation

Recent development of biochemical and molecular markers has complemented and hastened the process of information gathering therefore reducing the time taken in planning the best sampling strategies for conservation. The discovery that genes code for proteins, combined with the development of the techniques of protein electrophoresis led to the advent of biochemical markers which provided means of estimating genetic variation in natural populations (Crawford, 1990).

Among the markers available, isoenzymes have been used in a wide array of applications in plant biology such as population genetic structure, genetic diversity in natural populations and in breeding systems. In the context of conservation management, isoenzymes have been used to gather genetic information on endangered plant species to aid sampling for *ex situ* conservation (Scheon and Brown, 1995). Other applications include phylogenetic analysis (Swofford *et al.*, 1996) and ecological genetics (Azzouzi *et al.*, 1997). These investigations have involved many forest tree species (eg, Hamrick, 1992; Moran, 1992; Zimniak-Pryzybylska, 1995).

This advancement was revolutionized further by the advent of Restriction Fragment Length Polymorphism (RFLP) and the development of the Polymerase Chain Reaction (PCR) giving rise to DNA based markers.

These technologies assay the variation that exists at the DNA sequence level revealing the multiple forms (polymorphisms) that exist among individuals. A number of techniques have been developed based on the modern advances in molecular biology to evaluate genetic characteristics of pant populations. These include DNA sequencing (Murray, 1989), RFLP (Bostein *et al.,* 1980), Amplified Fragment Length Polymorphism (Vos *et al.*, 1995), Randomly Amplified Polymorphic DNA (Welsh and McClelland, 1990; Williams *et al.*, 1990), Microsattelites (Webber and May, 1989; Morgante and Olieveri, 1993) and Isoenzymes (Tanksley and Orton, 1983). The choice of an appropriate technique is determined by the aims of the study, the sensitivity and convenience of the technique, and the availability of resources among other factors (Karp, 1997). RAPD was chosen amongst the plethora of techniques essentially because it was immediately available for genetic analysis without the requirement of prior sequence information. In addition, it has easily identifiable variation and is relatively cheap compared to other techniques.

1.3 Justification

In Kenya, more efforts are now being directed towards the valuable, highly demanded indigenous forest tree species with the aim of promoting their propagation on-farms and in plantations, natural regeneration and conservation (both *in situ* and *ex situ*). The socio-economic and ecological importance of arid and semi arid lands (ASALs) as a national resource is also recognized. There is also an urgent need to incorporate indigenous tree and shrubs in reforestation programmes in the ASALs to make them part of the overall strategy for addressing environmental degradation problems.

Semi-arid lands are important because they (a) make up more than 50 % of Kenya's land area, (b) have the potential for increased resource exploitation, (c) they usually support the bulk of the rural populations, (d) have the potential to carter for some of the excess population of the already crowded high potential areas of the country and, (e) need rehabilitation from soil erosion and other environmental degradation.

Melia volkensii, which is endemic in drylands of East Africa, has emerged as a high potential tree. It is fast growing and tolerant to xeric conditions (Teel, 1985; Mabberly, 1997). This multipurpose tree is valued for both its timber and non-timber products the main one being to provide sawn timber

and poles (Stewart and Blomley, 1994). Currently, *Melia* populations are under increasing pressure from human activities. In common with other valuable tree species in the tropics, it has been overexploited because of its high quality timber and termite resistant poles. Habitat fragmentation and loss of the species natural population is also on the increase especially in the highly settled areas. Maintenance of genetic diversity is considered crucial for long-term survival and evolutionary response of populations to changes in the environment (Hueneke, 1991). These activities will reduce genetic diversity and adaptability of the species to the changing environment. In addition, genetic erosion would reduce the potential of the species improvement through selection.

Concerted efforts towards *in situ* and *ex situ* conservation are therefore necessary for preserving the stability of the species genetic structure. Lack of knowledge on species biology has been identified as a major obstacle in conservation (National Research Council, 1991). Although a number of studies such as drought tolerance and seed dormancy (Milimo, 1989; Milimo, 1994), have been carried out on *M. volkensii* detailed information on the genetic variation are lacking. In the present study, the RAPD technique was utilised to assess genetic variation in *Melia volkensii.*

The present work will assist in making reasoned decisions on sustainable management and conservation and utilization of *M. volkensii* genetic resources. To my knowledge, this is the first time molecular markers are being used in population genetic analysis of this species.

1.4 Hypothesis

- Diversity in ecology within the range of *M. volkensii* implies the existence of high levels of genetic variation in natural populations.
- Populations are adapted to the location of their origin and hence genetically differentiated.

1.5 General Objective

The present study aims at characterizing genetic variability within and between populations of *Melia volkensii* using molecular markers.

1.5.1 Specific objectives

- To develop a protocol for DNA isolation from *M. volkensii.*
- To assess the levels of genetic variation between and within populations of *M. volkensii.*
- To compare the levels of genetic variation between on farm and natural populations.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background information on *Melia volkensii*

2.1.1 Classification

The Meliaceae family is sub-divided into four sub-families and contains 51 genera and approximately 800 species. *Melia volkensii* belongs to the tribe Meliaeae of the Meliodeae sub-family. The genus contains three species of which *M. volkensii* is native to East Africa, *M. bambolo* native to Angola, and *M. azedarach* L. is native to South East Asia. *M. azedarach* is also widely planted in East Africa (Mabberley, 1997).

2.1.2 Seedling

Germination is epigeal; sclerotesta being ejected by the unfolding cotyledons. The two opposite primary simple leaves are stalked and developed from cotyledons. Shoots are green; leaves are alternatively arranged, elliptical, entire, fairly serrated and hairy. The taproot is fleshy and looks swollen (Milimo, 1994).

2.1.3 Mature Tree

The tree has been described as deciduous, open crowned and laxly-branched (Dale and Greenway, 1961; Riley and Bronkenshaw, 1988; Albrecht, 1993;

Beentje, 1994). Mature trees attain heights of between 6-20 m and a diameter of 25 cm. The bark is gray, fairly smooth but furrowed with age with pronounced vertical fissures and sometimes reticulated gray (Beentje, 1994).

2.1.4 Foliage

Leaves are pale bright green bi-pinnate with sub-opposite leaflets 3-7 per pinnae, up to 35 cm long and are densely hairy when young (Dale and Greenway, 1961). The leaflets are oval to lanceolate in shape and tapering to the apex. Their margins are entire or serrated, becoming almost glabrous when mature. The length is about 4-4.75 cm and the width could be half the entire length (Riley and Bronkeshaw, 1988).

2.1.5 Inflorescence and Flowers

The flowers are small, white fragrant in loose sprays. Inflorescence is congested and up to 12 cm long, auxilliary and on the older branchlets (Albrecht, 1993). The petals are 4-5 merous, white and free, sometimes curling backwards. The stamens are as many, but sometimes twice the number of petals and united into a tube.

2.1.6 Fruits and Seed

The fruit is drupe-like and oval in shape. The colour changes from green to pale yellow as the fruit matures (Milimo and Helum, 1989). The size of the fruit is 4 cm and has a thick and bony endocarp (Milimo, 1994)

2.2 Biology

2.2.1 Phenology and Life Cycle

Once established, *M. volkensii* grows fast to a deep-rooted, deciduous tree that sheds its leaves twice a year early in the dry season. New leaves flush before the rains towards the end of the dry season. Flowers and fruits are produced twice a year; fruit becoming ripe at the end of the dry season as the leaves emerge (Stewart and Blomley, 1994). Flowering and fruit setting take place at the age of 2.5 years (Stewart and Chirchir, 1992).

2.2.2 Reproductive Biology

Pollination mechanisms of *M. volkensii* are currently not clear. Since the tree is visited by bees and considering the flower in *M. volkensii* is similar to that of *M. azederach*, except for the colour, it is possible that insects are involved in the pollination of *M. volkensii* (Teel, 1985). Albrecht (1993) also reported that the species is insect pollinated.

Fecundity in this species is high with a seasonal yield of between 600-100,000 viable seeds per tree (Milimo, 1994) that are dispersed by giraffe, oryx and Lesser Kudu (Leuthold and Leuthold, 1972) and human beings (Shepherd, 1990). Disjunction in the distribution of *M. volkensii* and confinement within game parks decreases the potential for long distance seed dispersal by the game.

2.2.3 Propagation

Propagation is achieved using seedling cuttings, and natural regeneration (Stewart and Blomley, 1994). However, propagation especially through seedlings is difficult and time consuming because the species exhibits a long dormancy (Dale and Greenway, 1961; Milimo and Hellum, 1989).

2.3 Ecology

2.3.1 Distribution

The distribution of *M. volkensii* is shown in fig 2.1. The tree is endemic to semi-arid zones of East Africa (Dale and Greenway1961). It's native range extends from the southern slopes of the Pare Mountains (04°05'N, 37°41'E)

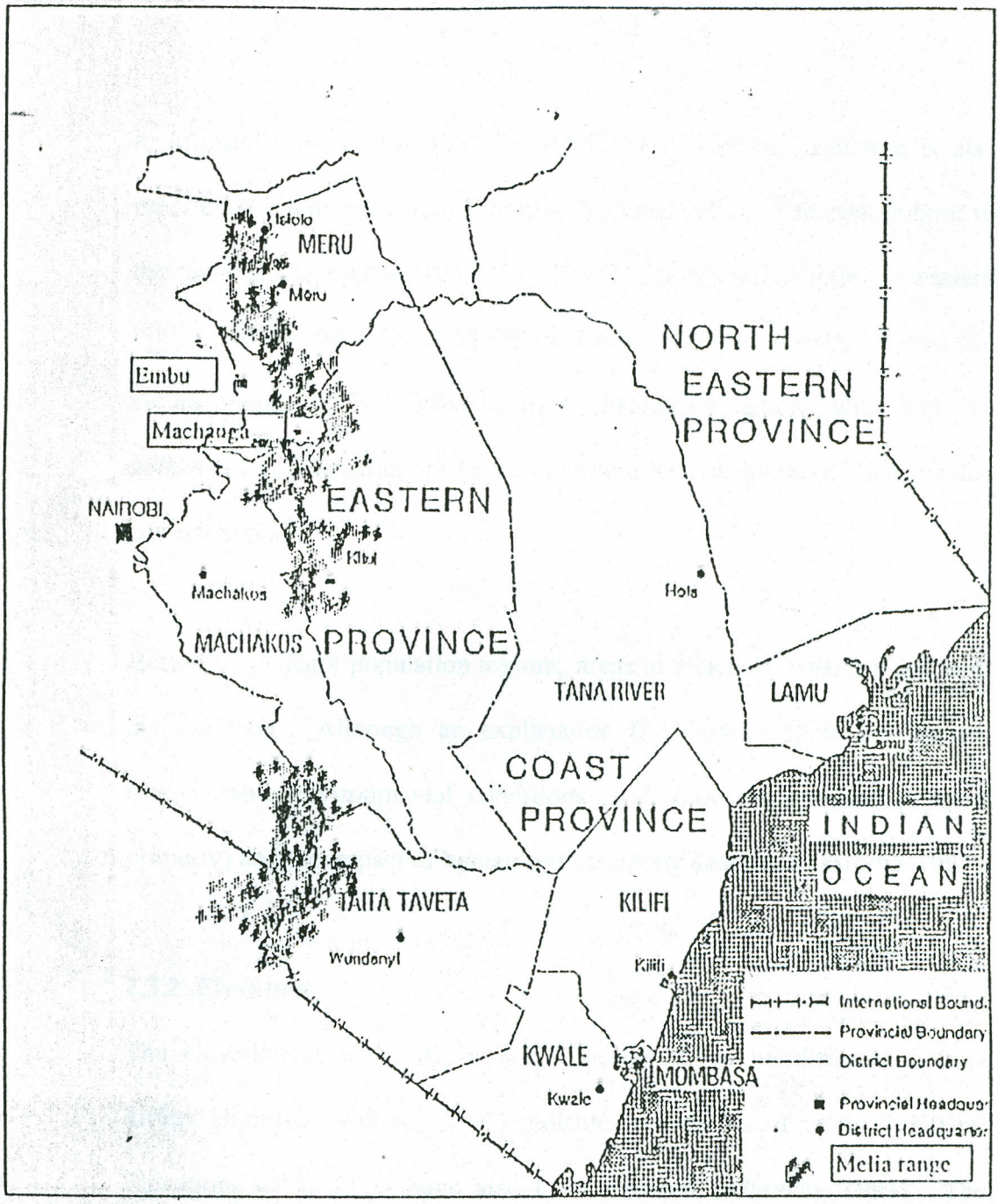

Fig. 2.1 Geographic distribution of *Melia volkensii* in Kenya

in Tanzania to El Wak (03°5'N, 41°52'E) in Kenya. The tree is also reported to occur in Eastern Ethiopia (Milimo, 1994). The eastern limit of the range is Lug Gunane (03°41'N, 42°35'E) in Somalia, while the western limit is Isiolo (00°21'N, 37°35'E) in Kenya (Milimo, 1994). Within the natural range of *M. volkensii*, three distinctive regions with high *M. volkensii* stand densities are found in eastern Kenya, southern Tanzania and eastern Somali.

Between the major population regions, areas in which *M. volkensii* is absent are common. Although an explanation for these gaps is not known, unfavourable environmental conditions (soil, moisture and soil physical property) and the impact of human settlement are suspected (Milimo, 1994).

2.3.2 Elevation

The elevation at which *M. volkensii* occurs given by different authors differs slightly. Milimo (1994) indicates elevations of up to 1,000 m. Elevations of 1,675 m have also been reported (Albrecht, 1993). The species can also grow at 0 meters above sea level (m.a.s.l.) as evident in the Kenyan coast at Lamu and Kwale (Milimo, 1994).

2.3.3 Soils

Most of the *Melia volkensii* range in East Africa is underlain by quaternary sedimentary and basement rocks. Carboniferous Permian and the Triassic rocks underlie small sections of the range, especially along the coast (Pratt and Gywnne, 1977). Three soil types arbitrary distinguished according to physical characteristics can be found within the range. These are sandy soils, clays and shallow storey soils (Pratt and Gwynne, 1977). Good drainage appears to be a common characteristic among most soils within this range although a few sites classified as imperfectly drained have been observed in parts of the range in Meru and Isiolo, Kenya (Milimo, 1994)

2.3.4 Climate

The climate in the range of *M. volkensii* is tropical, and since it spans only a short distance across the equator, there is little variation in seasonal changes in temperature (<5 °C; Milimo, 1994). The mean maximum for the hottest month does not exceed 37 °C and temperatures above 40 °C are rare (Pratt and Gwynne, 1977).

2.3.5 Rainfall

Mean annual rainfall in the *M. volkensii* range is variable ranging from 150-800 mm within the altitude range of 46-1,000 m a.s.l. Sites along the coast

of Kenya have one rainy season with maximum rainfall occurring in May and the minimum between January and February. Further inland, rainfall is bimodal with the first peak occurring between March and April and the second between October and November.

2.4 Utilisation

2.4.1 Agroforestry

Melia volkensii is among the most valued indigenous trees in the drylands (Kidundo, 1996; Stewart and Blomley, 1994). *In situ* conservation of wildlings is common (fig 2.2). The tree is compatible with most crops, *M. volkensii* is common in farmlands (fig 2.3).

2.4.2 Timber and Poles

Although *M. volkensii* is a multipurpose tree producing a range of products, timber is valued most as it provides cash to meet pressing needs. *M. volkensii* provides high quality timber that has a coarse structure and works easily and planes well. Its density is approximately 0.62 (Dale and Greenway (1961). Artisans compare it favorably with *Ocotea usambarensis* and *Vitex keniensis* while Dale and Greenway (1961) compare it with *Khaya* species. The heartwood is especially termite and decay resistant. Timber is converted *insitu* using pit sawers or contractors

using tractor mounted bench saws. One tree about 20 years old can generate as much as US $ 15 equivalent when sold by farmers (Stewart and Blomley, 1994). The timber is used locally for window and doorframes shatter, rafters and furniture while poles provide the corner and door posts of squire constructions, the central roof support of circular huts.

2.4.3 Beehives

Honey is highly valued both for sale and for brewing. *M. volkensii* is the major tree species used by farmers as it is easily worked and shaped. For similar reasons it is used for making acoustic drums, containers and mortars. In addition the flowers provide excellent bee forage.

2.4.4 Fodder

M. volkensii is a source of excellent fodder for livestock. The tree is pruned for fodder towards the end of the dry season a time when fodder is extremely scarce. Goats also eat the large fleshy drupes after they fall from trees. The fruit pulp is reported to contain 10 % crude fat, >12 % crude protein while the mature leaves are reported to contain 5 % crude fat and > 21 % crude protein (Milimo, 1990).

Fig. 2.2 ***Melia volkensii*** **growing at Mutomo in Kitui showing natural regeneration**

Fig. 2.3 ***Melia volkensii*** **growing at Mbololo showing intercropping**

2.4.5 Fuelwood

Branches looped from routine management are left to dry in the field before being used for firewood. Firewood and charcoal from *M. volkensii* produces smoke and is therefore said to be of poor quality.

2.4.6 Insecticide and Medicinal

Leaf preparations are used as flea and fly repellants especially those that attack goat (Riley and Brokensha, 1988). Various extracts from *M. volkensii* are widely reported to posses antifeedant and growth inhibitory effects on *Shistocera gregaria*-the desert locust (Rajab and Bentley, 1988), larvicidal and growth inhibitory effects against *Culex pipiens* (Sharook *et al.*, 1991) and *Anopheles arabiensis* a major malaria vector (Milimo, 1990). Traditionally, the Taitta have used preparations from *M. volkensii* for pain relief (Kokwaro, 1993).

2.5 Genetic variation in natural populations

Genetic variation occurs over time due to selection, mutation, gene flow and genetic drift. The forces in nature working to increase variation are gene flow and mutation while those that reduce it are selection and genetic drift (Hamrick, 1989). Levels of genetic variation differ significantly among species with different geographic ranges, life forms, and taxonomic affinities (Loveless, 1992). Cultivated taxa maintain higher polymorphism

and mean heterozygosity than tree species while woody perennials possess more variation at marker loci than short-lived plants (Loveless, 1992). Tropical tree species are predominantly outcrossing and populations are genetically structured, probably by spatial, ecological and phenological factors. Progeny arrays from individual adults are genetically variable, both within a single season and among different years, and individuals are also likely to differ in their reproductive contributions to the population in different seasons (Loveless, 1992).

2.6 Genetic variation in *M. volkensii*

The first attempt to study genetic variation in *M. volkensii* was by Milimo (1994). Isoenzyme electrophoresis and glass house screening experiments were used to screen for genotype variation. However, the Isoenzyme technique failed to adequately resolve genetic differences. Isoenzyme as a method of detecting variation in population-based studies is limited because it can only detect polymorphism at the coding sequences (Dawson *et al.*, 1993).

2.7 Importance of genetic variation

The genetic improvement of any organism depends on the existence, nature and extent of the genetic variability available for manipulation. The

partitioning of genetic variability between and within populations will influence the breeding strategy to be adopted (Chalmers *et al.*, 1992). An understanding of the underlying structure of genetic variation within and among plant populations is crucial for scientific approaches involving their conservation and utilisation (Dawson, 1995).

2.8 Analysis of genetic variation in plants

Prior to the development of biochemical and molecular markers, genetic resources were characterized by a combination of morphological and agronomic traits (Nei, 1975). This process is time consuming as many of the morphological descriptors can only be assessed at maturity (Wilde *et al.*, 1992). In addition, these characteristics may be influenced by environmental factors and may therefore not reflect the actual genetic similarities or differences (Dawson *et al.*, 1993).

2.9 Molecular markers

Biochemical and molecular techniques provide a powerful set of tools for the study of plant population genetics (Chalmers *et al.*, 1992). Isoenzymes are different molecular forms of the same enzyme and they can be separated by gel electrophoresis. These markers have been greatly applied in plant breeding, to characterize plant genetic resources (Chalmers, *et al.* 1992) and

in determining genetic relationships among breeding lines and populations (Tanksley and Orton, 1983).

With the advent of restriction enzymes and the polymerase chain reaction, assessment of genetic variation directly at the DNA level is possible and a wide range of techniques is now available.

2.9.1 Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLPs (Bostein *et al.*, 1980) are the original DNA markers developed in the late 1970's. The RFLP assay allows the detection of length polymorphisms in particular restriction fragments by hybridization with labeled probes. Such length polymorphisms can result from substitutions occurring in the DNA resulting in a sequence difference within a particular restriction site and a length difference in the fragment produced. Insertions or deletion of segments of DNA between two restriction sites may also occur changing the length of a particular fragment. RFLPs are extensively used in construction of genetic maps and have been applied successfully in genetic diversity assessments (Castagne *et al.*, 1994).

2.9.2 Polymerase Chain Reaction (PCR)

The development of the Polymerase Chain Reaction (PCR) (Mullis *et al.*, 1986) has revolutionized the analysis of nucleotide sequences. This

technique allows the *in vitro* amplification of DNA. It involves the annealing of oligonucleotides to homologous sequences in template DNA, followed by DNA polymerization primed by these oligonucleotides using deoxyribonucleotide triphosphates (dNTPs) as substrates. DNA amplification is brought about by repeated temperature recycling through; denaturation of double stranded DNA, annealing of primer to single stranded DNA target sequences and, extension of primers using target DNA as template (Brown, 1995).

The first experiment with PCR used the Klenow fragment of DNA polymerase I from *Escherichia coli*. However, Klenow activity is denatured by the high temperatures needed for denaturating the template DNA necessitating addition of new enzyme at the start of each cycle. The technique was revolutionized by the discovery of a heat-stable polymerase (*Taq* polymerase) isolated from thermophilic bacteria *Thermus aquaticus* (Saiki *et al.*, 1988).

2.9.3 PCR-RFLP

PCR-RFLP is a technique related to RFLP in which the restriction size differences define polymorphism between individuals, but the differences are visualized with locus specific PCR amplification products. This method

has been applied successfully in forest trees (Tsumura *et al.*, 1997; Harry *et al.*, 1998).

2.9.4 Amplified Fragment Length Polymorphism (AFLP) Analysis

Recently, AFLP markers have been developed (Vos *et al.*, 1995). The basic AFLP protocol consists of four successive steps involving; digestion with restriction enzymes, ligation to adapters, PCR amplification, and detection following electrophoresis on high resolution polyacrylamide gel (Glaubitz, 1995). Though a recent technique, AFLPs have been used widely in forest trees (Beismann, *et al.*, 1997, Ellis *et al.*, 1997, Muluvi *et al.*, 1999).

2.9.5 Single Strand Conformation Polymorphism (SSCP) and Denaturing Gradient Gel Electrophoresis (DGGE)

SSCP analysis (Hayashi, 1992) is an important method for detecting sequence differences in PCR products or restriction products of PCR products up to 400 bases long. Heating prior to their resolution on a cooled, non-denaturing polyacrylamide gel denatures PCR products. Single stranded DNA molecules develop secondary structures under denaturing conditions by folding upon themselves via internal complementary hybridization. A great variety of secondary structures are possible and the precise shape taken is highly sensitive to sequence differences. The different shapes produced often migrate differently on the non-denaturing

polyacryamide gel (Hayashi, 1992). Several studies in forest trees have utilised this technique (Watano *et al.*, 1995; Dumolin-Lapegue *et al.*, 1996; Bodenes *et al.*, 1997; Quijada *et al.*, 1997)

Like SSCP, DGGE (Myers *et al.*, 1987; Sheffield *et al.*, 1989) utilizes polyacryamide containing a gradient of increasing concentration-denaturing agents. PCR products are loaded in double stranded form but upon electrophoresis, reach a point in the gel where they begin to denature. Complete denaturation is prevented by the attachment of a 'GC clamp' to one end prior to loading (Sheffield *et al.*, 1989). The PCR products partially denature up to the GC clamps, starting from the lowest GC content. The partially denatured DNA molecules cannot migrate in the polyacrylamide, and form a light band at the point where they are partially denatured. PCR products of slightly different GC contents stop migrating at different positions in the gel. DGGE, has shown promise in its application in forest trees (Temesgen *et al.*, 1998).

2.9.6 Microsattelite Markers (Simple Sequence Repeats- SSRs)

Microsattelite markers consist of segments of DNA containing tandem repeats of simple motif sequences, usually one to five bases that are amplified by PCR (Webber and May, 1989; Morgante and Olieveri, 1993).

They are highly polymorphic due to the high number of alleles at a microsattelite locus, each having a different number of tandem repeats.

Application in tree species includes analysis of mating systems, paternity and patterns of gene flow. They are also useful in quality control in tree breeding programmes and for the certification of genetically improved seed and planting stock. The first microsattelites developed in trees were from *Pinus radiata* D. Don (Smith and Devey, 1994) and they have since been developed in many forest tree species including *Quercus* spp. (Dow *et al.*, 1995; Barret *et al.*, 1997; Isagi and Suhandono, 1997), *Eucalyptus* spp. (Byrne *et al.*, 1996) and *Pinus strobus* L. (Echt *et al.*, 1996, Dawson *et al.*, 1997).

2.9.7 DNA Sequencing

The most comprehensive analysis of genetic differences can be obtained by sequencing. With the advent of cycle sequencing methodology (Murray 1989), direct sequencing of PCR products is now fairly routine. However, this method is generally too expensive for population and conservation genetics applications for which large sample sizes are required.

2.9.8 Random Amplified Polymorphic DNA (RAPD) Analysis

The Random Amplified Polymorphic DNA (RAPD) assay (Welsh and Mclelland, 1990; Williams *et al.*, 1990) is based on the amplification of random DNA sequences by PCR using single primers of arbitrary nucleotide sequence (Bowditch *et al.*, 1993; Tingey and Tufo 1993; Williams *et al.*, 1993). The RAPD marker method detects DNA polymorphisms that result in the loss or gain of amplification product at a locus. Amplification can be lost owing to substitution within a primer-binding site or a large insertion between two primer-binding sites. Amplification can be gained from substitutions or insertions that create new primer binding sites hence bringing them within PCR amplifiable range of each other. Hence there are typically only two possible alleles at a RAPD marker locus represented by the amplified product (the present allele) and no band (the absence or null allele).

Williams *et al.*, (1993) described the RAPD reaction in details. To perform a RAPD reaction, a single oligonucleotide of arbitrary DNA sequence is mixed with a genomic DNA in the presence of a thermostable DNA polymerase and a suitable buffer and then subjected to temperature cycling conditions typical of the PCR. The products of the reaction depend on the sequence length of the oligonucleotide as well as the reaction conditions. At an appropriate annealing temperature, during the thermal cycle, the

single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (within a few thousand nucleotides). The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide binding sites on the genomic DNA.

The DNA amplification reaction is repeated on a set of DNA samples with several different primers under conditions that result in several amplified bands from each primer. Amplification products are separated electrophoretically in agarose gels giving banding patterns that can be scored for genetic variation (Sigurdson *et al.*, 1995). A single primer can be used to identify several polymorphism each of which maps to a different locus.

The technical ease of RAPD markers and their application to any species has led to their use in many studies in forest trees both in genetic linkage mapping (Tulsieram *et al.*, 1992; Grattapaglia and Sederoff 1994; Nelson *et al.*, 1994) and population genetic applications (Mosseler et al., 1992; Chalmers *et al.*, 1994; Isabel *et al.*, 1995; Nesbit *et al.*, 1995; Schierenbeck *et al.*, 1997).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant material

Leaf samples were collected from mature trees chosen at random from nine locations along the species natural distribution range in Kenya. The names of the populations sampled are shown in Table 3.1 and Fig 3.1. The sites where these populations are located differed from each other in terms of vegetation structure (species composition, richness and phenology), soil and climate (Pratt and Gwynne, 1977). Some of the populations chosen were from farmland while others were from National parks. Leaf samples were collected from 10 trees for each population bringing the total sample size to 90. To ensure adequate preservation, leaves were dried with silica gel in snap-top plastic containers (Milligan, 1992).

Table 3.1 ***Melia volkensii*** **leaf samples collected from 9 populations for RAPD analysis of genetic variation.**

Population Name	GPS readings	Altitude m	Population type	Population designation
Kitui	01°30', 30°50'	1 300	Farmland	A
Kibwezi	02°20', 38°57'	1 200	Farmland	B
Embu	00°48', 37°55'	1 050	Farmland	C
Isiolo	00°22', 37°35'	700	Farmland	D
Mbololo	03°20', 38°28'	1 166	Farmland	E
Galana	03°32', 37°20'	730	National park	F
Mwatate	03°30', 38°30'	900	National park	G
Meru	00°00', 37°36'	1 200	Farmland	H
Taveta	03°37', 37°44'	1 000	National park	I

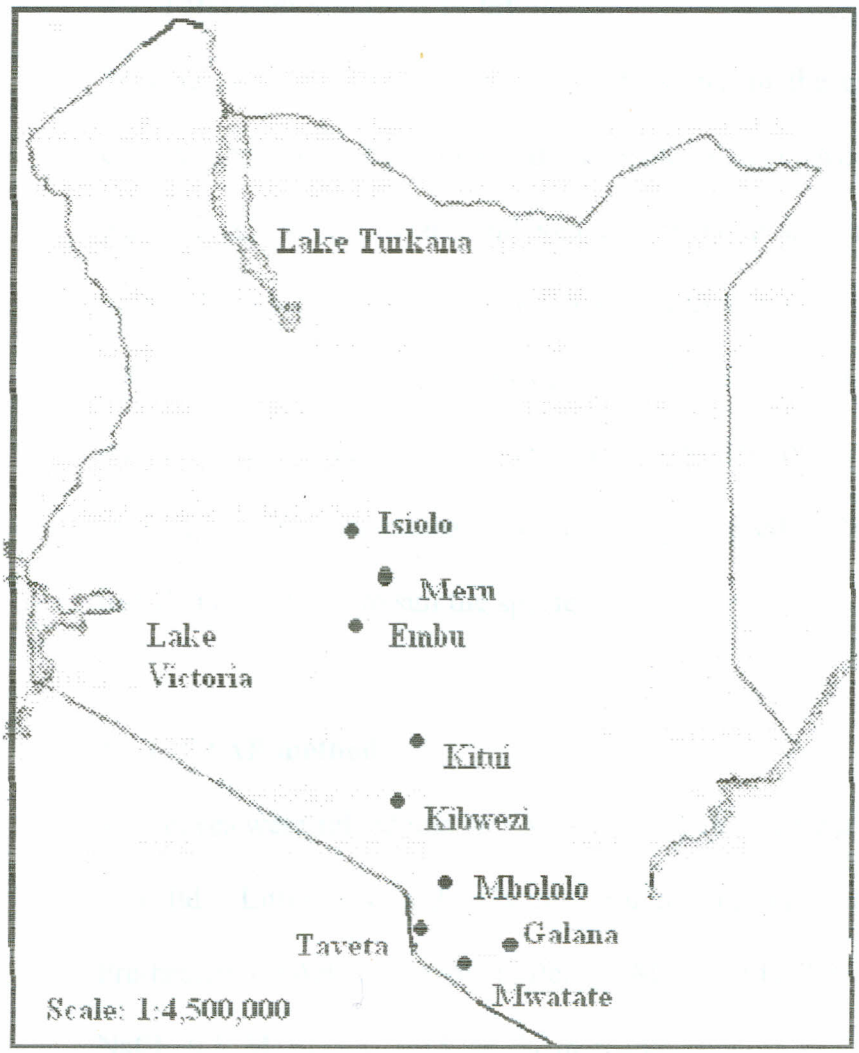

Fig. 3.1. ***Melia volkensii*** **leaf samples collected from 9 populations for RAPD analysis of genetic variation**

3.2 Total genomic DNA isolation

Previous workers have encountered difficulties in the extraction of good quality DNA from fresh or dried leaf material from woody species (Pyle and Adams, 1989; Rowland and Nguyen, 1993). It was therefore necessary to develop a protocol with which DNA of sufficient quality can be obtained for subsequent molecular analysis in *M. volkensii*. Two published protocols on DNA isolation, Murray and Thompson (1980) and Edwards *et al.* (1991), classified on the basis of the detergent used (SDS or CTAB) were tested and modified to suit the species.

3.2.1 CTAB method

The leaves were folded into two and punched by closing a 1.5 ml-microfuge tube lid. Little sand and polyvinyl polypyrollidone (pvpp) were added. Pre-heated CTAB extraction buffer (1 M Tris pH 7.5, 1.5 CTAB, 5 M NaCl, 0.5 M EDTA, 0.15 M % mercaptoethanol) was added to all the samples and the mixture vortexed for 5 seconds before incubating at 55 °C for 30 min. Following centrifugation (10 min at 10,000 g), 500 µl of the supernatant was transferred into a fresh microfuge tube and an equal volume of chloroform/isoamyl alcohol (24:1) added. The samples were mixed well by inversion and then centrifuged at 10, 000 g for 10 min. The aqueous phase (400 µl) was transferred to a clean microfuge tube and an equal volume of isopropanol added. The contents were mixed by inversion

and then centrifuged at 10, 000 g for 8 min to pellet the nucleic acids. The supernatant was poured off and the DNA pellets washed with 400 µl of 70 % ethanol by centrifuging at 10, 000 g for 1 min. The DNA pellets were dried and resuspended in 100 µl of TE buffer before storage at 4 °C.

3.2.2 SDS Method

Samples were collected using the lid of a sterile microfuge tube to pinch out a disc of leaf material. Extraction buffer (500 µl) consisting of 200 mM Tris HCl, pH 7.0, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS and 10 mM mercaptoethanol was added and the tissue macerated at room temperature using disposable grinders, with addition of (10-20 mg) insoluble polyvinyl pyrrolidone and sterile sand.

The samples were vortexed for 10 seconds and left for 45 min at room temperature. The extracts were centrifuged at 10,000 g for 10 min, 500 µl of the supernatant transferred to a fresh microfuge tube and an equal volume of chlorofom/isoamyl alcohol (24:1) added. The samples were mixed well by inversion to emulsify and then centrifuged at 10,000 g for 10 min.

The supernatant (400 µl) was transferred into a fresh microfuge tube and mixed with an equal amount of chilled isopropanol and left at room temperature for 2 min to precipitate the DNA. Following centrifugation at

10,000 g for 8 min, the supernatant was poured off and the pellet washed with 200 µl of 70 % ethanol by centrifuging at 10,000 g for 1 min. The ethanol was drained by inverting the tubes and the DNA pellet resuspended in 200 µl of TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA). The DNA was stored at 4 °C.

3.2.3 Modified SDS method

The method of Edwards *et al.* (1991) was adopted with some modifications to isolate total genomic DNA from *M. volkensii*. Samples were collected using the lid of a sterile microfuge tube to pinch out a disc of leaf material. Pre-warmed (60 °C) extraction buffer (500 µl) consisting of 1 M Tris HCl, pH 7.0, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS, 10 mM mercaptoethanol was added and the tissue macerated at room temperature using grinders, with addition of polyvinyl pyrrolidone (10-20 mg) insoluble and sterile sand. Following incubation in a water bath set at 60 °C for 30 min, DNA was extracted twice with 500 µl of chloroform/isoamyl alcohol (24:1) by centrifuging at 10,000 g for 10 mins. The aqueous layer (350 µl) was transferred to a clean microfuge tube and an equal volume of chilled isopropanol added. Samples were left at -20 °C for 30 min then centrifuged at 10,000 g for 8 min. The supernatant was poured off and the DNA pellets washed with 500 µl of 70 % ethanol by centrifuging at 10,000 g for 1 min. Ethanol was poured off and the pellet allowed to dry. The DNA pellet was

then resuspended in 100 µl of TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA) and stored at 4 °C.

3.3 DNA quantitation by ethidium bromide fluorescence

DNA yields were estimated by direct comparison with standard DNA concentrations in 1 % agarose gels buffered in 1 X TBE (89 mM Tris HCl pH 8, 89 mM boric acid and 2 mM EDTA) in horizontal electrophoresis apparatus. DNA (5 µl) was mixed with loading dye (0.25 % bromophenol blue, 0.25 % xylene, cyanol and 30 % glycerol), and 2µl of each of the series of DNA standard solutions (0, 2.5, 5, 20, 30, 40, 50 $\mu g ml^{-1}$). Gels were run at 150 V until the bromophenol blue moved 1 cm. DNA concentration was estimated from ethidium bromide stained gels and samples were diluted to a 10 $ng\mu l^{-1}$ working solution using sterile distilled water.

3.4 Spectrophotometric determination of DNA concentration

DNA measuring 5 µl was dissolved in 495 µl of 1 X TE buffer to make a 1:100 dilution. The optical density of the samples was measured at 260 nm and the concentration determined assuming an equivalent of 50 $mg ml^{-1}$ to 1 absorbance unit at 260 nm.

3.5 RAPD reaction

A total of 35 decanucleotide primers (Operon Technologies Inc.) were screened and evaluated for suitability in discerning polymorphism in *M. volkensii* populations. Of these, 8 were found to give good polymorphism and were used in subsequent analysis. Amplification was carried out in a 20 µl volume reaction mix, which contained 200 µM of each of the dNTPs (Life technologies), 1 X *Taq* polymerase buffer (Perkin Elmer), 3 mM $MgCl_2$ (Perkin Elmer), 0.2 µM primer (Life Technologies), 2.5 $ng\mu l^{-1}$ DNA and 0.75 unit's of *Taq* polymerase (Gold™; Perkin Elmer). Amplification conditions for *M. volkensii* were set as, 1 cycle of 15 min at 94 °C, 44 cycles of 1 min at 94 °C (denaturation), 1 min at 36 °C (annealing), 2 min at 72 °C (extension). A final 5 min extension (72 °C) was allowed to ensure full extension of all amplified products. The amplified products were maintained at 5 °C after termination of the experiment.

3.6 Gel electrophoresis

Amplification products were separated on 2 % agarose gel using 1 X TBE electrophoresis buffer (0.045 M Tris-borate and 0.001 M EDTA). DNA samples were mixed with 6 & gel loading dye (0.25 % bromophenol blue, 25 % Xylene Cyanol and 30 % glycerol). To assist in determining the sizes of unknown DNA fragments, 3µl of DNA marker (Life Technologies) in a loading buffer containing 10 mM EDTA, 0.04 % bromophenol blue and 5

% glycerol were loaded. Electrophoresis was carried out at 150 V and the gels photographed under UV illumination after staining with ethidium bromide (5 μgml^{-1}).

3.7 Data analysis

Various statistical methods were used in the analysis of genetic variation. Analysis was based on both individual and population frequency data.

3.7.1 Scoring of RAPD data

The presence and absence of RAPD bands were scored by visual inspection of the gel images. Each individual amplification product was considered to represent the dominant allele at a RAPD locus. The resulting data was transformed into a 1 (presence) or 0 (absence) matrix over all fragments scored.

3.7.2 Nei's unbiased diversity

Population allele frequency data and diversity values were calculated and analysed with POPGENE 1.31 (Yeh *et al.*, 1999) assuming Hardy-Weinberg equilibrium within populations and using Nei's unbiased statistic (Nei, 1987):

$$H = n\,(1 - \Sigma\,[p_i^2]\,/(n\text{-}1)$$

Where n = number of individuals analysed and p_i is the frequency if the ith allele. Diversity values were averaged across loci.

3.7.3 Nei's distance and cluster analysis

Genetic distance (D) between population frequency data sets was generated with POPGENE 1.31 (Yeh *et al.*, 1999) from the equations of Nei (1972):

$$D = \ln [\Sigma J_{XY}/(J_X J_Y)]$$

Where J_X, J_Y and J_{XY} are the arithmetic means of individual loci identities Σx_i^2, Σy_i^2 and $\Sigma x_i y_i$ over all loci respectively.

Cluster analysis based on Nei's genetic distance was undertaken using an unweighted pair-group method with arithmetic averaging (UPGMA; Sneath and Sokal, 1973) to generate phenograms.

3.7.4 AMOVA analysis

Components of variance attributable to differences between regions, between localities within regions and between individuals within localities were estimated by Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992) using Arlequin version 2000 (Schneider *et al.*, 2000).

Significance values were assigned to variance components based on the random permutation (5,000 times) of individuals.

CHAPTER FOUR

4.0 RESULTS

4.1 DNA isolation

The largest amount of good quality DNA was obtained from the leaves of *M. volkensii* using a modification of SDS extraction method described by Edwards *et al.* (1991). Modifications involved incubation in a water bath at 60 °C for 30 min and an extra chloroform/isoamyl alcohol extraction step. In addition, nucleic acids were precipitated using one volume of chilled isopropanol after incubation for 60 min at –20 °C and then resuspended in TE buffer. In this case, total DNA yields as measured by absorbance spectrophotometer were in the range of 1,000-3,000 μg ml^{-1} extracted from (0.02 g) of tissue. The average ratio of absorbance at 260 and 280 nm was 1.85.

4.2 Amplification products

Eight of the primers chosen generated a total of 72 bands out of which 38 were polymorphic. An example of the molecular profile generated by one of the primers (KP01) is shown in fig 4.1. Primers differed in the way they resolved polymorphism (Table 4.1). The number of polymorphic amplification products ranged from 2 (KP29) to 8 (KP01) while the size of the amplified products ranged from 150 to 2,000 base pairs. The lowest

percentage polymorphism was detected with primer KP29 (5.3%) while the highest was detected with KP01 (21.1%). No bands were found which were population specific. However some bands were found which occurred in high frequencies within populations from eastern province (Kitui, Kibwezi, Embu, Meru, Isiolo) and rare within coastal populations (Mbololo, Taveta, Galana, Mwatate). Conversely, some bands were common in the eastern populations but rare within coastal populations. The percentage polymorphic loci ranged from 21.1% (Galana) to 52.6% (Kibwezi).

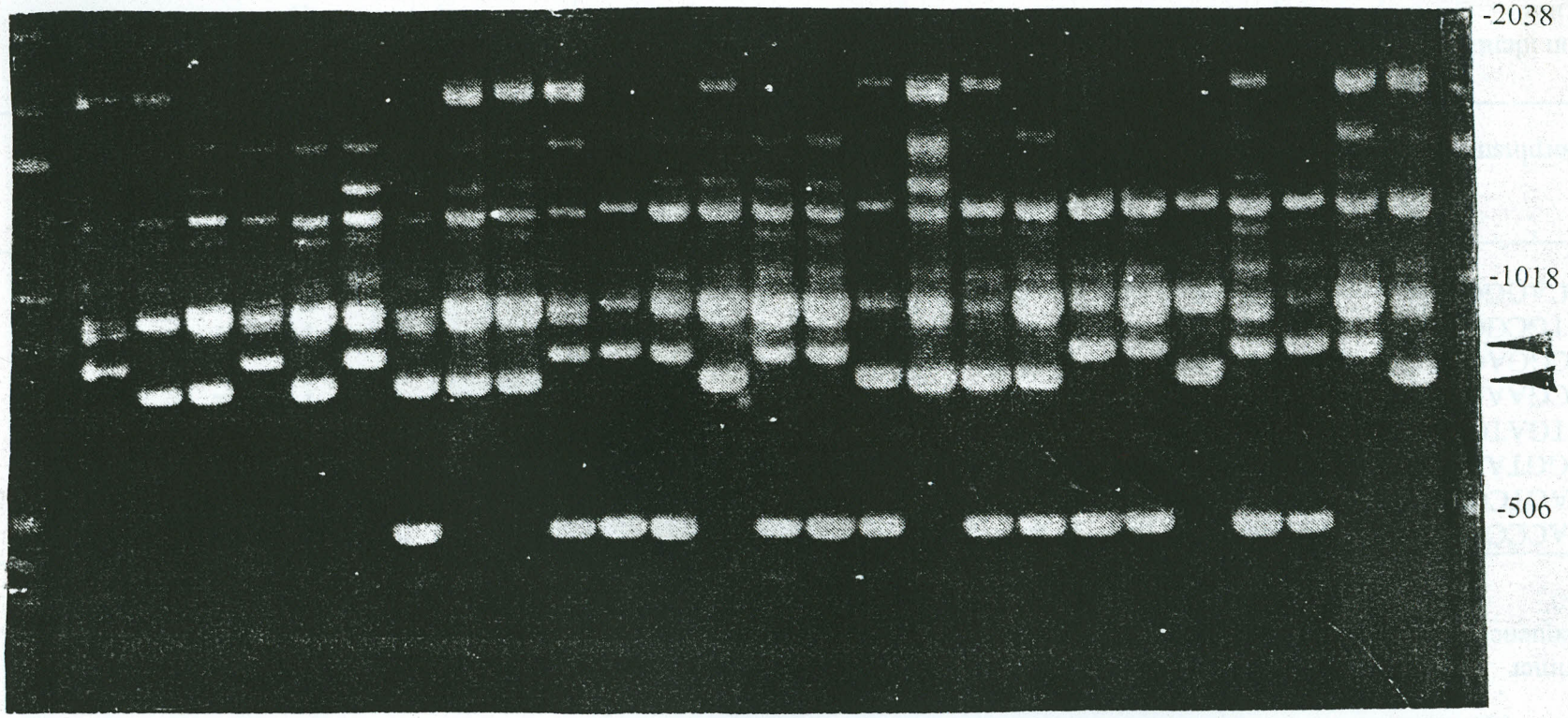

Fig. 4.1 An example of a RAPD profile generated using primer KP01. Some of the polymorphic bands scored are indicated with arrows. Lanes 1 and 28 are for DNA marker. Lanes 2-27 are samples studied in the order; 2-11 Kitui, 12-21 Kibwezi, 22-27 Embu.

Table 4.1 RAPD amplification products for 9 populations of *Melia volkensii* and the total number of bands generated {N(*T*)}, number of polymorphic bands {N(*P*)} and the % polymorphism (% P)

Primer No.	Primer Sequence 5'–3'	Product size (~bp) max-min	N (*T*)	N(*P*)	% P	No. of polymorphic products generated per population								
						Pop	A	B	C	D	E	F	G	H	I
KP01	GACCCCGGCT	2000-506	12	8	21.1		6	7	5	5	4	4	4	5	4
KP02	CAGTCGGGTC	1200-200	10	4	10.5		1	1	1	2	0	0	0	2	0
KP03	GGGTAACGCC	1100-250	10	5	13.2		2	2	2	2	1	0	0	1	0
KP04	GTGATCGCAG	1500-180	10	5	13.2		2	3	2	2	1	1	1	2	1
KP06	CCGAAGCCCT	1200-360	10	6	18.8		3	3	2	2	1	0	1	2	1
KP08	TCGGAGTGGC	1000-150	8	4	10.5		1	1	0	0	2	1	0	1	1
KP21	GTGCGGACAG	900-210	8	4	10.5		2	2	1	1	0	0	1	1	1
KP29	CCAGGCGCAA	640-300	4	2	5.3		1	1	1	1	2	2	2	0	2
Total			72	38			18	20	14	15	11	8	9	14	10
% polymorphism				52.8	11.3		47.4	52.6	36.8	39.5	28.9	21.1	23.7	36.8	26.3

Population identity

A	Kitui	F	Galana
B	Kibwezi	G	Mwatate
C	Embu	H	Meru
D	Isiolo	I	Taveta
E	Mbololo		

4.3 Genetic diversity

Nei's diversity estimates (Table 4.2) differed between populations. The Kibwezi population showed the highest level of diversity (0.1372) followed by the Kitui population (0.1196). The lowest level of genetic diversity was found in the Galana population (0.0663) followed by Mwatate population (0.0667). When diversity was compared between the regions (coastal and eastern) (Table 4.3) coastal populations had lower levels of genetic diversity (0.0697) than the eastern populations (0.1146). In addition, when genetic diversity was compared between farmland and natural populations, (Table 4.4) it was found that the highest average levels of genetic diversity were found in farmland populations (0.1075) compared to natural (0.069).

Table 4.2 Mean diversity estimates (*H*) for the 9 populations of *Melia volkensii* tested with 8 RAPD primers, based on Nei (1987) unbiased diversity estimates

Population Name	Population type	Region	*H*
Kitui	Farmland	Eastern	0.1196
Kibwezi	Farmland	Eastern	0.1372
Embu	Farmland	Eastern	0.0957
Isiolo	Farmland	Eastern	0.1131
Meru	Farmland	Eastern	0.1074
Mbololo	Farmland	Coastal	0.0718
Galana	National park	Coastal	0.0663
Mwatate	National park	Coastal	0.0667
Taveta	National park	Coastal	0.0740
Mean			0.0946

Table 4.3 Mean diversity estimates (*H*) for the eastern and the coastal populations of *Melia volkensii* tested with 8 RAPD primers, based on Nei (1987) unbiased diversity estimates

Eastern populations		Coastal populations	
Name	*H*	Name	*H*
Kitui	0.1196	Mbololo	0.0718
Kibwezi	0.1372	Galana	0.0663
Embu	0.0957	Mwatate	0.0667
Isiolo	0.1131	Taveta	0.0740
Meru	0.1074		
Mean	0.1146		0.0697

Table 4.4 Mean diversity estimates (*H*) for the farmland and natural populations of *Melia volkensii* tested with 8 RAPD primers, based on Nei (1987) unbiased diversity estimates

Farmland populations		Natural populations	
Name	*H*	Name	*H*
Kitui	0.1196	Galana	0.0663
Kibwezi	0.1372	Mwatate	0.0667
Embu	0.0957	Taveta	0.0740
Isiolo	0.1131		
Meru	0.1074		
Mbololo	0.0718		
Mean	0.1075		0.0690

4.4 Genetic relationships

A phenogram based on Nei's genetic distance is shown in fig 4.2. The neighbour joining analysis resolved the populations into two main clusters. One cluster consists of populations from the eastern region while the other cluster consists of populations from the coastal region. Within the coastal populations, two groups could be recognized one comprising of farmland populations while the other consisted of populations from the national park. Mwatate and Taveta populations were further grouped together. Among eastern populations, Kitui was grouped separately while the other populations from that region formed a unique cluster. The Kibwezi and Meru populations were further grouped together.

Genetic similarities and differences between the various populations is shown in Table 4.5. The highest similarity was found between Mwatate and Taveta populations (0.9635) followed by Kibwezi and Meru (0.9532). The lowest similarity was found between Mwatate and Kitui (0.8194) followed by Kitui and Galana (0.8315).

4.5 AMOVA analysis

The results of the AMOVA partitioning are shown in Table 4.6. Estimates of variance components within populations, between populations within regions (eastern and coastal), and between regions, calculated using

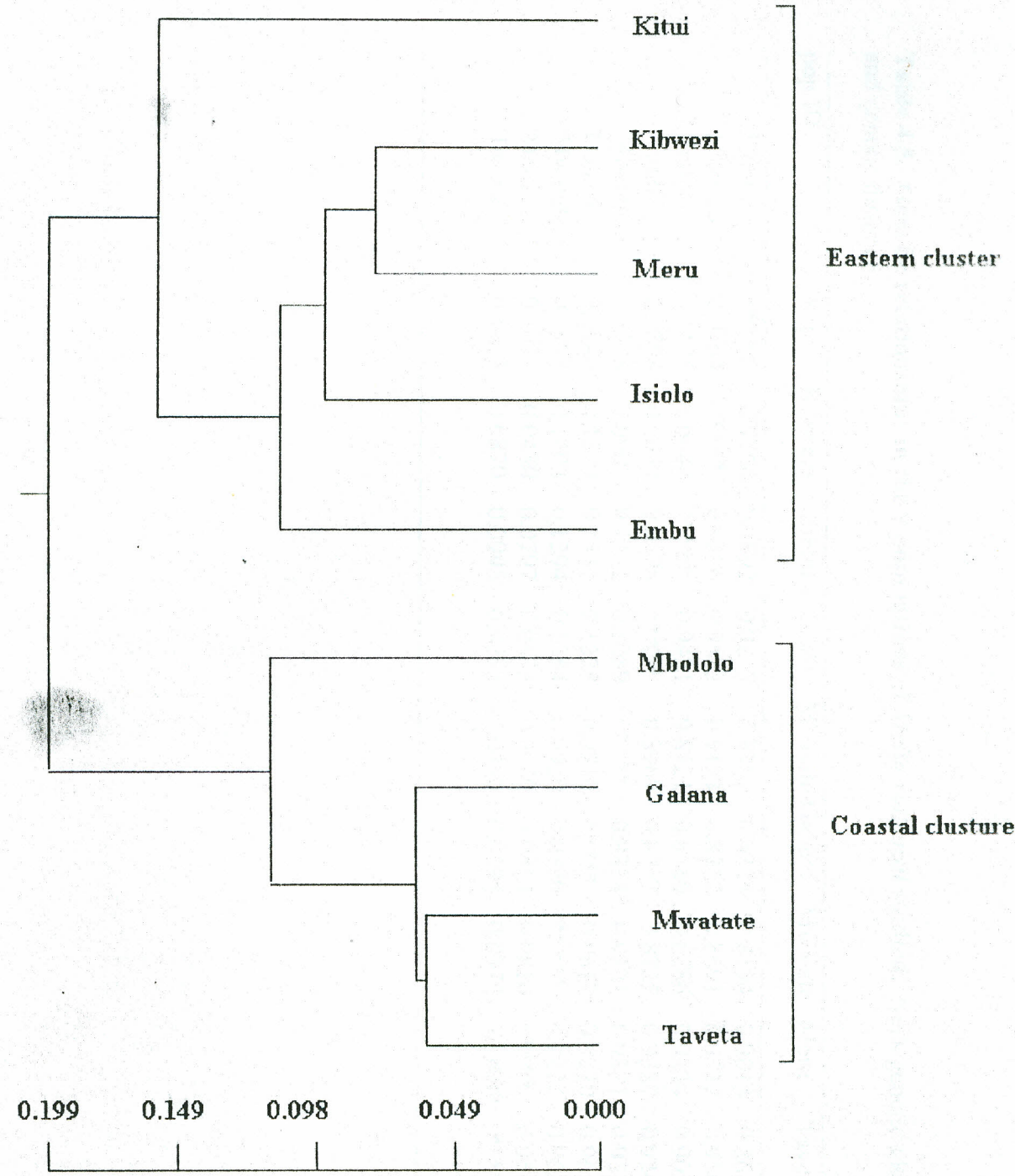

Fig 4.1. Phenogram based on Nei's (1978) genetic distance for 9 populations of *Melia volkensii* based on 38 RAPD markers.

Table 4.5. Genetic relationships in the 9 populations of *Melia volkensii* studied. Nei's genetic identity (above diagonal) and genetic distance

pop ID	Kitui	Kibwezi	Embu	Isiolo	Mbololo	Galana	Mwatate	Meru	Taveta
Kitui	*****	0.9264	0.8892	0.9233	0.8761	0.8315	0.8194	0.9013	0.8613
Kibwezi	0.0764	*****	0.9376	0.9475	0.9131	0.8712	0.8691	0.9532	0.9167
Embu	0.1175	0.0644	*****	0.9421	0.8921	0.8580	0.8786	0.9234	0.9053
Isiolo	0.0798	0.0539	0.0596	*****	0.8969	0.8757	0.8725	0.9387	0.9246
Mbololo	0.1323	0.0909	0.1142	0.1089	*****	0.9314	0.9121	0.9381	0.9531
Galana	0.1846	0.1379	0.1531	0.1328	0.0710	*****	0.9617	0.9220	0.9613
Mwatate	0.1992	0.1403	0.1294	0.1364	0.0920	0.0390	*****	0.9222	0.9635
Meru	0.1039	0.0480	0.0797	0.0633	0.0639	0.0813	0.0810	*****	0.9541
Taveta	0.1493	0.0870	0.0995	0.0784	0.0480	0.0395	0.0372	0.0469	*****

AMOVA (Excoffier *et al.*, 1992) revealed that most of the total variance could be attributed to variation within populations (75.4 %, $P < 0.0002$). However, a significant amount of variation (21.1 %, $P < 0.0002$) was attributable to variation between the eastern and the coastal regions. The small variance value of between population variation (3.5 %, $P < 0.0002$) indicates no structuring. When used to partition variation between farmland and natural populations, AMOVA revealed that (18.8 %, $P < 0.0002$) of the total variation is found between the two types of populations (farmland and national park).

Table 4.6. Analysis of molecular variance for *Melia volkensii* for the nine populations studied

Source of variation	d.f.	Variance component	% variance	*P* values
Between regions (Coastal & Eastern) Among groups	1	0.14	(21.12%)	< 0.0002
Between farmland & Natural populations Among groups	1	0.12	(18.8%)	< 0.0002
Among populations within groups	7	0.02	(3.49%)	< 0.0002
Within populations	81	0.48	(75.39%)	< 0.0002

CHAPTER FIVE

5.0 DISCUSSION, RECOMMENDATIONS AND CONCLUSIONS

5.1 Discussion

Isolation and purification of good quality DNA from plant tissue is fundamental for reliable and repeatable DNA fragment analysis. Difficulties in isolation and fractionation of nucleic acids from specific plant tissues have limited previous RAPD analysis (e.g. Kidwell and Osborn, 1992). Problems are caused by compounds extracted together with the DNA, especially polyphenols, proteins, carbohydrates, RNA and others, which decrease and interfere with the separation of DNA fragments during RAPD analysis (Couch and Fritz, 1990; Lodhi *et al.*, 1994; Mejjard, *et al.*, 1994).

In the present study it was possible to optimize a protocol for obtaining good quality DNA from the leaves of *M. volkensii*. The 1.85 absorbance value for the 260/280 ratio is well within the value expected for pure DNA of 1.8. Efficient disruption of plant tissue was found to be an important step in efficient extraction of DNA for *M. volkensii*. When more than 200 mgl^{-1} of extraction buffer was used, the DNA extraction yield was reduced. A dark brown contamination appeared during extraction. The oxidation material was probably phenoloxidase activity and a low pH in the extracting

solution (Steenkamp *et al.*, 1994). DNA extraction was performed with increased concentration of Tris-HCl (1M to the SDS buffer) and an additional extraction step using chloroform/isoamyl alcohol. Under these conditions, a highly pure total DNA was obtained. The increase in the total DNA obtained after the modification of the SDS method also confirmed earlier findings of Steenkamp *et al.* (1994). After addition of 1 volume of chilled isopropanol, some extracts did not precipitate and were thus kept at –20 ^{0}C for 60 minutes. This ensured sufficient precipitation of DNA pellets in all the samples.

Levels of genetic diversity within populations (H = 0.0946) estimated using Nei's (1987) diversity estimates compare to those found by Milimo (1994) using isoenzymes in three populations (Mbololo, Embu and Isiolo) of *M. volkensii*. Other workers using the RAPD approach in other tropical trees obtained similar results. For example Dawson *et al.* (1995) in the analysis of genetic variation in *Hordeum sponteum* populations in Israel found H to vary between 0.015 and 0.237. This genetic diversity is likely caused and maintained by effective gene flow within populations and high fecundity (Loveless and Hamrick, 1984; Hamrick and Godt, 1989). *M. volkensii* is also dispersed by ingestion by livestock and wild animals. Hamrick and Godt (1989) also found that trees dispersed by ingestion of seeds by animals are generally more variable within the populations.

M. volkensii is highly fecund with a seasonal yield of 100,000 viable seeds per tree (Milimo, 1994). Correlation between fecundity and genetic variation has already been documented. Highly fecund species are capable of a large variety of recombinant progeny with a potential for reaching and surviving in a wide variety of micro-habitats (Hamrick *et al.*, 1979). In addition, selection in highly fecund species is far more intense than in less fecund ones (Stebbins, 1958;Williams, 1975). However, the expected high genetic variation as a result of high fecundity might be compromised by the effects of seed dormancy that significantly reduces germination percentage (Milimo and Hellum, 1989) and therefore might effectively negate the assumption that species with high fecundity also have high genetic variation. The problem of high seed dormancy is further compounded by poor seedling recruitment. *M. volkensii* is also propagated asexualy by cuttings (stems and roots), and natural regeneration (coppicing). These modes of propagation may reduce the expected high diversity rates.

Diversity within populations from the national parks is low in comparison to farmland populations. This low polymorphim could be a consequence of the distribution of *M. volkensii* and population size. High population densities are found on-farm in the eastern province and the distribution becomes diffuse in the coast where only a few stands exist in the national parks. Distribution range and population size have been identified as major

correlates of within-population genetic variation in tropical tree species with restricted populations showing significantly less variation than those with broader distribution (Loveless, 1992). On the other hand, genetic diversity varies directly with population size (Travis *et al.*, 1996). The three populations (Mwatate, Galana and Taveta) are all in the Tsavo national park. Confinement of these populations in the national reserves may also compromise diversity due to decreased potential for long distance seed dispersal by game. Also worth of mention is the observation that the national park populations are also taken from the coastal populations which from the one farmland population (Mbololo), also taken from the coastal region seem to habour lower diversity estimates than the eastern region. Thus the lower diversity within the national park samples may be also a consequence of the region in which they were sampled rather than the population effects in the national parks relative to farmlands.

AMOVA results revealed more variation within populations (75.5 %) than between populations (24.5%). These values are entirely in agreement with what is expected of an outcrossing plant (Loveless, 1992), and are similar to those obtained by other workers, for example a study utilizing isoenzymes in *Cordia alliandra*, a neotropical tree species indicated that variation within populations from Central America accounted for 88 % of the total diversity recorded (Chase *et al.*, 1995). Hall *et al.* (1994) found that the

variation within populations of *Pentaclethra macroloba*, a tree species restricted to the Atlantic region within Costa Rica, accounted for 78.1 % of the total variation observed within this species.

However contrary to our findings, some workers obtained higher values for between population diversity. For example, Muluvi (1998) using RAPDs to study population genetics of *Moringa oleifera and M. stenopatela* obtained higher mean diversity between populations (69 %) than within populations. In Chalmers *et al.* (1992) on *Gliricidia sepium* and Harris *et al.* (1994) on *Leuceana leucocephala* using isoezymes, the diversity among populations was 60 % and 70 % respectively. The high between population variation in Muluvi, (1998) was attributed to the mixed mating system with a high proportion of selfing of the two *Moringa* trees. The association between breeding system and levels of genetic variation has been well documented. In general, most selfing species are characterised by higher variation among populations whereas predominantly outcrossed wind pollinated species exhibit less variation among populations (Loveless and Hamrick, 1984; Falk and Holsinger, 1991). The present results therefore suggest that *M. volkensii* is outcrossing and gene flow occurs over long distances. The present data does not lend support to the earlier hypothesis of Milimo (1994) who suggested that *M .volkensii* could be inbred. The mating system of *M. volkensii* nevertheless needs further investigation.

Although diversity between populations was lower than within-population diversity, highly significant ($P < 0.0002$) genetic difference among regions was detected in the AMOVA (Table 4.6). The significant degree of variation between the eastern and the coastal populations indicates a close association between ecogeograpical factors and RAPD variation as has been found in *Hordeum spontaneum* (Dawson *et al.*, 1993). In the case of *M. volkensii*, parent rock and rainfall could have influenced divergence. While the eastern populations of *M. volkensii* are underlain by quaternary sedimentary and basement rocks, the coastal ones are underlain by carboniferous, Permian and Triassic rocks (Pratt and Gwynne, 1977). Rainfall is also variable between the two regions as sites along the coast have one rainy season with the maximum rainfall occurring in May. Inland, rainfall is bimodal with the first peak occurring between March and April and the second one occurring between October and November (Pratt and Gwynne, 1977). In addition, although gene flow may occur between populations in the range, such movement between the coastal and the eastern populations is limited because of distance and disjunction in the distribution range of the species.

The UPGMA phenogram (Fig. 4.2) showed a differentiation in two groups basically corresponding to two geographic regions (coastal and eastern). This is the first study to demonstrate splitting in a plant species between the

eastern and coastal populations of Kenya. However, Mwangi (2001) using RAPDs to study genetic variation in *Prunus africana* found a split between the western and the eastern populations. This split was attributed to ecogeographical differences between these two regions (Mwangi, 2001).

Three coastal populations from the national park (Mwatate, Galana and Taveta) formed a cluster that excluded Mbololo. This is indicative of effective gene flow among the three populations and limited gene flow with Mbololo. Whereas the three populations are confined in the national reserves, Mbololo is a farmland population and gene flow with these populations is unlikely. The eastern populations formed similar clusters that further indicate effective gene flow within populations.

Given its geographical position, it is somewhat surprising that the Kitui population should form its own cluster. The difference in the Kitui population may lend support to the earlier morphological data, which showed the Kitui population to vary from others in growth, germination and leaf margin (KEFRI unpublished). However, this was not reflected in the RAPD profile by unique loci probably due to the sampling of the genome by the RAPD primers. RAPDs sample a random section of the genome while only only a few genes subject to varying levels of selection may

control morphological traits such as seed dormancy. The findings however need further investigation especially with a higher resolution technique.

5.2 Recommendations

These results have a number of implications on the conservation of *M. volkensii*. RAPD variation was found within populations suggesting that sampling from a few localities for either breeding or conservation could capture a large proportion of the variation within the species. Nevertheless significant variation was found between regions (eastern and coastal) and these have profound implications for conservation of *M. volkensii*. In particular, populations in these two regions need to be adequately conserved if the full breadth of genetic variation across the species range is to be maintained. Therefore, sampling should be done across each group's geographical range to ensure more representative sampling of genetic variation. Since the levels of genetic differentiation observed might be related to adoptive variation, structured progeny trials are required to assess the performance of different populations in the different regions for traits of interest such as timber and fodder.

Since natural stands continue to decline drastically and only a few still exist in the national parks (Shepherd, 1989; Riley and Bronkeshaw, 1998), efforts should be made to conserve the remaining stands *ex situ* to serve as living

orchards especially because present work has shown national park populations to be less diverse. On farm conservation strategy of *M. volkensii* need to be emphasized since most of the variation is found on farmland populations. Major emphasis should therefore be placed on propagation and conservation of wildlings on-farm. Interestingly, farmland populations seemed to habour at least as much diversity as those from national parks. This may be a consequence of the way in which farmland samples are taken, in that they may represent a good sample of the local variation. The possibility and benefits of using a *circa situ* (i.e. farmers are encouraged to use the species material of local provenance) approach to conservation need to be encouraged. Such a strategy would mean that the farmers have the benefit of globally maintaining species diversity. In addition, populations where diversity is low especially within the coastal populations, (Galana, Taveta, Mwatate and Mbololo) material from more diverse populations (Kibwezi and Kitui) can be used to make fresh infusions.

A big sample size could not be obtained in some populations especially in the national parks. There were also cost constraints in analyzing many samples. A relatively small sample size of 10 individuals per population was used. Small sample sizes however are prone to variation and there is therefore need for a much bigger sampling. In addition, current work

investigated variation in 9 *Melia volkensii* populations in Kenya. Further research is needed to determine genetic variation within the species entire range (Ethiopia, Tanzania and Somalia).

Further work need to be done on populations, which were very divergent to determine if there are important quantitative traits, associated with those populations. In addition, mating systems and gene flow, which are of central importance to conservation biologists (Chase *et al.*, 1996) need to be investigated.

5.3 Conclusion

A basic protocol for DNA isolation in *Melia volkensii* was optimized and RAPDs provided new insights into the patterns of genetic variation within and between *M. volkensii* populations. This knowledge of the genetic structure can be considered an essential first step in the development of a sound conservation strategy for *M. volkensii* genetic resources.

Significant amount of variation was observed between the eastern and the coastal populations of *M.volkensii* and therefore populations in these two regions need to be fully conserved to maintain the full breadth of genetic diversity. National park populations were found to be less diverse and these need to be conserved *ex situ* to serve as orchards.

REFERENCE

Albrecht, J. 1993. Tree seed handbook of Kenya. Kenya Forestry Seed Centre, Muguga Kenya.

Azzouzi, K., Vekemans X., Meerts, P and Lefebrre, C. 1997. Allozyme variation in calciocolous and silicolous populations of *Heliantheemum numnularium*. *Belgian Jounal of Botany* **129,** 101-106.

Barret, C., Lefort, F., and Douglas, G.C. 1997. Genetic characterization of oak seedlings, repicomic, crown and micropropagated shoots from mature trees by RAPD and microsatellite PCR. *Sciantia Horticulturae* **70**, 319-330.

Bawa, K.S. and Krugman, S.L. 1991. Reproductive biology and genetics of tropical trees in relation to conservation and management In: Gomez-Pompa, A., Whitemore T.C. and Hadley M. (eds) *Rain Forest Regeneration and Management.* UNESCO and Pathenon Press, Paris, MAB series **6,** pp. 119-136.

Beentje, H. 1994. Kenya Shrubs and Lianas. National Museums of Kenya. Nairobi.

Beismann, H., Baker, J.H.A., Karp, A and Speck, T. 1997. AFLP analysis sheds light on the distribution of two *Salix* species and their hybrids along a natural gradient. *Molecular Ecology* **6**, 989-983.

Bodeness, C., Labbe, T., Pradere, S. and Kremer, A. 1997. General Vs local differentiation between two closely related white oak species. *Molecular Ecology* **6**, 713-724.

Boistein, D., White, R.L., Skolnick, M. and Davis, R.W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* **32**, 314-331.

Bowditch, B.M., Albright, D.J., Williams, J.G.K. and Broun, M.J. 1993. Use of randomly amplified polymorphic DNA markers in comparative genome studies. *Methods in Enzymology* **224**, 294-309.

Brown, T.A. 1995. Gene Cloning: an introduction Chapman and Hall London U.K.

Byrne, M., Marquez-Garsia, M.I., Uren, T., Smith, D.S. and Moran, G.F. 1996. Conservation and genetic diversity of microsatellite locus loci in the genus Eucalyptus. *Australian Journal of Botany* **44**, 331-341.

Castagne, R. G., Maga, Perenzin, M. and Henn, M. 1994. RFLP-based genetic relationships of Einkon wheats. *Theoretical and Applied Genetics* **88**, 818-823.

Chalmers, K.J., Newton, A.C., Wough, R., Wilson, J. and Powell, W. 1994. Evaluation of the extent of variation in mahoganies (Meliaceae) using RAPD markers. *Theoretical and Applied Genetics* **89**, 504-508.

Chalmers, K.J.; Waugh, R.J., Sprent, J.L., Simons, A.J. and Powell, W. 1992. Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. Maculata* using RAPD Markers. *Heridity* **69**, 465–472.

Chase, M.R., Boshier, D.H. and Bawa, K.S. 1995. Population genetics of *Cordia alliodara* (Boranginaceae), a neotropical tree. Genetic variations in natural populations. *American Journal of Botany* **82,** 468-475.

Chase, M., Kessel R. and Bawa, K. 1996. Microsatelitte markers for population and conservation genetics of tropical trees. *American Journal of Botany* **83**, 51 – 57.

Crawford, D.J. 1990. Plant Molecular Systematics; Macromolecular Approaches. John Wiley and Sons, New York. Pp. 338.

Couch, J.A. and Fritz, P.J 1990. Isolation of DNA from plants high in polyphenolics. *Plant Molecular Biology Reporter* **8,** 8-12.

Dale, I.R, and Greenway, P.J. 1961. Kenya Trees and shrubs. Buchanan's Kenya Estate Limited, London, UK.

Dawson, I.K. 1995. Molecular biology and population genetics of the tropical tree legume *Gliricidia*. PhD Thesis, Dundee University, U.K.

Dawson, I.K., Chalmers, K.J., Waugh, R. and Powell, W. 1993. Detection and analysis of genetic variation in *Hordeum spontaneum* population from Israel using RAPD Markers. *Molecular Ecology* **2**, 151 – 159.

Dawson, I.K., Simons, A.J., Waugh, R. and Powell, W. 1995. Diversity and genetic differentiation among sub-populations of *Gliricidia sepium* revealed by PCR-based assays. *Heredity* **74**, 10 – 13.

Dawson, I.K., Waugh, R., Simons, A.J. and Powell, W. 1997. Simple sequence repeats provide a direct estimate of pollen-mediated gene dispersal in the tropical tree *Gliricidia sepium* . *Molecular Ecology* **6**, 179-183.

Dow, B.D., Ashley, M.V. and Howe, H.F. 1995. Characterization of highly variable $(GA/CT)_n$ microsatellites in the bur oak, *Quecus macrocarpa*. *Theoretical and Applied Genetics* **91**, 179-183.

Dumolin-Lapegue, S., Bodenes, C. and Petit, R.J. 1996. Detection of rare polymorphisms in mitochondrial DNA of oaks with PCR-RFLP combined to SSCP analysis. *Forest Genetics* **3**, 227-230.

Echt, C.S., Maymarquardt, P., Hseih, M. and Zahorchak, R. 1996. Characterization of microsatellite markers in eastern white pine. *Genome* **39**, 1102-1108.

Edwards, K., Johnson, C. and Thomson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* **19**, 1349.

Ellis R.P., McNicol, J.W., Baird, E., Booth, A., Lawrence, P., Thomas, B. and Powell, W. 1997. The use of AFLPs to examine genetic relatedness in barley. *Molecular Breeding* **3**, 359-369.

Excoffier, L., Smouse P.E. and Quattro J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 449-491

Falk, D.A. and Holsinger, K.E. 1991. Genetics and conservation of rare plants. Oxford University Press, Oxford.

Glaubitz, J.C. 1995. Applications of molecular markers to forest genetics: gene expression, genetic linkage mapping, and genetic diversity. PhD Thesis, University of British Columbia, Vancouver.

Grattapaglia, D. and Sederoff, R. 1994. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross mapping strategy and RAPD markers. *Genetics* **137**, 1121-1137.

Hamrick, J.L. 1989. Isoenzymes and the analysis of genetic structure in plant populations. In: Soltis D.E. and Soltis P.S. (eds) *Isoenzymes in Plant Biology*. Dioscorides Press Portland , OR, USA., pp. 87-105

Hamrick, J.L. 1992. Distribution of genetic diversity in tropical tree population: implications for the conservation of genetic resources. Paper to: IUFRO S2.02-08 conference, Breeding tropical trees, Cali and Cartagena, Columbia. 9-18 October, 1992.

Hamrick, J.L. and Allard R.A. 1972. Microgeological variation in allozyme frequencies in *Avena barbata*. *Proceedings of National Academy of Science* USA **69**, 2000-2004.

Hamrick, J.L. and Godt, M.J.W. 1989. Allozyme diversity in plant species. In: Brown A.H.D., Clegg M.T., Kahler A.L. and Weir, B.S. (eds) *Plant Population Genetics, Breeding and Genetic Resourses*. Sinauer, Sounderland, 4363.

Hamrick, J.L., Linhart, Y.B. and Mitton, J.B. 1979. Relationships between characteristics and elecrophoretically detectable genetic variation in plants. *Annual Review of Ecological Systems* **10**, 173-200.

Harris, S.A., Hughes, C., Abbott, C. and Ingram, R. 1994. Genetic variation in *Leucaena leucocephala* (Lam) de wit. (Leguminosae: mimosoidae). *Silvea Genetica* **43**, 159-167.

Harry, D.E., Temesgen, B. and Neale, D.B. 1998. Codorminant PCR-based markers for *Pinus tadeata* developed from mapped cDNA clones. *Theoretical and Applied Genetics* **97**, 327-336.

Hayashi, K. 1992. A method for detection of mutations. *GATA,* 973-979.

Hueneke, F.L. 1991. Ecological implication of genetic variation in plant populations. In: Falk, D.A. and Holsinger, K.E. (eds) *Genetics and*

Conservation of Rare Pants. Oxford University Press, New York, NY, pp31-44

Huff, D.R., Peakall, R. and Smouse, P.E. 1993. RAPD variation within and among natural populations of outcrosing Bufallograss [*Buchloe dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics* **86**, 927 – 934

Isabel, N., Beauleu, J. and Bousquet, J. 1995. Complete congruence between gene diversity estimates derived from genotypic data at enzyme and RAPD loci in black spruse. *Proceedings of the National Academy of Sciences, USA* **92**, 6369-6373.

Isagi, Y. and Suhandono S. 1997. PCR primers amplifying microsatellite loci of *Quercus myrsinifolia* Blume and their conservation between oak species. *Molecular Ecology* **6**, 897-899.

Karp, A. and Edwads, K.J. 1997. Molecular techniques in the analysis of the extent of distribution of genetic diversity. In: Ayed W., Hodgkin, T., Jordet, A. and Rao, V.R. *Molecular Genetic Techniques for Plant Genetic Resources* (eds) Report of an IPGRI workshop 9th October 1995, Rome Italy.

Kidundo, M. 1996. Participatory technology development and nursery propagation of *Melia volkensii* Gurke: A potential agroforestry tree species for semi-arid Kenya. Mphil Thesis, University of Wales, Bangor.

Kidwell, K. K. and Osborn, T.C. 1992. Simple plant DNA isolation procedures. In: Beckmann, J.S. and Osborn, T.C. (eds) *Plant Genomes Methods for Genetic And Physical Mapping.* Kluwer Academic Publishers, London pp.114

Kokwaro, J.O. 1993. Medicinal plants of East Africa 2nd edition. Kenya Literature Bureau, Nairobi.

Leuthold, B.M. and Leuthold W. 1972. Food habits of giraffe in Tsavo National Park, Kenya. *East Africa Wildlife Journal* **10**, 129-141.

Lodhi, M.A., Gw Ye Weeden, N.F. and Reisch, B.I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Plant Molecular Biology Reporter* **12,** 6-13.

Loveless, M. D. 1992. Isoenzyme variation in tropical trees: Patterns of genetic organization. *New Forests* **6**, 67-94.

Loveless, M.D. and Hamrick, J.L. 1984. Ecological determinants of genetic structure in populations. *Annual Review of Ecological Systems* **15,** 65-95.

Mabberley, D.J. 1997. The Plant Book a Portable dictionary of the vascular plants. 2nd Edition Cambridge University Press.

Mejjard, M. Vedel, F. and Ducreaux, G 1994. Improvement of DNA preparation and of PCR cycling in RAPD analysis of marine macroalgae. *Plant Molecular Biology Reporter* **12,** 101 –105.

Milligan, B.G. 1992. Plant DNA isolation. In Hoelzel, A.R. (eds) *Molecular Genetic Analysis of Population.* Oxford, UK IRL Press. pp. 59 – 88

Milimo, P.B. 1989. The control of germination in *Melia volkensii* seeds. Msc. Thesis, Department of Forest science, University of Alberta, Canada.

Milimo, P. B. 1994. Mechanisms of Drought Resistance in *Melia volkensii* and *Melia azedarach.* PhD Thesis, Australian National University, Canberra.

Milimo, P.B. 1990. New uses for *Melia azedarach* and *Melia volkensii. Forestry Newsletter* 10, Australian Center for International Agricultural Research, Canberra.

Milimo, P.B. and Hellum, A.K. 1989. Dormancy in the seeds of *Melia volkensii* Gurke. *East African Agriculture and Forestry Journal.* **54,** 111-122.

Moran, G.F. 1992. Pattern of genetic diversity in Australian tree species. *New Forests* **6**, 49-66.

Morgante, M. and Olieveri, A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal* **3**,175-182.

Mosseler, A., Egger, K.N. and Hughes, G.A. 1992. Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers. *Canadian Journal of Forestry Research* **22**, 1332-1337.

Mullis, K., Faloona, S. Scharf, S., Saiki, R., Horn,G. and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro*: The polymerase

chain reaction. *Cold spring Harbor Symposim Quanitative Biology* **51**, 263-273.

Muluvi, G.M. 1998. Molecular ecology and population genetics of two multipurpose trees, *Moringa oleifera* Lam. and *Moringa stenopetula* (Bark.F.). Cuf. PhD Thesis, University of Dundee, Dundee, UK.

Muluvi, G.M., Sprent, J.I., Saranzo N., Provan, J., Odee, D., Folkland, G., McNicol, J.W. and Powell, W. 1999. Amplified Fragment Length Polymorphism analysis of genetic variation in *Moringa oleifera* Lam. *Molecular Ecology* **8**, 463-470.

Murray, M.G. and Thomson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-4321.

Murray, V. 1989. Improved double stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Research* **17**, 8889.

Mwangi, A. 2001. Genetic variation in the threatened medicinal tree *Prunus africana (Hook)* in Kenya and implications for the genetic

management of the species. Msc. Thesis. Kenyatta University, Kenya.

Myers, R.M., Maniatis, T., and Lerman, L.S. 1987. Detection and localization of single base changes by denaturing gradient gel electrophoresis. *Methods in Enzymology* **155**, 501-527.

National Research Council. 1991. Managing global genetic resources. Forest Trees National Academy Press, Washington D.C. pp 228.

Nei, M. 1972. Genetic distance between populations. *American Naturalist* **106,** 283-292.

Nei, M. 1975. Molecular Population Genetics and Evolution. North-Holland Publishing Company Oxford.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583 – 590.

Nei, M. 1987. Molecular Evolution Genetics, Columbia University Press, New York, USA.

Nelson, C.D., Kubisiak, T.l., Stine, M. and Nance, W.L. 1994. A genetic linkage map of long leaf pine (*Pinus palustris* Mill.) based on random amplified polymorphic DNA. *Journal of Heridity* **85**, 433-439.

Nesbit, K.A., Potts, B.M., Vaillancourt, R.E., West, A.K. and Reid, J.B. 1995. Partitioning and distribution of RAPD variation in a forest tree species *Eucalyptus globlus (*Myrtaceae). *Heridity* **74**, 628-637.

Pratt, D.J. and Gwynne, M.G. 1977. Rangeland management and Ecology in East Africa. Hodder and Stoughton, London. 310 pp.

Pyle, M.M. and Adams, R.P. 1989. *In-situ* preservation of DNA in plant specimens. *Taxon* **38**, 574 – 581.

Quijada, A., Liston, A., Robinson, W. and Alvarez-Buylla, E. 1997. The ribosomal ITS region as a marker to detect polymorphism in pines. *Molecular Ecology* **6**, 995-996.

Rajab, M.S. and Bentley, M.D. 1988. Tetranoortriterpenes from *Melia volkensii. Journal of Natural Products* **51 (5),** 840-844.

Riley and Brokenshaw 1988. The Mbeere in Kenya, Volume II, Botanical identities and uses. Institute of Anthropology, University Press of America, USA

Rowland, L.J. and Nguyen, B. 1993. Use of polyethylene glycol for purification of DNA from leaf tissues of woody plants. *Biotechniques* **14**, 735 – 736.

Saiki, P.K., Genfond, D.H., Stoffel, S., Scharf, S.J., Higuchi, R. Horn, B.T., Mullis K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplifications of DNA with a thermostable DNA polymerase. *Science*, **239**, 487 – 491. Hall, London.

Scheon, D.J. and Brown, A.H. 1985. Maximizing genetic diversity in core collections of wild relatives of crop relatives of crop species. In: Hodgkin T., Brown A.H.D., Van Hintum, J.L. and Morales, E.A.V. (eds) *Core Collections of Plant Genetic Resourses*. John Wiley and Sons, Chichester, pp. 55-76.

Schierenbeck, K.A., Skupski, M, Lieberman, D. and Lieberman, M. 1997. Population structure and genetic diversity in four tropical tree species in Costa Rica. *Molecular Ecology* **6**, 137-144.

Schneider, S., Kneffer, J.M., Roessli, D. and Excoffier, L. 1997. Arlequin Ver. 2000. A software for population genetic data analysis. Genetics and Biometry Laboratory, University of Geneva.

Sharook, Balan, K.G. Jiang, Y. and Rembold, H .1991. Insect growth inhibitors from two tropical Meliaceae. Effect of crude seed extracts on mosquito larvae. *Journal of Applied Entomology* **5**, 425–430.

Sheffield, V.C., Cox, D.R., Lerman, L.S. and Myers, R.M. 1989. Attachment of a 40- base pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single base changes. *Proceedings of the National Academy of Sciences, USA* **86**, 232-236.

Shepherd, G. 1990. Trees in the farming system of Mount Kenya. In: Tarltone G.L. (ed) Forestry: A Multiple-Use Enterprise, *Proceedings of the Thirteenth Commonwealth Forestry Conference*, Rotorua, Newzealand, pp. 224-230.

Sigurdson, V., Anamthawat-Jonsson, K. and Siurgeirsson, A. 1995. DNA fingerprinting of *Populus trichocarpa* clones using RAPD markers. *New Forest* **10**, 197-206.

Smith, D.N. and Devey M.E. 1994. Occurance and inheritance of microsatellites in *Pinus radiata. Genome* **37**, 977-983.

Sneath, P.H.A. and Sokal, R.R. 1973. Numerical Taxonomy. Freeman San Francisco, United States of America.

Stebbins, G.L. Jnr. 1958. Variation and evolution in plants. Columbia University Press, New York. pp 463.

Steenkamp, J., Wiid, Y., Lourens, A. and Van Helden, P. 1994. Improved method for DNA extraction for *Vitis vinifera* . *American Journal of Euology and Viticulture* **45**, 102-106.

Stewart, M. and Blomley T. 1994. Use of *Melia volkensii* in a semi-arid agroforestry system in Kenya. *Commonwealth Forestry Review* **73**, 128 – 131.

Stewart, M. and Chirchir R.K. 1992. Dryland research in the Embu-Meru- Isiolo Forestry Project Kenya, KEFRI/ODA.

Sun, M., Wong, K.C. and Lee, J.S.Y. 1998. Reproductive biology and population genetic structure of *Kandelia candel* (Rhizophoraceae) a viviporous mangrove species. *American Journal of Botany* **85**, 1631-1637.

Swofford, D.L., Olsen, G.J., Waddell, P.J. and Hillis, D.M. 1996. Phylogenetic inference. In: Hillis D.M., Moritz C. and Marble B.K. (eds) *Molecular Systematics*. Sinauer Associates, Sounderland, Massachusetts, 407-514.

Tanksley, and S.D., Orton, T.J. 1983. Isoenzymes in plant genetics and breeding. Elsevier, Amsterdan.

Teel, W. 1985. A pocket directory of Trees and Seeds in Kenya. Published by Kenya Non-Governmental Organizations, Nairobi, Kenya. pp151.

Temesgen, B., Neale, D.B and Harry, D.E. 1998. Sequence-tagged markers for loblolly pine (*Pinus taeda* L.) revealed by denaturing gradient gel electrophoresis. *Abstract, plant and animal genome VI Conference, San Diego 18-22 January 1998.* (http://probe.nalusola.gov:8300/pag/6/review/446.html.).

Tingey, S.V., and del Tufo, J.P. 1993. Genetic analysis using random amplified polymorphic DNA markers. *Plant Physiology* **101**, 349-352.

Travis, S.E., Maschinski, J. and Keim, P. 1996. An analysis of genetic variation in *Astragalus cremnophylax* var. *Cremnphylax*, a critically endangered plant, using AFLP markers. *Molecular Ecology* 5, 735–745.

Tsumura, Y., Suyama, Y., Yoshimura K., Shirato. N and Mukai, Y. 1997. Sequence-tagged sites (STDs) cDNA clones in *Cryptomenia japonia* and their evolution as molecular markers in conifers. *Theoretical and Applied Genetics* **94**, 764-772.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Freijters, A., Pot, J., Peleman, J., Kuiper, M. and Zebeau, M. 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acid Research* **23**, 4407-4414.

Watano, Y., Imaszu, M. and Shimizu, T. 1995. Chloroplast DNA typing by PCR-SSCP in *Pinus pumila*, *P. parriflora* var. *pentaphyla* complex (Pinaceae). *Journal of Plant Research* **108**, 493-499.

Webber, J.L. and May. 1989. Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. *American Journal of Human Genetics* **44**, 388-396.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. *Nucleic acids Research* **18**, 7213-7218.

Wilde, J., Waugh, R. and Powell, W. 1992. Genetic fingerprinting of *Theobroma* clones using RAPD markers. *Theoretical and Applied genetics* **88**, 871–877.

Williams, G.C. 1975. Sex and Evolution. Princeton. NJ: Princeton University Press pp. 200.

Williams, J.G.K., Hanafey, M.K., Rafaski, J.A. and Tingey, S.V. 1993. Genetic analysis using random amplified polymorphic DNA markers. *Methods in Enzymology* **218**, 704-740.

Williams, J.G.K., Kubelik, A.R., Livak, K.I., Rafalski, J.A. and Tingey, S.V. 1990. DNA-Polymorphism amplified by arbitrary primers as useful as genetic markers. *Nucleic Acids Research* **18**, 6531 – 6535.

Williams, T. J. 1991. International Aspects of Biodiversity. *The Forestry Chronicles* **68,** 454-458.

Yeh, F.C., Yang, R.C. and Boyle, T. 1999. Popgene Version 1.31. University of Alberta, Canada.

Young, A. and Merriam, H.G. 1992. The effect of forest fragmentation on genetic variation in an *Acer saccharinum*. (March sugar maple) population. Paper to: *International Symposium on Population Genetics and Gene Conservation of Forest Trees*. IUFRO Working Parties S2.04-05, S2.04-01 and s2.02.00. Carcaus Maubuisson, France. 24-28 August 1992.

Zimniac-Przybylska, Z. and Przybylska, J. 1995. Electrophoretic seed globulin patterns and species relationships in Vicia section *Faba* (Fabaceae). *Journal of Applied Genetics* **36**, 299-312.