ANALYSIS OF MATING SYSTEM AND GENE FLOW PATTERNS OF *MELIA VOLKENSII* WITHIN CLONAL SEED ORCHARD IN KIBWEZI USING MICROSATELLITES

ROTICH KIPKOECH JAPHET (BSC. AGED AND EXTENSION)

I56/CE/34136/2016

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE IN MASTER OF SCIENCE (BIOTECHNOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY.

SEPTEMBER, 2025

DECLARATION

This thesis is my original work and has not been submitted for award of a degree or any other award in any other university.

Signature ……... Date …….

Rotich Kipkoech Japhet (BSc. AGED and EXTENSION)
I56/CE/34136/2016

SUPERVISORS

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

Signature……. Date…….

Dr. George Ochieng Asudi
Biochemistry, Microbiology and Biotechnology Department,
Kenyatta University

Signature…… Date……

Dr. Stephen Omondi
Forest Genetics and Tree Improvement Department,
Kenya Forestry Research Institute.

DEDICATION

To my mother Elizabeth Maina who always kept my hopes alive, my lovely wife who is always a pillar of encouragement and prayers then my daughter Millycent and son Jayden. You are indeed a blessing to me, thanks all for your immeasurable support.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my supervisors Dr. Stephen Omondi of KEFRI and Dr. George Asudi for their immeasurable support and professional guidance during the study. Most sincere thanks goes to Dr. Omondi for always giving this study a priority and making sure that all the reagents were availed on time and ensuring I get to learn new concepts and techniques in modern molecular biology research, I'm grateful for your mentorship sir. Sincere gratitude to Dr. Asudi for vast knowledge and experience in molecular biology work and going through my write-up to ensure that it's always up to standard, Indeed you were a blessing to me and I learnt a lot from you.

I acknowledge the joy of working with the staff at Biotechnology lab at KEFRI Headquarters, Mr. Charles Oduor whose experience in DNA extraction came in handy and Asenath Adienge who really assisted me during DNA quantification and PCR.

My sincere appreciation goes to my brothers and sisters for their unwavering financial and emotional support they offered me during the course of this study, be blessed abundantly.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

ASAL	Arid and semi-Arid Land
CPT	candidate plus trees
CTAB	Cethyl-trimethyl Ammonium Bromide
DNA	Deoxyribonucleic acid
JICA	Japan International Cooperation Agency
KEFRI	Kenya Forestry Research Institute
MELIA	*Melia volkensii*
MIN	Time in minutes
PCR	Polymerase chain reactions
RPM	Revolutions per minute
RAPD	Random Amplified Polymorphic DNA
S	Time in Seconds

ABSTRACT

Melia volkensii (M. volkensii) is an indigenous tree species that grows and develops well in Kenyan Arid and semi-arid lands (ASALs). There has been a drastic decline in the *M. volkensii* population because of the overexploitation and uncontrolled harvesting of the natural stands where large and best trees are utilized for charcoal and timber, creating a huge demand for quality planting materials. *Melia volkensii* improvement programme began in 2012 to address the sharp decline in their genetic diversity, provide farmers with improved seeds for commercial plantations in dry lands and produce drought-tolerant and fast-growing trees adaptable to ASALs. Scions from the best *M. volkensii*, also known as candidate plus trees (CPTs), were collected to establish the first generation of clonal seed orchard to increase the breeding population and genetic resources of *M. volkensii.* The distributed materials from a tree seed orchard should always be of the recommended genetic quality with no or minimal contamination from unimproved wild stands surrounding the orchard. Therefore there is a need to determine the level and patterns of gene flow among the elite trees grown in a seed orchard to maintain random outcrossing in order to minimize loss of diversity. The study was carried out using the *M. volkensii* trees grown at the Kenya Forestry Research Institute seed orchard in Kibwezi in Makueni County. In total, 15 populations which comprised of 618 *M. volkensii* trees consisting of four hundred and twenty three open pollinated progenies from ten clones, ninety eight candidate plus trees and ninety seven individuals from the orchard background population were studied. Genomic DNA was isolated by CTAB method and amplified by multiplex PCR reactions using primers previously developed by Hanaoka *et al.*, 2012. The amplified DNA fragments were screened by capillary electrophoresis on the ABI 3500 genetic analyzer. Parameters of genetic diversity were determined using GenAlEx 6.503; MLTR program was used to determine the mating system in the orchard based on the mixed mating model. The population had a mean heterozygosity of 0.712, inbreeding coefficient of -0.035, fixation index of 0.068, a high number of migrants (Nm = 3.952), with high single (1.014) and multi-locus (1.2) estimates of the outcrossing rates. This indicates an exclusive out-crossing mating system and existence of gene flow in *M. volkensii*, possibly promoting inter-breeding and genetic diversity among the trees in Kibwezi seed orchard, which is vital for their future survival. Paternity analysis implicitly assigned 334 out of 423 progenies (78.96%) to 77 out of 98 potential (78.57%) seed orchard clones, with the unassigned 89 progenies (21.04 %) not matching any of the 98 seed orchard clones thought to be derived from the background population. The panmictic design of the seed orchard and flowering synchrony with the background population could have contributed to the pollen introgression in the orchard therefore regular studies should be done across the other blocks of the orchard to find out the extent of contamination and then come up with suitable mitigation which might include thinning and a review of the current isolation distance of 200 metres between the orchard and the background population to ensure the integrity of the seed source.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Only 6.3% of Kenya is under forest, with arid and semi-arid lands (ASALs) accounting for 80 % of the land (Mganga *et al*., 2015; FAO, 2020). Despite this minimal forest coverage, wood fuel accounts for the greater percentage of energy sources in Kenyan households placing intense pressure on forest resources. The already harsh climatic conditions in ASALs are expected to escalate due to the sustained climate change evidenced by a permanent shift in the traditional climate patterns (Matsushita *et al.,* 2021). It is projected that in a century, the average temperature will increase by 3.5°C throughout the entire East Africa region (Almazroui *et al*., 2020). Besides, effects of climate change in Kenya is already being experienced through prolonged droughts, unpredictable rainfall patterns accompanied by too-high temperatures, making it to initiate adaptation programs on climate change and desertification in ASALs (Mganga *et al*., 2015). The country's National Forest Program 2016-2030 recommends expanding forest cover by planting commercial trees in ASALs and establishing a tree improvement programme for the drylands to help combat climate change (MENR, 2016).

One of the indigenous tree species that grows and develops well in Kenyan ASALs is *Melia volkensii* (*M. volkensii*). Under good management and proper care, its investment can empower farmers in those areas economically, making it one of the highly valued trees recommended for planting (Wekesa *et al.,* 2012, 2019). This tree species found in ASALs of Eastern Africa grows widely on sandy, sandy loam or sandy clay soils with

good drainage (Orwa *et al*., 2009; Kamondo *et al*., 2016). The multipurpose tree species is particularly famous in Kenyan ASALs because it provides high-quality, durable and attractive timber, which is resistant to termite attack (Mulatya, 2000; Orwa *et al*., 2009; Kamondo *et al*., 2016).

Its valuable wood is used in furniture making, while the branches are a good source of fencing poles, firewood and general-purpose timber. The green leaves, developing fruits and flowers are nutritious sources of livestock fodder during the dry seasons. The tree flowers exude valuable bee forage nectars and other chemical substances that repel flies, ticks and fleas in livestock (Al Sharook *et al*., 1991; Orwa *et al*., 2009; Kamondo *et al*., 2016). This hardwood tree species also grows fast, producing high-value timber with a high market price in a relatively short duration, fewer than 20 years (Mwangi and Mukiama, 1989; Kamondo *et al*., 2016), making the majority of farmers invest in its production. Averagely, an 11-year-old mature *M. volkensii* tree sells at about Ksh 2,734 at the farm, but upon conversion to timber, produces an average of 140 feet of timber of various sizes. In 2018, the mean annual income from Melia enterprise in the counties of Kitui, Taveta, Makueni and Embu was Ksh 97,175 (USD 800) (Ochieng *et al*., 2018), which has likely increased since the last seven years.

However, Melia has a relatively slow regeneration rate, especially under wild or unmanaged habitats, which can be overcome by proper management. Its seedlings are also poorly established in the wild, mainly due to heavy seed predation, low germination rates without pre-treatment and harsh environmental conditions in ASALS (Kamondo *et*

al., 2016). Therefore, the tree seeds are pre-treated to improve their germination and regeneration. Though *M. volkensii* has moderate coppicing ability and can regenerate from cut stumps, it cannot regenerate vigorously as other indigenous tree species. Further, the tree exhibits high growth diversity, with some trees shorter and densely branched, making them unsuitable for quality timber (Kamondo *et al*., 2016; Ochieng *et al*., 2018). Over the past few years, there has also been a drastic decline in its population because of the overexploitation and uncontrolled harvesting of the natural stands where large and best trees are utilized for timber, creating a huge demand for quality planting materials (Stewart and Blomley, 1994).

Uncontrolled harvesting also results in increased homozygosis due to the mating of trees with small genetic variation (Runo *et al.,* 2004), eventually leading to genetic erosion and thus, severely threatening the tree as a potential dryland plantation species (Wekesa *et al*., 2019). Analysis of nine populations of *M. volkensii* using random amplified polymorphic DNA (RAPD) has also indicated a lower genetic diversity in the populations within the natural habitats than those in the farmlands (Runo *et al*. 2004). Therefore, selecting the best trees and conserving them in *ex-situ* is the most promising intervention towards the sustainable development of Melia as a plantation species in drylands (Kidundo, 1997; Tedd, 1997; Mulatya, 2000; Muturi *et al*., 2003).

Melia volkensii improvement programme began in 2012 to address the sharp decline in their genetic diversity, provide farmers with improved seeds for commercial plantations in drylands and produce drought-tolerant and fast-growing trees adaptable to ASALs

(Matsushita *et al.*, 2021). Scions from the best *M. volkensii* or candidate plus trees (CPTs), were collected to establish the first generation of clonal seeds to increase the breeding population and genetic resources of *M. volkensii* orchards, followed by evaluating their progenies to develop the second generation of clonal seed orchard. All CPTs were assigned a unique name, code and number and subsequently genotyped for identity (Luvanda *et al*., 2015; Ochieng *et al*., 2018). Two large clonal seed orchards, each measuring 11 ha in Kitui and Kibwezi, were established between 2010 and 2013 from the CPT clones (Kamondo *et al*., 2004). Seeds from these two clonal orchards were used to establish eight F1 progeny trials, out of which four were the main progeny test sites located in Tiva in Kitui county, Kibwezi in Makueni county, Marimanti in Tharaka Nithi county, Kasigau in Taita Taveta county (Luvanda *et al.,* 2015).

The mating system in flowering plants is important as it directly determine the evolutionary potential by influencing the genetic diversity (Barrett and Harder, 2017; Hodgins and Yeaman, 2019) and therefore it can affect the expected genetic gain through breeding (Ivetic *et al*., 2016; Tambarussi *et al.,* 2018). *Melia volkensii* is a monoecious insect-pollinated tree distributed in the ASALs of Eastern Africa (Orwa *et al*., 2009). Recently, Hanaoka *et al.* (2012) used simple sequence repeat (SSR) markers to estimate genetic parameters in three natural populations of *M. volkensii* and observed positive fixation index values in most of the loci, implying the presence of mixed mating patterns in the species (Wang *et al*., 2014). Similar findings were also observed in the diversity and structure of 380 *M. volkensii* trees from 13 natural populations that represented its species distribution range in Kenya. The study also detected high inter-population genetic

differentiation with three possible gene pools, namely Northern, Central and Southern, where conservation and breeding programs can concentrate (Omondi *et al*., 2018). Therefore, understanding the mating patterns and systems in the newly established *M. volkensii* seed orchards is necessary for sustained production and constant supply of good-quality seeds (Frankham, 2015).

1.2 Problem statement

Melia volkensii has been prioritized as an indigenous timber species (Kamondo *et al*., 2016; Ochieng *et al.,* 2018). Natural stands of Melia have been heavily exploited for timber and wood fuel, with most trees now grown on farms (Mwangi and Mukiama, 1989). Despite heavy financial investment and a highly publicized campaign to encourage farmers in ASALs to adopt the cultivation of *M. volkensii* as a plantation species, little progress has been made (Matsushita *et al*., 2021). The slow uptake of Melia enterprise was attributed partly to the unavailability of quality planting materials and variability observed in unimproved Melia stands (Kamondo *et al.,* 2016). Past surveys show a high demand for Melia's timber because of its high market price compared to most other indigenous tree species (Wekesa *et al*., 2012, 2019).

Unimproved wild stands exhibit variations with some trees branching densely at shorter heights of 1–2 metres, producing timbers with lots of dead knots and narrow stem diameter that is unsuitable for conversion into quality timber for use (Kamondo *et al*., 2016). Some unimproved Melia trees also tend to have uncharacteristically slow growth rates. Even though past studies show that superior clones of this tree species still exist, they are in the wild, which cannot be accessed easily by farmers who require planting

materials due to the expansive nature of the geographical area where these trees grow well (Kamondo *et al*., 2016; Ochieng *et al*., 2018).

Melia volkensii growing belt is a heterogeneous habitat where the trees must adapt to different prevailing harsh environmental conditions for their survival (Omondi *et al.,* 2015). Because of this, elite trees were identified all over the Melia growing belt and subsequently used to conserve the gene pool in a seed orchard (Matsushita *et al.*, 2021). Initiation of seed orchards ensures a constant supply of superior seeds and seedlings for the farmers' full adoption of the *M. volkensii* enterprise (Ochieng *et al*., 2018). There is also a need to constantly analyse the mating patterns and genetic diversity to check on the free flow of genes within an orchard to eliminate the possibility of founder effect and genetic drift in the resulting population (Gaiotto *et al.,* 2003; Pannel, 2015). The analysis can also help to evaluate the possible pollen introgression to eliminate the possibility of dilution of good attributes of the elite trees in the offspring (Campbell, 2015; Kariuki *et al*., 2016).

1.3 Justification

The distributed materials from a tree seed orchard should always be of the recommended genetic quality with no or minimal contamination from unimproved wild stands surrounding the orchard. Understanding mating patterns in a newly established orchard is crucial for ensuring long-term genetic integrity, productivity and sustainability of forest genetic improvement programs (El-Kassaby *et al.,* 2014). The estimation of actual versus the expected parental contribution is vital for assessing the effectiveness of orchard design because non-random mating and unequal genetic contribution among the parents

can lead to genetic drift and reduced effective population size (N_e) (Gaiotto *et al.,* 2003; Campbell, 2015) Pollen introgression further complicates genetic composition by potentially introducing maladaptive traits (Funda *et al.,* 2008). There is also a need to ensure random outcrossing within the orchard to minimize the loss of diversity (Frankham, 2015) and evaluate the level and patterns of gene flow among the elite trees grown in a seed orchard (Anderson, 2016).

To achieve this, diversity studies of the mating patterns within a newly established tree seed orchard using morphological, biochemical or genetic markers should ascertain that the products from the orchard are true to the expected standards (Wekesa *et al*., 2019). However, using morphological traits is unsuitable in *M. volkensii* because it will take considerably longer to confirm the presence in their progenies (Wang *et al.,* 2019). Compared to morphological markers, genetic markers generate important information about an individual's genetic variation and are found throughout the genome, making them more preferred in shedding light on plant evolution and genetic improvement (Hayward *et al*., 2015).

The present study used microsatellites as they are efficient in the investigation of the genetic diversity and population structure of *M. volkensii*. Microsatellites are also vital in the assessment of parentage and population dynamics of the tree species, studies on gene flow and mating systems (Omondi *et al*., 2015). In contrast, other markers such as RAPD are dominant and potentially population-specific, less polymorphic and are very sensitive to set laboratory conditions and protocols, giving varied results (Farooq *et al*., 1994,

1995). Microsatellite markers can be used to analyse plants of different ages at different seasons or growing in variable ecological conditions, using a few tissues, such as a leaf or a bud. In comparison to morphological identification, use of microsatellite is more objective as it diminishes genotyping error, enhancing the regulation of trading in forest reproductive materials (Hanaoka *et al*., 2012; Omondi *et al.*, 2010, 2015, 2018, 2019). This method can also reduce bureaucracy and provide an opportunity for fast certification of forest genetic materials.

1.4 Hypotheses

1. *Melia volkensii* seedlings obtained from Kibwezi seed orchard are not genetically diverse.
2. There is limited gene flow resulting in restricted mating among clones of *M. volkensii* in the Kibwezi seed orchard.
3. There is no pollen introgression from the unimproved background population of *M. volkensii* in the Kibwezi seed orchard.

1.5 Objectives

1.5.1 General objective

To investigate whether the design of *M. volkensii* clonal seed orchard in Kibwezi permits random mating among the clones or families for the production of frequent, abundant and superior *M. volkensii* seeds.

1.5.2 Specific objectives

1. To evaluate the genetic diversity of *Melia volkensii* trees in the Kibwezi seed orchard.

2. To analyse the gene flow and mating patterns among clones of elite *Melia volkensii* trees within the Kibwezi seed orchard.
3. To investigate the extent of pollen introgression from an unimproved background population of *Melia volkensii* in the Kibwezi seed orchard.

1.6 Significance and anticipated outputs

Key to the afforestation and plantation programme is an ideal tree seed orchard that produces frequent, abundant and improved seeds. For a constant supply of improved seeds and seedlings from the orchard, the overall design of the orchard should permit random mating among the clones or families of trees (Barrett, 1998). Mating patterns within trees in the orchard may vary in space and time, therefore affecting the dissemination of pollen throughout the orchard (Thakur *et al*., 2016). It is thus necessary to carry out a study that will estimate if a seed orchard for *M. volkensii* is designed in a manner likely to permit the free flow of genes by allowing random cross-pollination among *M. volkensii* trees within the seed orchard and at the same time if its products are not contaminated with pollen from unimproved *M. volkensii* growing as neighbouring wild stands (Ochieng *et al*., 2018; Matsushita *et al.*, 2021). This is to enhance the continuous and sustainable supply of quality seeds and seedlings (Barrett, 1998).

The results will ensure distribution of materials of recommended genetic quality with no or minimal contamination from unimproved wild stands surrounding Kibwezi seed orchard of *M. volkensii* (Campbell, 2015; Kamondo *et al*., 2016). The information generated from this study will also be vital to tree breeders, scientists, policy makers and farmers in the forestry sector and can be customized and assimilated into improvement of other tree species.

CHAPTER TWO

LITERATURE REVIEW

2.1 Botany of *Melia volkensii*

Melia volkensii belongs to the family Meliaceae or Mahogany and genus Melia (Orwa *et al*., 2009). The family Meliaceae has a total of eight species, many of which are native to Asia and Australia. Only *M. volkensii Gürke* is native to East Africa (Dale and Greenway, 1961; Orwa *et al*., 2009). It is a fast-growing multipurpose and deciduous tree, growing up to 20 m with a stem diameter of about 125 cm (Fig. 1) under intensive management, with a grey and moderately smooth bark when mature (Orwa *et al*., 2009; Hanaoka *et al*., 2016).

Melia has bright green compound leaves with distinct 3-7 lobed leaflets measuring up to 36 cm. The leaflets are lanceolate and have smooth lamina, a pointed apex and serrated margins. This monoecious tree has small, scented flowers with white petals and five whorls, which freely curl backwards. Androecium and gynoecium are similar in number and enclosed in a tube-like structure. Mature fruits are green and oval, measuring about 4 cm in length, but when ripe, the very thick and fleshy fruit endocarp is yellow-green and bony (Orwa *et al*., 2009; Kamondo *et al*., 2016). Seeds are ripe when the green fruit changes to yellow-green and has a soft pulp. Flowering and fruiting do not follow a season but can occur 2-3 times annually, depending on the rainfall patterns, with fruits on the same branch showing different stages of ripeness. The scented flowers are insect-pollinated, mainly by the honey bee, *Apis mellifera* (Hymenoptera; Apidae), although the tree can also self-pollinate (Orwa *et al*., 2009).

Figure 2.1: A picture of Melia candidate plus tree, showing a straight stem with reduced branching habit (Ochieng *et al.*, 2018).

2.2 Geographical distribution of *Melia volkensii*

Melia volkensii is a high-value tree growing naturally as a wild tree in ASALs of the eastern and northern regions of Kenya, to parts extending into southern Somalia and from western parts of coastal Kenya extending to the north of Tanzania (Mulatya, 2000; Broadhead *et al*., 2003; Orwa *et al.*, 2009). It grows best at an altitude of 150–1,700 m above sea level (m.a.s.l) with an annual mean rainfall of 300–800 mm in well-drained sandy soils (Orwa *et al*., 2009; Kamondo *et al*., 2016; Ochieng *et al.*, 2018). Presently, the tree species is found in several counties of eastern and northern Kenya (Fig. 2),

growing in the rangeland as a forest tree or in farms as one among the planted tree species (Tedd, 1997; Broadhead *et al*., 2003). Due to its expansive ecosystem, Melia is often referred to by the use of different names by the natives in different habitats, including *Bamba* by the Borana and Somali, *Kirumbuta* by the Digo and Taita, *Makau* by the Kamba and Tharaka and *Mkowe* by the Taveta (Kamondo *et al*., 2016; Ochieng *et al*., 2018).

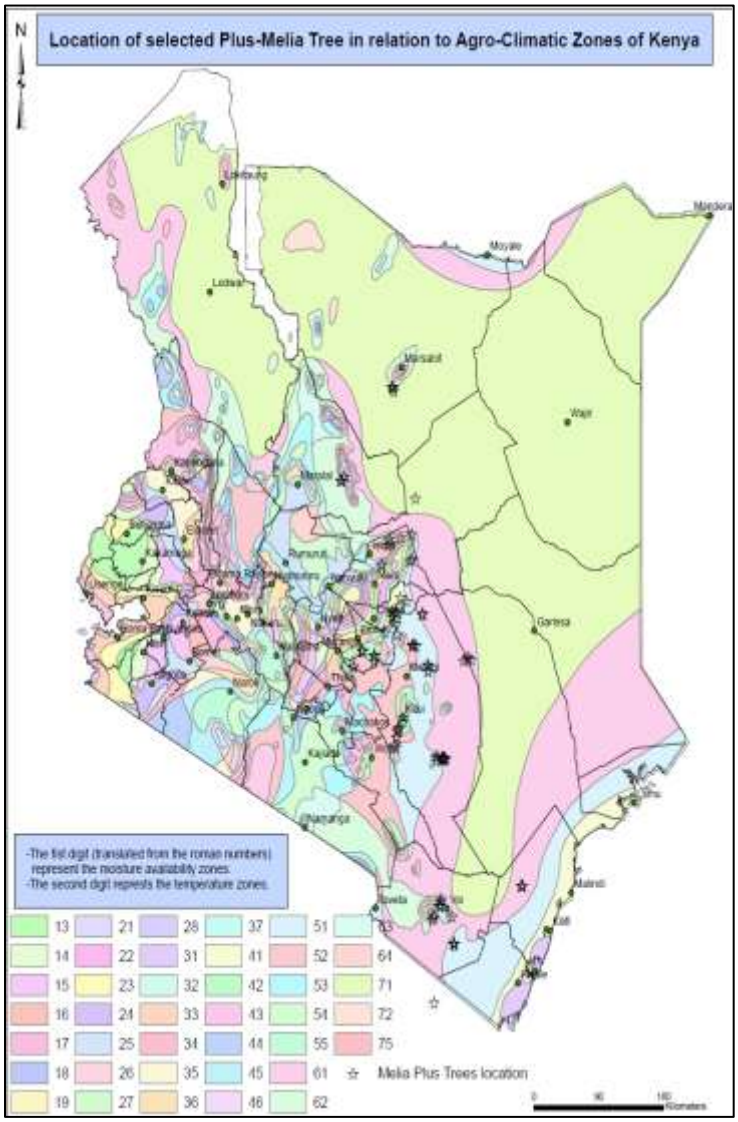


Figure 2.2. Map of Kenya showing the areas of distribution of Melia trees (Ochieng *et al.*, 2018).

2.3 Importance of *M. volkensii*

Melia volkensii is a highly recommended tree for agroforestry and afforestation programs in the ASALs due to its drought tolerance, resistance to fungal diseases, fast growth rate and production of high-quality termite-resistant timber (Mulatya, 2000). It is highly valued as a source of wood, producing timber in short periods of 10 to 15 years, thus making it a fast-growing tree compared to other hardwood trees (Mabberly, 1997). The tree often branches lightly, forming an open crown at the top, an ideal shade for livestock and humans (Luvanda *et al*., 2015). The attractive timber from this tree is used to make furniture, mortars and acoustic drums (Mwangi and Mukiama, 1989), while the stems are converted to traditional beehives or used to produce wood carvings. The tree leaves are good sources of organic mulch, green-leaf manure and livestock fodder (Kidundo, 1997; Orwa *et al*., 2009; Kamondo *et al*., 2016; Ochieng *et al*., 2018), especially for the browsers. The *M. volkensii* flowers are a valuable source of nectar for the bees and beautification, while its low tree-crop competition, under good management, makes it suitable for intercropping (Luvanda *et al*., 2015).

2.4 Propagation of *Melia volkensii*

To propagate *M. volkensii*, yellow ripened fruits with brown patched pericarps are hand-picked from the tree crown using loop shears to cut branchlets bearing fruits or shaken with a Y-forked stick. The picked fruits are sorted to remove black, over-mature, dark-green immature, undersized or rotten fruits (Kamondo *et al.*, 2016). The fruits that fall independently are also not propagated as they could be immature or diseased. The gathered and sorted fruits are then stored in woven bags to allow air circulation to avoid rotting and loss of viability in fruits or temporarily stored under shade before processing

(Ochieng *et al*., 2018). The fruits are then immediately de-pulped by a pestle and mortar or placed on a piece of timber and hit with a plank (Luvanda *et al*., 2015). Seeds are nipped to break the sharp tips and soaked in cold water for 24 h, or the seed coat is split longitudinally (Ochieng *et al*., 2018).

This breaks dormancy, allowing water and air into the seed embryo to induce germination. The obtained nuts are then thoroughly washed in plenty of water and sun-dried for at least two days (d). Viable seeds are extracted from sun-dried nuts using a flexible Melia nutcracker (Lugadiru, 2004), a sharp knife or a plank and sown immediately within 30 d in sand sterilized using 2% (wt/vol) sodium hypochlorite and sprayed with 5g/l Ridomil Gold^{TM} (Syngenta, Kenya) to control fungal diseases in a nursery bed (KEFRI, 2013). The nursery bed used is usually raised and covered by a clear polythene bag or a non-mist propagator packed with 15-20 cm sterile river sand and closed tightly to maintain high humidity (Wekesa *et al*., 2019). Low temperatures experienced in June and July and during rainy seasons are not favourable for the germination of *M. volkensii*. Seeds germinate within 3-6 d, 3 d, after which they are pricked out to minimize seedling mortality and deformation (Luvanda *et al.*, 2015). The potted seedlings are managed in the nursery by shading, watering and root-pruning to avoid carrot-like roots (KEFRI, 2013).

2.5 Mating systems in *Melia volkensii*

Understanding pollination mechanisms is crucial for effective propagation and orchard management. The mating systems can also vary among species and individual populations within a species (Matshushita *et al*., 2021). In flowering plant populations,

genetic diversity and evolutionary potential are heavily dependent on the mating system (Barrett and Harder, 2017; Hodgins and Yeaman, 2019) because it can affect the expected genetic gain through breeding (Shuster, 2009; Ivetic *et al*., 2016; Tambarussi *et al.*, 2018). In plants, mating systems can be predominantly selfing or outcrossing, mixed (selfing and outcrossing), obligate or facultative apomixes and intra-gametophytic or haploid selfing (Liengsiri *et al*., 1998). Flowering plants predominantly outcross under natural conditions, which helps them to maintain high genetic diversity within populations (Savolainen *et al.*, 2007; Serrote *et al.*, 2020).

In contrast, repeated self-pollination may lead to a decline in genetic diversity of a population but ensures reproduction under instances of limited pollen cloud. However, extreme selfing can lead to low germination rates and progenies with poor growth due to inbreeding depression (Govindaraj *et al.*, 2015; Care, 2020). *Melia volkensii* is a monoecious, insect-pollinated and self-compatible tree species (Orwa *et al*., 2009) that flowers typically during the rainy season, thereby attracting various pollinators. In *M. volkensii*, the use of outcrossed seeds is highly recommended for both forestry and tree breeding to avoid effects of inbreeding depression (Matsushita *et al*., 2021).

DNA markers have for a long time been used to directly or indirectly estimate the plant mating systems. Recently, studies performed by using DNA markers have revealed that various ecological and environmental conditions influence the outcrossing and selfing rates in plants (Chandrawati *et al.*, 2017; Tambarussi *et al.*, 2018). Hanaoka *et al*. (2012) and Omondi *et al*. (2018) used SSR markers to estimate genetic diversity in the natural

populations of *M. volkensii* and showed positive values of fixation index in most of the SSR loci, confirming the presence of mixed mating patterns in the species. Using these outcrossed seeds is ideal for forestry and tree breeding to avoid the effects of inbreeding depression (Thakur *et al*., 2016).

In addition, understanding the actual mating patterns and systems in newly established *M. volkensii* orchards is important to sustain the production of good-quality seeds (Pierre-Olivier, 2012). This exchange of genetic materials also results in various degrees of outbreeding or outcrossing, where, in most cases, there is 95% outbreeding, 5 – 95% mixed mating and less than 5% selfing (Matsushita *et al*., 2021). The genetic composition of the population defines how genes are spread from one generation to another and is directed by the mating system of the species (Moeller *et al*., 2017). For proper germplasm conservation and plant diversity studies, including *M. volkensii*, in-depth knowledge of its mating system is a key component for molecular breeding and phylogenetic analyses (Jayusman *et al.,* 2018). For instance, individuals which outbreed will retain a great diversity because of the large gene pool, with the family structure being completely heterozygous (Campbell, 2015; Pannell, 2015; Omondi *et al*., 2010, 2015, 2018, 2019). Diversity in the genetic constitution of an organism also improves the quality of its progeny and yields in a breeding programme, while self-pollination results in a decline in genetic diversity or the extinction of a species (Anderson, 2016).

2.6 *Melia volkensii* breeding and tree improvement

Melia volkensii tree breeding programs were established to select and breed tree varieties with improved genetic quality from the best individuals within the best populations based

on the wide variations in growth rate, stem form and wood quality (Ochieng *et al*., 2018). *Melia volkensii* could be bred naturally, where trees best adapted to a particular local environment were favoured or artificially selected (phenotypic selection) based on physical characteristics that favoured trees with desirable traits suited for specific planting purposes and intended end uses. Subsequent selection and further improvement are based on evaluating the selected trees' genetic worth based on the performance of their respective progenies in well-designed and replicated trials termed progeny testing (Kariuki *et al*., 2016). *Melia volkensii* breeding started with selecting candidate plus trees (CPTs) from the tree's natural habitat to establish two seed orchards and eight progeny trials, which were assessed for adaptability to different ecological conditions and selection against inferior materials before disseminating to farmers (Kamondo *et al.*, 2016).

A series of phenological and physiological studies was also used to develop a drought tolerance index to assess the *M. volkensii* tree's response to drought and to match appropriate genotypes to different ecological conditions or regions. Weather stations were put up in Kibwezi (S2°18′33.624″ E38°01′36.0084″) and Kitui (S1°21′24.0732″ E38°00′47.7″) seed orchards for *M. volkensii* and the already established four progeny test sites to collect real-time biophysical data of the place and then correlate it to the tree growth pattern (Ochieng *et al*., 2018).

2.7 Seed orchard design

A properly designed seed orchard allows maximum random outcrossing among clones and ensures the isolation of all related clones (El-Kassaby *et al*., 2014). This is attained

by a randomized permuted design, which helps to eliminate selfing by promoting outcrossing of randomly planted CPTs (Ivetic *et al*., 2016), resulting in the best quality planting materials (Gniech-Karasawa, 2016). Randomized permuted design is necessary because it enhances cross-pollination among diverse genotypes, reduces the risk of inbreeding and helps to achieve a more effective population (N_e). This is particularly valuable in *M. volkensii* clonal orchard to limit a few superior genotypes that may dominate reproduction if not spatially separated (Poltri *et al.,* 2003). Within an orchard, studies on tree breeding which aim to improve stand volumes, wood quality, environmental adaptations (Yan *et al*., 2023) and breeding for pests and disease resistance are done. Such improvements are performed through several progenies and clonal-based tests.

A tree breeding program typically starts with the mass selection of phenotypically superior genotypes or CPTs from planted forests (KEFRI 2013), followed by the growth of the offspring or vegetative propagation materials obtained from the CPTs in a seed orchard to obtain progenies, which are used to establish progeny test sites as a valuable source of materials for genetic trials (Luvanda *et al*., 2015). The best genotypes can be selected from these tests and then vegetatively propagated through grafting or cuttings to develop a second-generation seed orchard for improved seed production. If clonal propagation is easier, scion gardens comprising the best genotypes are established and their cuttings are mass-produced to be used directly in clonal plantations (Hodge and White, 1993). The seed orchard system is also widely used by forest tree breeders for a continuous supply of quality seeds (Ochieng *et al*., 2018).

2.8 Characterization of plant germplasm

2.8.1 Use of morphological traits

Morphological descriptors, such as seed colour, shoot height, internode length, leaflet length, and taproot length, can be used to identify plants with desirable characteristics and those possessing identifiable genetic polymorphisms that allow manipulation to develop superior plant varieties (Asudi *et al.,* 2010; Guo *et al*., 2014). These traits are heritable alongside other vital agronomic characteristics that play a key role when indirectly selecting plants for practical breeding. Studies have shown that trees with high genetic diversity are the most suitable for breeding as they respond well to genetic improvement (Kamondo *et al*., 2004; Lavanya *et al.,* 2008; Chandrawati *et al*., 2017). However, relying on phenotypic characteristics to improve tree variety may take a considerably longer period (about 8-20 years), depending on whether the parents were of the best quality and subjected to environmental influence (Todorovska *et al*., 2005). Molecular markers have therefore brought relief by enhancing the selection of parents in plant breeding and shortening the interval taken to obtain quality offspring in breeding trials or crop improvement studies (Bahadur *et al*., 2015; Markam *et al.*, 2018).

2.8.2 Molecular markers

Living organisms have DNA fragments called molecular markers associated with specific loci in the non-coding regions of the genome, making them selectively neutral and able to reveal variations due to mutations (Fowler and Mooney, 1990; Nadeem *et al*., 2018). Plant breeders use the markers to target important physiological traits in plants (Hayward *et al*., 2015). The markers are also found throughout the genome and are not affected by

developmental stages of the plant, making them more preferred in shedding light on plant evolution and genetic improvement (Kim *et al.,* 2015).

Molecular markers have made it possible to analyze genetic variation within species and determine similarities or distances between individuals and populations. These markers can be classified as DNA sequence-, hybridization- or PCR-based, with the latter preferred over the others since they are unlimited to distinct species and does not need prior genetic sequences from the target species (Martins-Lopes *et al*., 2007; Liu *et al*, 2016; Barboza *et al*., 2018). In addition, an ideal molecular marker should be heritable and found throughout the genome, co-dominant, polymorphic, and produce results from a small quantity or degraded DNA (Guasmi *et al*., 2012). The common PCR-based markers used in diversity studies include RAPD, single-nucleotide polymorphisms (SNP), short tandem repeats (STR), ISSR (inter simple-sequence repeats (ISSR), amplified fragment polymorphism (AFLP) and the variable number of tandem repeats (VNTRs).

In comparison to morphological markers, genetic markers generate important information about an individual's genetic variation and are, thus, useful in the conservation of genetic resources (Horecky *et al.,* 2018; Vir *et al*., 2016). For example, the analysis of nine populations of *M. volkensii* using RAPD markers found that the levels of genetic diversity in *M. volkensii* grown in eastern parts of Kenya ranged from 0.0663 to 0.1372, with a mean value of 0.0946 (Runo *et al*., 2004). The study also found that the *M. volkensii* trees growing in the Eastern region exhibited high genetic diversity amongst their populations compared to those in the Coastal areas. This large genetic variation

observed in trees indicates high levels of genetic erosion on the coast compared to the trees growing in the Eastern region.

However, among the PCR-based markers, the microsatellites, especially the STRs or simple sequence repeats (SSR), are the markers of choice for many molecular studies as they can provide valuable and very informative signals (Govindaraj *et al*., 2015; Omondi *et al*., 2015, 2016a, b, 2018, 2019). Microsatellites are random tandem repeats of nucleotide motifs, consisting of 2-6 bp in the genomes of all eukaryotes. The tandem repeats of simple sequences of di, tri, tetra or penta motifs of nucleotides derive their names from the fact that density gradient centrifugation separates DNA fragments with repetitive nucleotides into upper satellite fractions (Todorovska *et al*., 2005). Polymorphism in SSRs is also due to the varied repeat copy numbers specific to each individual, which alter the repeat array length. Polymorphism makes SSRs suitable for analysing genetic variation and comparing individuals or populations. A gene is polymorphic if its allele frequency is less than or equal to 0.95.

Microsatellites can also be transferred between related species to allow simultaneous amplification of several DNA sequences in the same reaction tube. The DNA can be in small amounts or of low quality, provided the right quality of primers and the reaction conditions (Lorenz, 2012; Asudi *et al*., 2013; Campbell, 2015). Plant breeders have widely used microsatellites in quantitative trait loci (QTL) mapping, analyzing germplasms, genetic linkage map constructions, and marker-assisted selection. These markers can also be automated for high efficiency and genotyping throughput (Horecky

et al., 2018). Initially, observing traits like tree height, growth rate, stem diameter, and the size of the bole formed the basis of breeding and selection in *M. volkensii* (Palma-Silva *et al*., 2015).

However, this is not possible in some cases as these traits might have been suppressed by biotic or abiotic factors, making their expression impossible. Genetic markers can easily overcome this obstacle by identifying the presence or absence of genes associated with useful traits at the molecular level are applied in breeding programs to conserve the genetic resources of many organisms (Farooq and Azam, 2002; Omondi *et al.*, 2018). Omondi *et al.* (2018) utilised SSR markers to study 380 individuals from 13 remnant natural populations of *M. volkensii* across Kenya using nuclear SSR markers and detected higher genetic diversity. Hanaoka *et al.* (2012) also reported high genetic diversity levels with high inter-population genetic differentiation that structured into three gene pools namely Northern, Central and Southern. These results provide a platform where conservation and breeding programs can concentrate on.

2.8.3 Measurements of genetic diversity in plants

Genetic measurements of plant diversity in a population are essential for understanding how variable and resilient the population is (Nayanakantha *et al.*, 2010; Omondi *et al.*, 2010) and can also help in conservation biology, breeding programs and ecological studies. The key genetic metrics used to assess plant diversity are allelic richness (A), heterozygosity, percentage polymorphic loci (%*P*), fixation index (F_{st}), inbreeding coefficient (F_{is}), genetic distance and effective population size (Li *et al.*, 2013; Markam *et al.*, 2018). Allelic richness measures the number of different alleles per locus and

indicates the overall genetic variation in a population (Omondi *et al.*, 2015). Heterozygosity is measured as observed heterozygosity (H_O) and expected heterozygosity (H_e), where H_O is the proportion of individuals that are heterozygous at a locus, while H_e is the probability that two alleles selected randomly from the population are different. High heterozygosity indicates high genetic diversity (Govindaraj *et al.*, 2015; Jayusman *et al*., 2018), while the %*P*, which is the percentage of loci with more than one allele, measures the variable proportion of the genome.

Fixation index measures the genetic differentiation between populations, with values close to zero signifying little differentiation while values closer to 1 demonstrating high differentiation (Frankham *et al.*, 2011; Kim *et al.*, 2015). Fixation index is useful when comparing genetic diversity across populations, while F_{is} indicates the level of inbreeding in a population, with positive values suggesting inbreeding and negative values suggesting outbreeding (Rousset, 1997; Yan *et al*., 2023). Further, the Nei genetic distance measures genetic divergence between individuals or populations and is used in clustering and phylogenetic analysis (Takezaki and Nei, 1986), while N_e shows the number of individuals that contribute genes equally to the next generation. Smaller N_e indicates higher genetic risk and loss of diversity due to increased genetic drift, reduced genetic diversity, increased fixation of deleterious alleles and higher inbreeding (Omondi *et al*., 2010; Ochieng *et al.*, 2018).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The research was carried out using the *M. volkensii* trees grown at the KEFRI seed orchard in Kibwezi in Makueni County, mainly maintained for the tree improvement programme. Kibwezi is an ASAL area that receives a small amount of rainfall throughout the year, averaging about 300-600 mm of precipitation. The temperature averages about 23°C, with the highest in February and the lowest in July (Mganga *et al*., 2015).

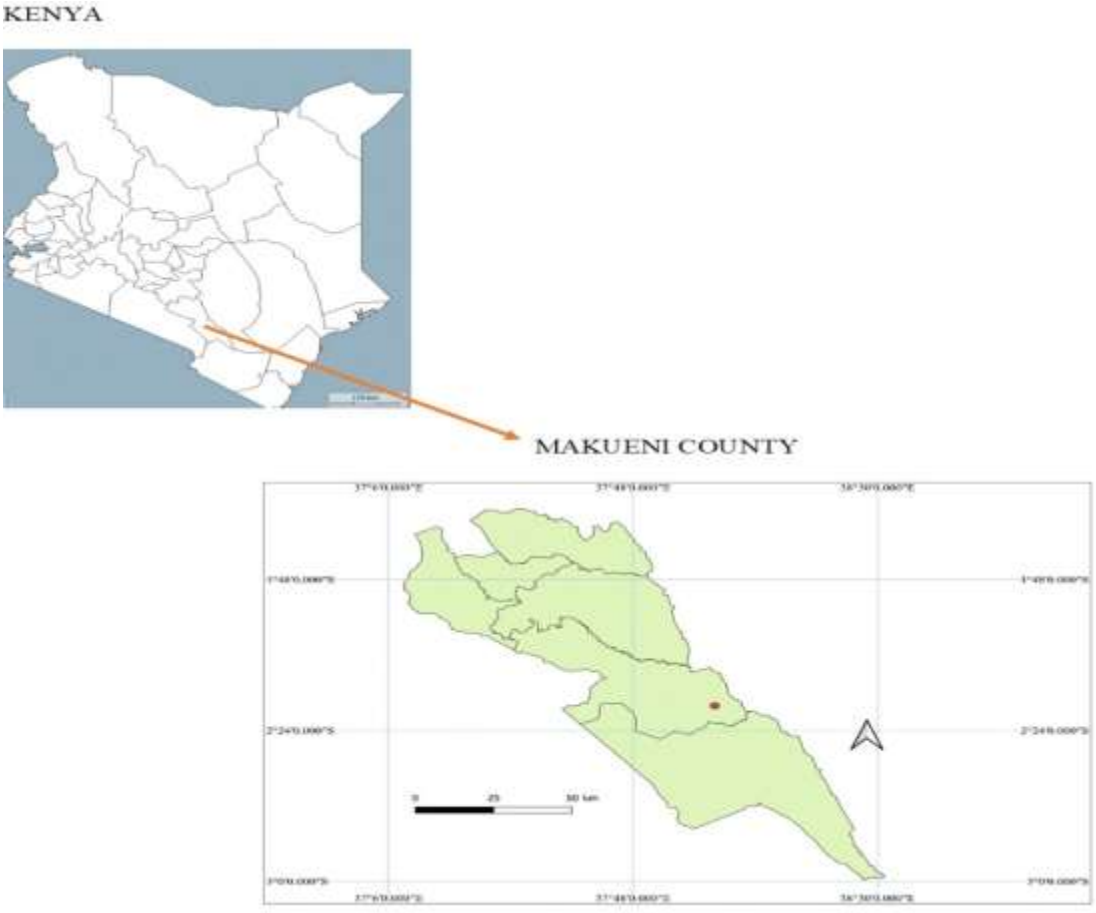


Figure 3.1: Map of Kenya showing the study area of Kibwezi in Makueni county (Source: Google Maps).

3.2 *Melia volkensii*

Kibwezi *M. volkensii* seed orchard is a first-generation clonal seed orchard established from 100 elite trees or CPTs selected from various regions of natural stands and those planted on private farms in ASALs between 2012 and 2013 (Ochieng *et al*., 2018). The characteristics of the elite trees included stem straightness, tree form, growth vigour and resistance to disease and pests such as powdery mildew and nematode attack, which are common in the seedling stage (Kamondo *et al*., 2016). The selection of CPTs started with a reconnaissance visit to sites of the natural occurrence of *M. volkensii*, followed by the actual selection within the mature *M. volkensii* populations of at least 30 mature trees (Kariuki *et al*., 2016). The selection of an individual CPT involved identifying the potential candidate tree and assessing its superiority over the five nearest neighbours or check trees (KEFRI, 2013).

All the geographic coordinates of the CPTs were taken by a global positioning system. *Melia volkensii* rootstocks were raised at the KEFRI regional research centre in Kitui for 90 days and grafted using scions obtained from every CPT. In total, 80 scions were obtained from each of the selected plus trees, with 72 grafted successfully onto *M. volkensii* rootstocks by September 2012 using the top grafting method (Ochieng *et al*., 2018). The nursery routine management practices were carried out until the seedlings were 90 days old. The grafted seedlings were then used to establish two seed orchards in December 2013 at Kitui KEFRI Regional Research Centre and in Kibwezi. In each orchard, 100 clones of plus trees, each with five ramets, were planted in six blocks,

totaling 3000 seedlings per site and 500 trees per block with a plant-to-plant spacing of 6 x 6 m in a predetermined position (Kamondo *et al*., 2016).

3.3 Sample size

Fifteen populations of 618 *M. volkensii* trees were sampled. Clones 1, 12, 18, 22, 35, 36, 39, 4, 5 and 60, with 47, 46, 44, 34, 35, 47, 32, 48, 45 and 47 samples each, respectively, were collected from the first-generation clonal seed orchard of *M. volkensii* located in Kibwezi, previously established from 100 elite trees or CPTs. Additionally, 20, 23, 28 and 26 mature wild trees surrounding the orchard in the South, East, West and North directions, and 98 CPTs located outside the 1st generation clonal seed orchard were also randomly sampled to analyse for possible pollen contamination. Five tender leaves from each mother plant and neighbouring wild stand tree were hand-picked and put in a paper envelope and labelled according to the population, sample number and the direction from which it was collected in relation to the *M. volkensii* seed orchard. The leaf samples were put in a nylon bag with silica gel to absorb moisture and then stored at -4 °C before use.

Only mature fruits of the 423 clones from block one of the seed orchard at Kibwezi were hand-picked from *M. volkensii* trees that had consistently produced seeds for the past three years to ensure sources with high chances of germinating (KEFRI, 2013), then put in a well-labelled paper envelope. The fruits were then de-pulped, and their hard endocarps were broken with a sharp knife to release the seeds. Extracted seeds were kept separately in a paper envelope, indicating the clone number. Seeds from each clone were then germinated in sterile sand (autoclaved at 120 °C) inside a glasshouse at KEFRI headquarters (S1°12′56.4696″ E36°38′44.772″). At 90 days old, two healthy leaves were

picked from each randomly selected seedling, put in a paper envelope and labelled according to the clone name and tree number, then used in the genetic study.

3.4 DNA isolation from *Melia volkensii* leaf tissues

The genomic DNA was obtained from 0.2 g of dry leaf tissues of all the clone seedlings, neighbouring plants and CPTs using a cetyl trimethyl ammonium bromide (CTAB) method as described by Hanaoka *et al*. (2012). The leaf tissues were ground into powder in a 2,000 µl microcentrifuge tube with a round ball using a mixer mill (Retsch® MM400). The crashed leaf tissue was mixed with a 500 µl isolation buffer comprising 10 % polyethylene glycol, 0.1 M Tris-HCl (pH 8.0), 0.35 M mannitol, 0.01 M EDTA (pH 8.0) and 0.5 % β-mercaptoethanol.

The mixture was thoroughly vortexed and then centrifuged at 10,000 rpm at 4 °C for 3 min, followed by the subsequent removal of the supernatant isolation buffer. This step was then repeated four times using 800 µl of the isolation buffer until a less viscous supernatant was formed, which was then mixed with 800 µl of CTAB buffer containing 2 % CTAB, 0.1 M Tris HCl, 1.4 M NaCl and 0.2 M EDTA. The solution mixture was then gently vortexed and incubated at 65 °C for 1 h, followed by 30 min at 37 °C and in an oscillating water bath. The resulting mixture was centrifuged at 14,000 rpm at room temperature for 10 min, and the upper aqueous phase was mixed with an equal volume (~800 µl) of chloroform: isoamyl alcohol, prepared in the ratio of 24:1. The solution mixture was mixed thoroughly by gentle inversion for 10 min and centrifuged at 14,000 rpm at room temperature for 10 min.

The DNA was then precipitated using 60 µl of 3 M NaOAc and 600 µl of ice-cold isopropanol, followed by gentle inversion and centrifugation at 15,000 rpm at 4 °C for 5 min. The DNA pellet was mixed with 800 µl of 70 % ethanol, followed by the flipping of the tube and centrifugation at 15,000 rpm at 4 °C for 5 min to wash the DNA pellet. The supernatant was discarded, and the DNA pellet was air-dried for 45 min, eluted in 300 µl DNase-free water, and then stored at 4 °C (Hanaoka *et al*., 2012).

3.5 Confirmation of DNA presence and quantity using gel electrophoresis

The presence and quality of DNA were determined in a 1% (wt/vol) agarose gel electrophoresis prepared using 1 g agarose, 100 ml of 0.5X TBE buffer and stained with 3.5 µl SYBR green dye (Invitrogen^{TM} 10000x in DMSO). The mixture was then put in a conical flask, boiled to form a clear, transparent solution, and cooled in running tap water. The mixture was poured out carefully into an already set gel tray with a castor comb and then covered in a cardboard box to solidify. The solidified gel with the comb was submerged in an electrophoretic tank containing 0.5X TBE buffer. For each sample, 2 µl of the DNA was mixed with 2 µl of ultrapure H_2O, and 1 µl of bromophenol blue and directly loaded into the already cast gel wells. A total of 44 samples were loaded at any given time, with the first and the last wells reserved for the 1 kb gene ruler (Thermoscientific^{TM}) used for sizing the fragments. The gel was run for 40 min at 50 V to allow DNA fragments to migrate and be visualized in a UV transilluminator (ATTA-E GRAPH) at 320 nm before being photographed.

3.6 Amplification of the *Melia volkensii* DNA

All the DNA samples were amplified using 12 microsatellite markers previously developed for *M. volkensii*. The 12 *M. volkensii* primers were previously designed and developed by using Primer3Plus computer software (Untergasse *et al.*, 2007; http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Hanaoka *et al*., 2012). The forward primers for each marker were labelled at the 5´ ends using fluorescent dyes, including 6FAM (blue), PET (red), VIC (green) and NED (yellow) (Table 3.1) for screening by capillary electrophoresis on the ABI Prism and 3500 genetic analyzer (Applied Biosystems). The primers were reconstituted, and 20 µM of each was combined into three different multiplex mixes, each consisting of four pairs based on the strength of the fluorescent dyes and fragment sizes (Table 3.1), and then kept at -20 °C until use. The first multiplex mix group included bcmv30, 43, 55 and 68, the second group composed of bcmv58, 79, 119, and 132, whereas the third set consisted of bcmv75, 97, 119, and 141 (Table 3.1).

Multiplex PCR reactions were performed using a 96 well low profile PCR plate on a Veriti 96 Well Thermal Cycler (Applied Biosystems) in a final reaction volume of 6 µl, comprising of 3 µl Multiplex PCR kit (Qiagen, Inc), 0.48 µl of each Multiplex primer mix (Applied biosystems), 0.52 µl PCR H_2O, 1 µl Q solution (Quiagen, Inc) and 1 ng of the template DNA. All PCR reaction preparations were performed on ice with primers (Table 3.1) covered with aluminium (Al) foil to protect the fluorescently labelled oligonucleotides from degradation by light. The thermal cycling conditions included a single cycle of initial denaturation at 95 °C for 15 min; followed by 35 cycles of

denaturation at 95 °C for 30 s, annealing at 57 °C for 90 s and extension at 72 °C for 1 min; and a final extension at 60 °C for 30 min to ensure all the products polymerized (Hanaoka *et al*.2012).

Table 3.1: Characteristics of 12 *Melia volkensii* primers used for PCR with their fluorescent labels and allele size ranges

DDBJ accession	Locus	Primer sequence (5'-3')	Repeat motif	Allele size
AB674473	bcmv030	F: CCCTATTCATTGTCCCTCCA***PET** R: GTTTCTTGTCCTCCTGGAATTCTGTGC	$(CT)_{19}$	142-162
AB674474	bcmv043	F: AGAGAAGACAGATCCCCCAGT***NED** R: GTTTCTTCAACACAACACAACACAGCAA	$(CT)_{18}$	216-246
AB674475	bcmv055	F: CAACCATGGTGTCGAGAAGA***FAM** R: GTTTCTTGCTTAATTTGCCTGTGCAT	$(AG)_{23}$	170-210
AB674476	bcmv058	F: CTAGACCAGCCCCAAGAACA***NED** R: GTTTCTTTCAAGGGCTTCTTCTGAATC	$(AC)_{9}$	212-222
AB674477	bcmv068	F: CCAATGTTGTTCAACTATATGAGGTC***VIC** R: GTTTCTTGAATTTCGAAGAGTGCCAAAA	$(TC)_{22}$, $(AC)_{14}$	159-195
AB674478	bcmv075	F: GGAACCCCAATTTAGGAACT***FAM** R: GTTTCTTGCTTGGTGAAAACCATAGA	$(AC)_{11}$	182-216
AB674479	bcmv079	F: CCGTGTAAGAGTGCCAAATC***FAM** R: GTTTCTTCTTGGAGGTGAGATCAAGTG	$(TA)_{5}$ $(TG)_{17}$	179-195
AB674480	bcmv097	F: TCTGTTGGTGGTGTTGTCAC***PET** R: GTTTCTTAATGTGGATGCAAGCAGTG	$(TC)_{22}(AC)_{10}$	151-199
AB674481	bcmv119	F: CAAGCACACACAAGGATTTG***NED** R: GTTTCTTGGCAACTCTCAGGTATCAA	$(GA)_{21}$	220-230
AB674482	bcmv129	F: GTTGTTTGTGGGTGTGTGTT***PET** R: GTTTCTTGAGAGAGAACCCAAAGGAAAA	$(TC)_{7}$ $(AC)_{12}$	139-171
AB674484	bcmv132	F: GTGCAGTGTCCATGTTGAAG***VIC** R: GTTTCTTGACATTTTCTCTGCAAGGTCA	$(TG)_{14}$	169-198
AB674485	bcmv141	F: TTCCCTCAGCATTAAGGTGT***VIC** R: GTTTCTTCAGGCAAAGGAAGGTAGGTA	$(TA)_{9}$ $(TG)_{8}$	164-204

Source: Hanaoka *et al*. 2012

3.7 Gel electrophoresis of PCR products

The amplicons were wrapped in Al foil and stored at -20 °C before gel electrophoresis was carried out as described in section 3.4 with 5 µl of each amplicon using a 1% (w/v) agarose gel in TBE buffer. To each PCR product, 3 µl of sample loading buffer was

added and mixed by pipetting before loading the mixture into preformed sample wells on the cast gel. The samples were run alongside a 1 kb DNA ladder at 40V for 40 min and then viewed via a UV transilluminator and photographed. Each amplified SSR fragment was visualized as a distinct band.

3.8 Capillary electrophoresis and fragment scoring

The amplified DNA fragments were screened by capillary electrophoresis on the ABI 3500 genetic analyzer (Applied Biosystems). The capillary electrophoresis runs were post-PCR co-loaded, where a range of 0.5 µl and 1 µl of the FAM, NED, PET and VIC labelled amplicons were mixed with their corresponding 9.0 µl capillary electrophoresis cocktail prepared from 1 ml of HI-DI formamide and 12 µl of Genescan^{TM} 500LIZ^{TM} size standard (Applied Biosystems) for 96 reactions. The DNA fragments were denatured and size fractioned by capillary electrophoresis on the ABI Prism 3730 automatic DNA sequencer (Applied Biosystems). Genotyping was carried out by capillary electrophoresis using ABI PRISM 3730 (Applied Biosystems), a fluorescent-based capillary detection system that uses polymer as the separation matrix at KEFRI Headquarters.

The GeneMapper v3.7 software (Applied Biosystems) was used to size the patterns of the peaks with an internal Genescan-500 LIZ size standard, followed by the analysis of the amplified fragments. This facilitated accurate sizing of microsatellite alleles to within ±0.3 bp. Primers were optimized by running different ratios of PCR products and choosing the one giving the best signal profile (Signal /noise ratio and relative fluorescent units (RFU). Size calling, including peak detection and fragment size matching, was

performed using the GeneMapper Bins, which represent a fragment size or base pair range and dye colour that define an allele, and were constructed from the reference data.

3.9 Data analysis

3.9.1 Marker validation and genetic diversity estimates

All 12 markers were highly reproducible and formed unambiguous, distinct bands that were included in the genetic parameters calculation. Genepop v. 4.7.5 web version (Raymond and Rousset 1995) was used to validate microsatellite markers using the neutrality and independence test, which are important precursors to population structure assessments. Deviations from the Hardy-Weinberg equilibrium (HWE) proportions in each Melia population and locus were approximated from the exact test based on a Markov chain iteration using a burn-in of 1000 and 100 batches with 1000 iterations per batch in Arlequin v 3.5 (Excofer and Lischer 2010). Significant values were obtained using Fisher's method by combining the probabilities of exact tests.

The microsatellite genotypic linkage disequilibrium (LD) among pairs of loci was evaluated using Fisher's exact test of Hardy–Weinberg proportion for multiple alleles. Pairwise tests for LD were estimated to obtain chi-square (χ^2) distribution per marker pair, degrees of freedom and original *P* values. Multiple tests in the detection of LD and HWE were corrected using the false discovery rate approach by using the Benjamini–Hochberg correction, which, in contrast to the Bonferroni correction, has lower incidences of false negatives. Complementarily, Bonferroni correction was used to

confirm the significance tests of LD among 12 pairs of loci (*P*-values are under LD [$P < 0.001$]).

Other parameters of genetic diversity, including H_o, H_e, and F_{is}, were determined using GenAlEx 6.503 software as described by Peakall and Smouse (2012). The *%P* (95% criterion), the total number of alleles (N_a) and the effective number of alleles (A_e) were estimated as described by Kalinowski *et al.* (2007) on CERVUS v3.0 software and POPGEN v1.32 (Yeh and Boyle, 1996). Evidence of null alleles was tested by use of Micro-Checker 2.2 as described by Van Oosterhout *et al*. (2004), while genetic differentiation between groups (F_{st}) was tested by use of the GENEPOP v4.0 program (Raymond and Rousset 1995). The F_{st}, which is a measure of significance of the difference between genetic groups, was determined by permutation tests of 10,000 on Arlequin v3.5 (Excofer and Lischer 2010).

3.9.2 Population structure and genetic differentiation

Wright's F-statistics (F_{st}, F_{is} and F_{it}) values were evaluated after testing for deviations from the HWE for each population and calculated following the randomization method using 1000 permutations over loci for a 95% confidence interval by utilizing the program MSA v 4.05 (Dieringer and Schlötterer, 2003) and confirmed in FSTAT v 3.9.3.2 (Goudet, 2002). To assess the degree of admixture and genetic populations within the trees, the population structure of the 618 samples from the *Melia* collection was inferred by using a model-based Bayesian clustering of individuals program in Structure software v. 2.3.4 (Pritchard *et al*., 2000). Structure analyses were conducted with K = 2 as the most

likely number of genetically distinct Melia groups based on the Evanno method's plot of K against ΔK (Evanno *et al*., 2005; Figure 4.2).

Structure Harvester Web version: v0.6.94 was used to calculate the optimal value of K (Earl and Vonholdt., 2012) using the ΔK criterion (Evanno *et al*., 2005). Simulations were run with K ranging from 1–10, with 10 iterations per value of K. The study presumed an admixture model and independent allele frequencies using a burn-in value of 50,000 and 100,000 Markov Chain Monte Carlo (MCMC) iterations for each run (Pritchard *et al*., 2000). The results from the Structure were exhibited as coloured bar charts using membership proportions or Q-values (clone/population) for each individual in the *Melia* population. Clustering was also assessed using bootstrapped Nei's genetic distance matrices obtained using the MSA v4.05 software (Dieringer and Schlötterer 2003) and used to construct the neighbour-joining (NJ) tree in Phylip v3.698 package. A 2-level (among and within population of trees) analysis of molecular variance (AMOVA) was done using GenAlEx to get insights into the partitioning of the microsatellite variance within and between genetic units.

3.9.3 Evaluation of mating system and pollen introgression into the orchard

The mating systems, gene flow patterns and level of pollen introgression within the *M. volkensii* clonal seed orchard were evaluated using the 12 microsatellite markers with 423 open-pollinated progenies from the 10 clones. Determination of parental gametic contribution, selfing rate and the extent of pollen contamination were obtained after conducting pedigree reconstruction to assign each seed's male parents. The MLTR

program (web version) (Ritland, 2002) was used to determine the mating system in the orchard based on the mixed mating model as described previously (Ritland and Jain, 1981). Maximum likelihood procedures were carried out to estimate multi-locus outcrossing rate (t_m), single-locus outcrossing rate (t_s), outcrossing rate between related individuals (t_m-t_s) and the correlation of paternity or proportion of full sibs among outcrossed progeny as described previously (Ritland, 1996).

Individuals were resampled within families, and pollen gene frequencies were constrained to equal ovule frequencies. The number of pollen donors contributing to each family or neighbourhood size and the inbreeding coefficient of maternal parents were estimated as described (Ritland, 1989, 2002), while the multi-locus and single-locus outcrossing rate values were compared to assess the degree of bi-parental inbreeding. Standard deviations of the estimates were calculated by bootstrapping with 1,000 bootstraps.

The coefficients of co-ancestry among families within and between populations were obtained using the FSTAT program (Goudet, 1995). Parental analysis of the 423 open-pollinated progenies from the 10 clones was carried out using standard paternity testing methods (Gaiotto *et al*., 2003) with the Cervus 3.0.7 web version (Marshall *et al.*, 1998) to assign their paternity. The assumptions of the mixed mating model analysis included no selection or mutation between fertilization and progeny assay, independent segregation of alleles at different marker loci, homogeneity of both the outcrossing rate and the pollen pool composition over maternal genotypes (i.e. random mating for

outcrosses), and over maternal trees themselves if mother genotypes were inferred from their progeny genotypes, which is the case in the present study.

The parentage analysis was simulated with a minimum of 10 typed loci, 10,000 offspring and 220 candidate fathers. Other parameters used included sampling of 0.999, 0.85 and 0.01 of the candidate fathers, typed loci, and mistyped loci, respectively. The output simulation parameters included use of delta to determine confidence, with 75% as the relaxed confidence level and 95.00% as the strict confidence level. Known parents or mother plants (98) were included as candidate parents.

CHAPTER FOUR

RESULTS

4.1 Marker diversity of Melia

The 12 microsatellite markers used in this study were highly polymorphic, ranging from 0.388 in bcmv075 to 0.849 in bcmv141 and generating 142 alleles. The total number of alleles per locus ranged from five in the loci bcmv058 and bcmv129 to 18 in locus bcmv043. The highest mean number of alleles (N_a) per locus was 10.00 in bcmv043, with a mean of 6.878. Of the 142 alleles, 22 were privately found only in a single population/genotype among a broader collection of populations/genotypes with frequencies ranging from 0.005 – 0.036 in 10 loci. The locus bcmv068 had the highest number of private alleles (four), while the four loci, bcmv043, bcmv079, bcmv119 and bcmv132, had one private allele each. The effective number of alleles A_e averaged 3.587 per marker, and ranged from 1.821 in bcmv075 to 5.654 in bcmv141 (Table 4.1).

The marker diversity analysis also showed high values for the average H_o of 0.712, which differed slightly from the average H_e of 0.683. The locus bcmv141 marker had the highest heterozygosity values; H_o = 0.863 and H_e = 0.816, respectively, with the least H_o and H_e found in bcmv075. The number of heterozygotes in all markers was consistently near the overall average H_e. The mean of genetic differentiation, F_{st} was 0.068, suggesting a very low differentiation for the sampled *Melia* trees used in this study. The F_{is} from observed changes in a mixed- mating population is estimated from the fitness of selfed individuals relative to outcrossed individuals. As expected, the average F_{is} was

negative (-0.035), given the high number of heterozygotes observed. All markers had negative F_{is} values except bcmv030 and bcmv075, with the locus bcmv075 generally showing lower genetic metrics except for F_{is} and F_{it}. The *Nm* also ranged from 1.443 to 6.839, with a mean of 3.952 (Table 4.1).

Table 4.1: Summary of microsatellite allele data and diversity across all 618 Melia samples.

Locus	Number of alleles	Private alleles	N_a	A_e	H_o	H_e	uH_e	*PIC*	F_{is}	F_{it}	F_{st}	*Nm*
bcmv030	10	3	5.467	3.074	0.649	0.668	0.677	0.644	0.029	0.071	0.044	5.420
bcmv043	18	1	10.000	4.598	0.810	0.757	0.768	0.807	-0.070	0.014	0.079	2.923
bcmv055	15	2	9.067	4.361	0.805	0.763	0.774	0.769	-0.055	-0.017	0.035	6.839
bcmv058	5	0	4.333	2.931	0.693	0.649	0.658	0.629	-0.068	-0.019	0.046	5.181
bcmv068	15	4	7.000	3.330	0.724	0.652	0.661	0.693	-0.110	-0.025	0.077	2.996
bcmv075	7	3	2.533	1.821	0.336	0.439	0.445	0.388	0.233	0.347	0.148	1.443
bcmv079	9	1	5.933	3.336	0.723	0.672	0.682	0.693	-0.076	0.010	0.080	2.880
Bcmv097	17	3	8.933	4.141	0.795	0.745	0.756	0.749	-0.067	-0.022	0.042	5.715
bcmv119	10	1	7.200	3.698	0.747	0.713	0.723	0.749	-0.047	0.028	0.072	3.218
bcmv129	5	0	4.333	2.695	0.686	0.618	0.627	0.620	-0.109	-0.020	0.080	2.869
bcmv132	14	1	8.067	3.410	0.710	0.698	0.708	0.719	-0.017	0.046	0.062	3.778
bcmv141	17	3	9.667	5.654	0.863	0.816	0.828	0.849	-0.058	0.002	0.057	4.157
Mean	**11.833**	**1.833**	**6.878**	**3.587**	**0.712**	**0.683**	**0.692**	**0.692**	**-0.035**	**0.035**	**0.068**	**3.952**

N_a = Number of different alleles, A_e = the number of effective alleles = 1/(Sum pi^2), H_o = observed heterozygosity (No. of Hets/*N*), H_e = expected heterozygosity (1 – Sum pi^2), uH_e = unbiased H_e ((2*N* / (2*N* – 1)); *PIC* = polymorphism information content; F_{is} = (mean H_e – mean H_o)/H_e; F_{it} = (H_t – H_o)/H_t; F_{st} = (H_t – *mean* H_e)/H_t; *Nm* = [(1/F_{st})/4; H_t = total H_e; pi = frequency of the ith allele for the population; sum pi^2 = sum of the squared population allele frequencies.

4.2 Genetic diversity and differentiation

The A_e is a key measure of genetic diversity that reflects how alleles are distributed at a given locus in a population, with a higher A_e indicating greater diversity and more balanced allele frequencies. Unlike allelic richness, A_e accounts for the frequency of alleles by focusing on the common alleles while discounting very rare ones. The A_e

across loci ranged from 2.818 to 4.348 in CPTs (candidate plus trees), suggesting moderate to high allelic diversity within the *M. volkensii* orchard population. This also indicates a fairly and even distribution of alleles, which is beneficial for maintaining adaptability in dryland planting environments.

Though the measures of genetic diversity varied slightly among the CPTs, clones and the trees neighbouring the orchard, the loci were polymorphic across all groups (%P = 100). Among the populations, the CPTs showed the highest number of different alleles (N_a = 9.750), a slightly higher number of effective alleles (A_e = 4.348) and Nei's diversity estimate (D) of 0.740. The genetic diversity also varied among clones, with the lowest values as follows: A_e = 2.818, H_e = 0.600 for clone 60 and N_a = 6.167 for clone 4, while the highest values were: A_e = 4.169, H_e = 0.732 for clone 5 and N_a = 7.667 for clone 36 (Table 4.2). The mother plants also had the highest number of private alleles (AP), signifying that the orchard has undergone genetic differentiation from its source population.

Table 4.2: Estimates of diversity based on the 15 *Melia* populations.

Population	Sample size	N_a	A_e	H_O	H_e	uH_e	F	AP	%P
Clone 1	47	6.833	3.432	0.742	0.652	0.659	-0.144	0.167	100.00%
Clone 12	46	6.417	3.030	0.699	0.618	0.625	-0.131	0.167	100.00%
Clone 18	44	6.750	3.614	0.760	0.705	0.713	-0.073	0.083	100.00%
Clone 22	34	6.417	3.476	0.737	0.677	0.688	-0.080	0.000	100.00%
Clone 35	35	6.667	3.523	0.717	0.677	0.687	-0.056	0.083	100.00%
Clone 36	47	7.667	3.906	0.702	0.725	0.733	0.052	0.083	100.00%
Clone 39	32	6.667	3.540	0.682	0.688	0.699	0.011	0.000	100.00%
Clone 4	48	6.167	3.321	0.708	0.674	0.682	-0.048	0.000	100.00%
Clone 5	45	7.500	4.169	0.711	0.732	0.740	0.039	0.000	100.00%
Clone 60	47	6.250	2.818	0.656	0.600	0.606	-0.102	0.083	100.00%
CPTs	98	9.750	4.348	0.743	0.740	0.743	-0.002	0.833	100.00%
East	20	6.417	3.677	0.735	0.678	0.696	-0.077	0.083	100.00%
North	23	6.583	3.529	0.714	0.681	0.696	-0.049	0.083	100.00%
South	28	7.000	3.691	0.684	0.692	0.705	0.029	0.167	100.00%
West	26	6.083	3.738	0.684	0.698	0.712	0.034	0.000	100.00%
Mean		**6.878**	**3.587**	**0.712**	**0.683**	**0.692**	**-0.040**		**100.00%**

N_a = Number of different alleles, A_e = number of effective alleles = 1/(sum pi^2), H_O = observed heterozygosity (No. of Hets/N), H_e = expected heterozygosity (1 – sum pi^2), uH_e = unbiased H_e ((2*N*/(2*N* – 1)) * H_e), F = fixation index = (H_e – H_O)/H_e = 1 – (H_O/H_e), AP = private alleles, *%P* = percentage of polymorphic loci; pi = frequency of the ith allele for the population; sum pi^2 = sum of the squared population allele frequencies.

4.3 Population structure analysis and differentiation results

STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000) was used to assess the degree of admixture and genetic populations of the trees. The optimal K-value was used to enumerate the number of clusters of the 618 trees based on the 12 microsatellite loci by plotting the number of clusters (K) against delta K (Evanno *et al.* 2005). This indicated a sharp peak at K = 2 (Figure 4.2), which was used in the current study. Simulations were run with K ranging from 1–10, with 10 iterations per value of K. The study presumed an admixture model and independent allele frequencies using a burn-in value of 50,000 and 100,000

Markov Chain Monte Carlo iterations for each run (Pritchard *et al*. 2000). The results from the Structure were exhibited as coloured bar charts using membership proportions or Q-values (clone/population) for each tree.

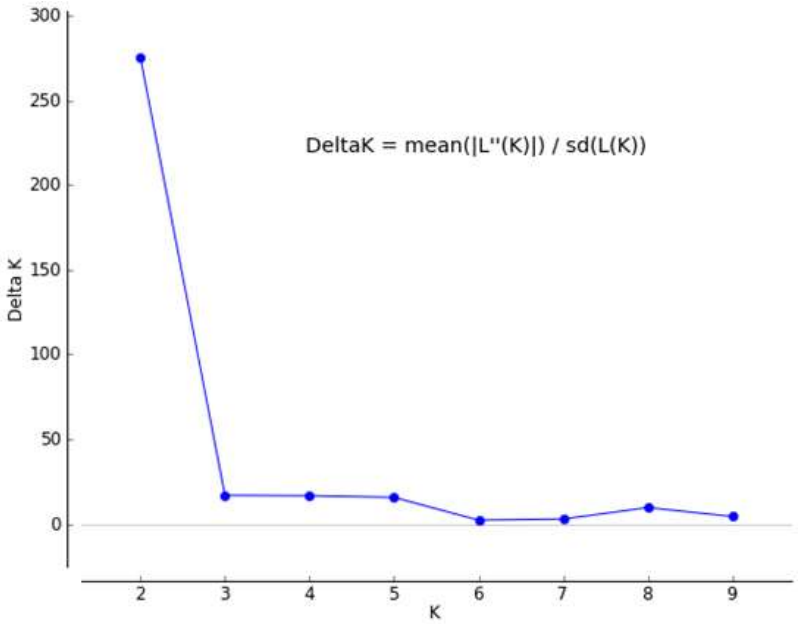


Figure 4.1: The plot of the number of clusters (K) against delta K by the Evanno method (Structure Harvester Web version: v0.6.94). The highest peak at K = 2 represents the best value of K.

The structure of the Melia population inferred by the Bayesian algorithm divided the 618 tree samples into two major clusters (K = 2; Figure 4.2) based on the threshold of 0.5 for the membership probabilities (Q-values). Cluster one (red) comprised of individuals from clones 4, 18, 22 and trees neighbouring the orchard, while cluster two (green) was characterized by individuals from clones 5, 12, 18, 35, 36, 39 and 60. *Melia* individuals from mother plants and clone 1 showed low Q-values below 0.5 and could not be placed into any specific clusters. These clusters show an admixture of individuals among the clones, suggesting an ongoing gene flow in these populations (Figure 4.2).

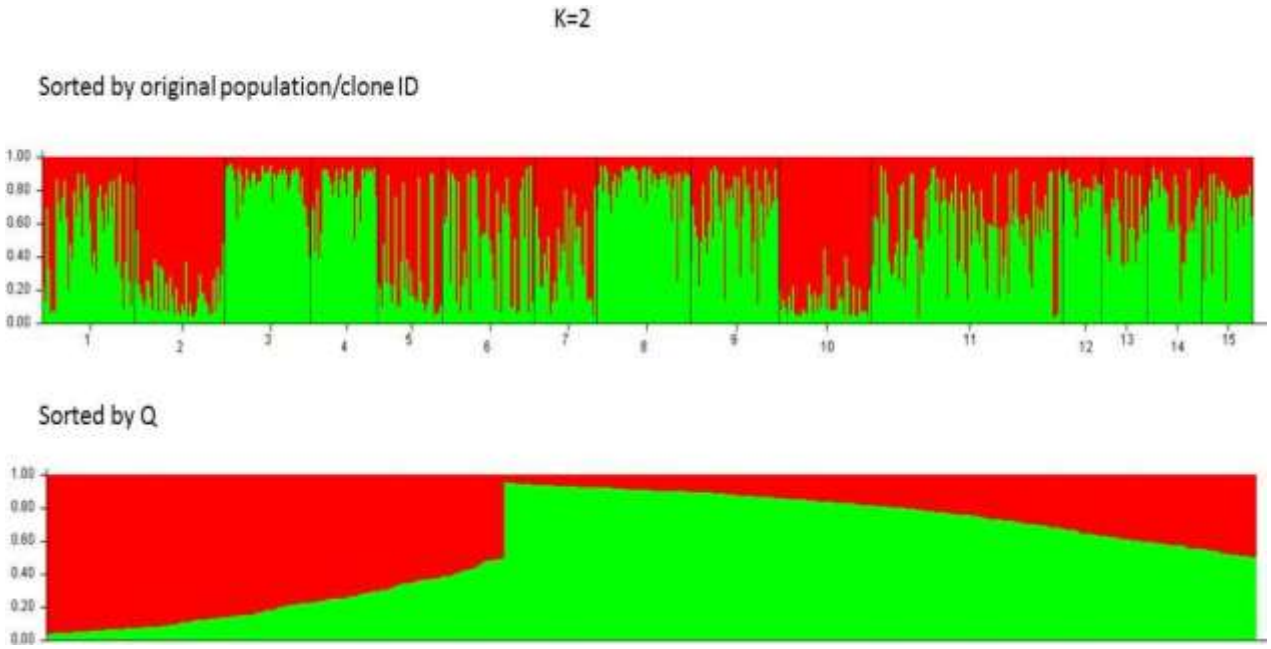


Figure 4.2: Population structure of the 618 Melia tree individuals based on membership probability (Q-values), the coloured box plots represent two inferred clusters.

Pairwise estimates of Nei's genetic distance ranged from 0.03 between clone 60 and clone 12 to 0.439 between clone 60 and clone 18, with an average of 0.175 (Table 4.3). The Nei genetic distances among the neighbouring trees (East, North, South and West) were also low, ranging from 0.051 between North and South trees, to 0.095 between West and North trees (Table 4.3). Clones 4 and 22 also showed a close relationship with each other, with a genetic distance of 0.052 (Table 4.3).

Table 4.3: Pairwise population matrix of Nei's genetic distance among 15 populations of *Melia volkensii*

	Clone 1	Clone 12	Clone 18	Clone 22	Clone 35	Clone 36	Clone 39	Clone 4	Clone 5	Clone 60	CPTs	East	North	South	West
Clone 1	0.000														
Clone 12	0.234	0.000													
Clone 18	0.343	0.393	0.000												
Clone 22	0.241	0.366	0.213	0.000											
Clone 35	0.225	0.050	0.283	0.286	0.000										
Clone 36	0.220	0.115	0.156	0.169	0.071	0.000									
Clone 39	0.217	0.092	0.210	0.218	0.051	0.061	0.000								
Clone 4	0.292	0.350	0.184	0.052	0.273	0.144	0.208	0.000							
Clone 5	0.219	0.202	0.132	0.130	0.136	0.051	0.115	0.099	0.000						
Clone 60	0.285	0.030	0.439	0.399	0.079	0.127	0.101	0.405	0.240	0.000					
CPTs	0.146	0.165	0.140	0.153	0.105	0.057	0.089	0.146	0.059	0.182	0.000				
East	0.195	0.357	0.195	0.114	0.258	0.160	0.181	0.125	0.112	0.371	0.108	0.000			
North	0.263	0.260	0.207	0.145	0.190	0.115	0.125	0.139	0.100	0.265	0.098	0.070	0.000		
South	0.243	0.277	0.158	0.130	0.196	0.115	0.128	0.135	0.091	0.288	0.088	0.052	0.051	0.000	
West	0.247	0.285	0.151	0.140	0.204	0.108	0.151	0.135	0.095	0.307	0.069	0.077	0.095	0.075	0.000

Further, the neighbour-joining (NJ) dendrogram constructed from 100 bootstrap samples of Nei's genetic distances showed two clades (Figure 4.3). Clone 1 clustered with CPTs in the NJ dendrogram with the remaining 13 populations forming the other cluster (Figure 4.3). The Melia trees from the neighbouring farms are closely related and they formed their own sub-cluster with the East and West groups showing the closest genetic relationship (0.077) than with the rest (Figure 4.3). Clones 4 and 22 showed a close relationship with each other whereby the genetic distance was 0.052 (Table 4.3). Similarly clones 12 and 60 also had close relationship with a genetic distance of 0.030 (Table 4.3)

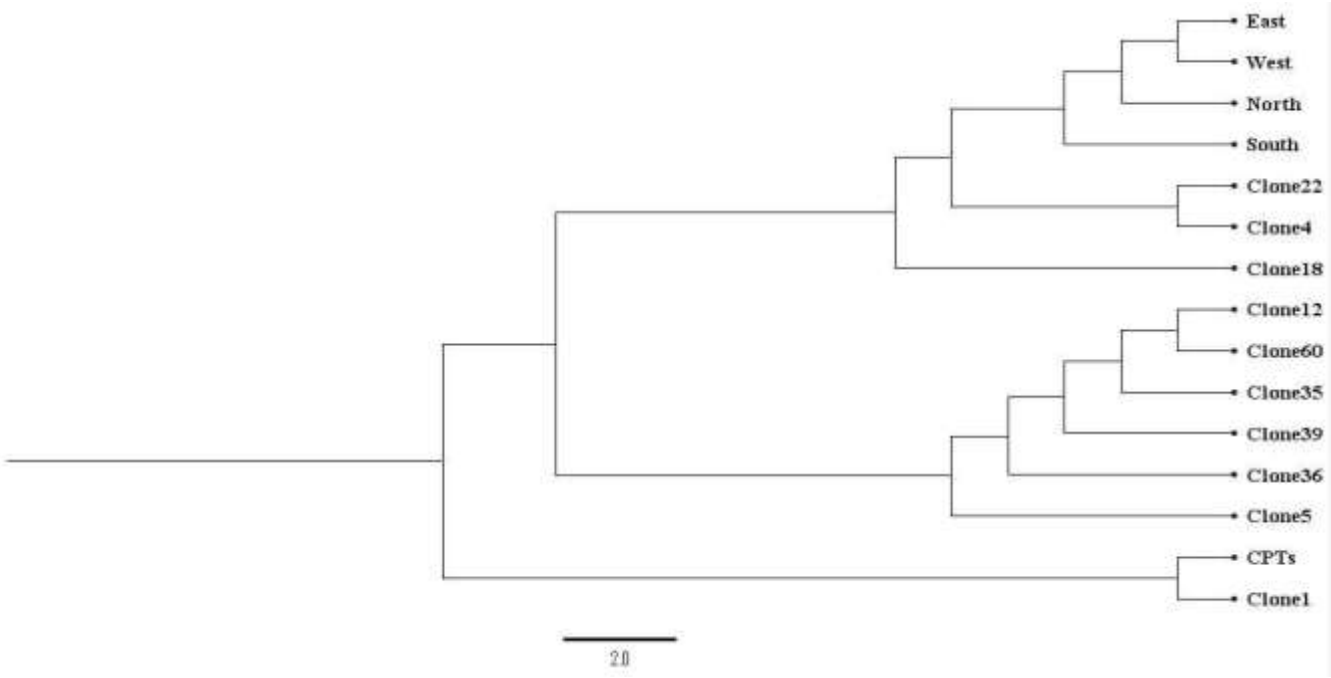


Figure 4.3: Clustering of the Melia CPTs, clones and the neighbouring plants by the neighbour-joining phylogenetic tree based on Nei's standard genetic distances.

4.4 Population differentiation by AMOVA

Population differentiation was performed to demonstrate the extent to which different clones of *M. volkensii* exhibit genetic variation or divergence. According to AMOVA analysis, the structuring of variances showed that most of the genetic variation was partitioned within the populations of *M. volkensii,* while only 5.73% of the variation existed among the population groups. The F_{st} value calculated from AMOVA variances indicated a significantly lower genetic differentiation among the groups (Table 4.4), which is typical for most predominantly outcrossing species such as *M. volkensii*.

Table 4.4: Summary of AMOVA analysis

Source of variation	d.f	Sum of squares	Variance components	Percentage of Variation			
Among Pops	14	339.342	0.248 Va	5.73%	Stat	Value	*P* value
Within Pops	1221	4978.215	4.077 Vb	94.27%			
Total	1235	5317.557	4.325	100%	F_{ST}	0.057	0.001

4.5: Evaluation of mating systems and pollen gene flow within the Melia seed orchard

Gene flow indicates how widespread pollination events are within a given environment. The analysis of the mating system of the progenies in the orchard based on the mixed mating model showed high single- and multi-locus out-crossing rates in *M. volkensii,* with lower bi-parental inbreeding and multilocus parental inbreeding coefficient. The probable number of pollinators comprised about 45.454% of the population with a low

coefficient of co-ancestry (Table 4.5). The observed high outcrossing rate suggests effective gene flow.

Table 4.5: Multilocus estimates from MLTR for the entire populations

Families	Offsprings	Statistics										
		F_m	T_m	T_s	$t_m - t_s$	r_t	R_s	$r_{p(s)}$	$r_{p(m)}$	$r_{p(s)} - r_{p(m)}$	*1/rp*	Θ
10	423	0.167 (0.108)	1.200 (0.119)	1.038 (0.130)	0.162 (0.083)	-0.200 (0.032)	0.562 (0.389)	-0.200 (0.001)	0.022 (0.069)	-0.222 (0.069)	45.454 %	0.045 (0.097)

The standard deviations in parentheses were estimated from 1000 bootstraps. F_m is the multilocus parental inbreeding coefficient; t_m is the multi-locus out-crossing rate; t_s is the single locus out-crossing rate; t_m-t_s = bi-parental inbreeding; r_p = multi-locus correlation of out-crossed progeny; r_s = correlation of selfing among loci; *1/rp* = probable number of pollinators and Θ = coefficient of co-ancestry.

4.6 Parentage analysis

The parentage analysis was tested for 423 progenies (Clones 1, 4, 512, 18, 22, 35, 36, 39 and 60), with 220 possible candidate fathers and typed with at least 10 loci. The average proportion of sampled candidate fathers was 0.999. The paternity analysis implicitly assigned 334 out of 423 progenies (78.96%) to 77 out of 98 CPTs (78.57%) in the clonal seed orchard. The unassigned 89 progenies (21.04%) did not match any of the 98 clones and were considered to be derived from donors outside the orchard (Table 4.6). The number of progenies assigned to paternal clones varied from 1 to 51, with an average of 5.805. The highest paternal clonal contribution was 11.409% of the total assigned progenies, and the top 10 paternal parents were collectively sired with 52.349% of the assigned progenies.

Table 4.6: Table showing results of observed and expected percentage of paternity analysis

Level	Confidence (%)	Critical Delta	Assignment		Assignment rate	
			Observed	Expected	Observed	Expected
Strict	95	2.44	201	(170)	48%	(40%)
Relaxed	75	0.33	334	(367)	79%	(87%)
Unassigned			89	(56)	21%	(13%)
Total			423	(423)	100%	(100%)

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

All the 12 microsatellite markers used to determine the genetic diversity in *M. volkensii* were highly polymorphic, generating 142 alleles and just a few private ones. The polymorphic information content (*PIC*) indicates the ability of a marker to distinguish between different genotypes, with values greater than 0.5 being very informative, 0.25 to 0.5 reasonably informative, and less than 0.25 slightly informative (Avval, 2017; Serrote *et al.,* 2020). The *PIC* values of all the microsatellite markers except bcmv075 were higher than 0.6, indicating that the chosen loci were very informative, diverse and could fully segregate (Bostein *et al.,* 1980; Omondi *et al*., 2010, 2015) all the individual Melia trees in the population. However, locus bcmv075 had low PIC value of 0.388, indicating that the marker did not generate sufficient information from the locus to distinguish between genotypes.

The effective number of alleles A_e showed an average of 3.587 per marker, with a slightly higher average observed heterozygosity (H_O) than the average expected heterozygosity (H_e). The gene diversity or H_e ranges from 0-1, with 1, indicating frequent alleles and 0, the absence of genetic diversity (Gwarinda *et al*., 2021). The mean H_O of most clones were also slightly higher than their H_e and the unbiased H_e (uH_e), implying high genetic diversity and population admixture in Melia trees that were used to establish the seed orchard, which is also common in trees with outcrossing mating systems (Omondi *et al.,* 2015; Anderson, 2016).

The results also indicate that individuals from the Block one orchard are more heterozygous than would be expected under HWE as a result of random mating or cross pollination among the different Melia individuals or populations (Markam *et al.*, 2018). The uH_e estimates the probability of heterozygosity of an individual in a population at a particular genetic locus, providing an unbiased estimate of genetic diversity (Pruett and Winker, 2008). The H_o was also higher than H_e in all loci and populations, except in bcmv30 and bcmv75, clones 36, 39 and 5, and plants located in the south and west directions, indicating some bi-parental inbreeding within the two loci in these populations. Overall, the mean F_{is} was negative, revealing insignificant inbreeding in the population (Guo *et al*., 2014; Omondi *et al*., 2015).

The high genetic diversity in the *M. volkensii* seed orchard at Kibwezi also corroborates with results from other populations of cross-pollinating plants such as *Swietenia macrophylla*, *Acacia tortilis* and *A. senegal* that often exhibit a high level of heterozygosity and genetic variation (Novick *et al*., 2003; Omondi *et al*., 2010, 2015). The high heterozygosity also suggests an exclusive out-crossing mating system in *M. volkensii*, possibly promoting inter-breeding and genetic diversity of trees in the Kibwezi seed orchard.

Melia volkensii is endemic to arid and semi-arid habitats with diverse and harsh climatic conditions in eastern Kenya (Jaetzold and Schmidt, 1983; Orwa *et al*., 2009; Kamondo *et al*., 2016), in which sufficient genetic diversity is vital for its adaptation. The relative heterogeneity of the habitat also explains the presence of effective alleles and high gene

diversity in *M. volkensii* clones as was suggested for *Prunus sibirika* with a wide and spatial distribution in the heterogeneous dryland of China (Li *et al.,* 2013; Wang *et al*., 2014). These moderate to high values of genetic diversity among the clones may be attributed to the history of expansive areas where CPTs or the ramets of *M. volkensii* clones were obtained from and their spatial distribution since trees with broad geographic distribution and long lifespans usually exhibit high levels of genetic diversity (Omondi *et al*., 2010, 2016b, 2019).

Tropical tree species, particularly those with outbreeding mating systems, long distant gene flow patterns, and growing in heterogeneous habitats such as dry-land ecosystems, also exhibit similar results (Wang *et al.* 2014). Therefore, the high genetic diversity in *M. volkensii* could also be attributed to significant levels of gene flow among populations through pollen movement or cross-pollination and seed dispersal (Matsushita *et al.*, 2021).

Fixation index (F_{st}) is a measure of population differentiation due to genetic structure and values above 0.15 are considered significant to differentiate among populations (Frankham *et al*., 2002). The low F_{st} value measured in Melia (F_{st} = 0.068) is typical for most predominantly outbreeding species, such as *A. tortilis* and *A. senegal* (Omondi *et al.*, 2015, 2019), suggesting a low to moderate differentiation (Rousset, 1997) for Melia trees used in this study.

The low F_{st} value also indicates existence of gene flow and other selective pressure among the *M. volkensii* clones in the orchard. The hierarchical distribution of molecular variance revealed significant genetic differentiation within the groups, with much of the variation partitioned within the groups, and a little among the groups of Melia. The F_{ST} value calculated from the AMOVA variances also indicated a significantly low genetic differentiation among the groups, further supporting low values measured in individual Melia trees. High genetic differentiation among populations is unique to most selfing species while less variation among populations is exhibited predominantly by outcrossing wind-pollinated woody species (Shuster, 2009).

Past studies on *M. volkensii* structured the Kenyan populations into the Northern, Central and Southern gene pools as a result of the fragmentation of the vast population of Melia trees due to human settlements and the development of roads and huge dams, interfering with the gene flow from the southern to the northern region by agents of seed dispersal such as elephants who are the major dispersers of *M. volkensii* seeds (Omondi *et al*., 2018). The current study structured the 15 *M. volkensii* population into two gene pools comprising of red (clones 4, 18, 22 and trees from the neighbouring farms) and green (clones 5, 12, 35, 36, 39 and 60), the reduction in gene pool is attributed to reduction in population size, founder effect and gene flow (Anderson, 2016; Omondi *et al*., 2010, 2015) For any species, the gene pool is dynamic and changes over time as a result of mutation, gene flow, genetic drift and natural selection (Nayanakantha *et al*., 2010; Frankham *et al.,* 2011).

The neighbour-joining phylogenetic tree also clustered the populations into two major groups, with CPTs and clones 1 clustering independently and the other 13 populations forming the other cluster. The lowest genetic distance was observed between clones 12 and 60 signifying a high genetic similarity and close relationship between the two; the highest genetic distance was between clones 18 and 60 (0.439).

The outcrossing rate principally determines the mating system and patterns of pollen donors and their contributions to the genetic diversity of the offspring (Shuster, 2009). Multi-locus outcrossing rate (t_m) values can be used to assess the level of outcrossing whereby values less than 1.0 show some level of selfing, less than 0.5 signify mixed or predominantly selfing mating pattern and values around 1.0 is observed in predominantly outcrossing population which is the case in present study (Savolainen *et al.*, 2007; Omondi *et al*., 2010). Coefficient of co-ancestry quantifies the genetic relatedness among two individuals whereby coefficients of 0 indicate no relatedness and coefficient of 1.0 signify identical twins. The coefficient of co-ancestry in the present study was 0.045 indicating that a first degree of relatedness exists among the population (Zhou *et al*., 2021).

The high multi- locus (t_m) and single-locus (t_s) outcrossing rates observed indicate a mixed mating pattern with a predominantly outcrossing breeding system in Melia, while the low difference between t_m and t_s shows the absence of bi-parental inbreeding in the tree in the orchard. The estimated correlation of paternity (r_p = 0.022) was also low indicating that many Melia progenies did not share the same paternal genitors. The self-

pollination rate can vary over a long time and is rare in a high-density, morph-ratio unbiased population of trees due to adequate gene flow throughout the population (savolainen *et al*., 2007). The insignificant self-mating in *M. volkensii* trees is attributed to its phenology and the availability of mating partners in the Kibwezi seed orchard.

For flower phenology, a relatively lengthy intra-morph bloom overlap may affect the degree of assortative mating in trees (Bai *et al.,* 2007; Zhou *et al.*, 2021), though its role in preventing self-mating in *M. volkensii* was not determined in the current study. The multi-locus parental inbreeding coefficient (F_m) within progenies was non-significantly low, showing a lack of inbreeding in Melia, with 45.45% of pollen sources (males) contributing to the fertilization of the progenies. This moderate pollen cloud diversity and low selfing rate in the Kibwezi seed orchard could be due to the panmictic design and layout of Melia in the seed orchard by breeders, with the closely related trees grown far from each other to rule out any incidence of self-pollination and guard diversity of the progenies (Bai *et al.,* 2007; Zhou *et al.*, 2021). Generally, the results show that *M. volkensii* naturally and predominantly outcross to minimize inbreeding and its depression and maintain high genetic diversity (Barrett and Harder, 2017; Hodgins and Yeaman, 2019), positively affecting genetic gain (Ivetic *et al.*, 2016; Tambarussi *et al*., 2018).

The paternity analysis assigned the majority of the progenies (78.57%) to 77 of the 98 potential seed orchard clones, with the unassigned 89 progenies (21.04%) not matching any of the seed orchard clones and are thought to be derived from unimproved background population. Similar findings were observed in an elite population of

Eucalyptus dunnii earmarked for establishment of a seed orchard (Poltri *et al*., 2003) whereby the probability of exclusion (Pe) was 0.9998 and the combined power of exclusion was greater than 99.98% (Ochieng *et al*., 2018). This is an indicator of existence of adequate gene flow taking place. This also points out the success of each molecular pattern which was assigned to each of the 98 genotypes designated as mother plants and the robust information sufficient to ensure molecular traceability for each of these materials at the different stages of nursery production and or field trials.

Compared to literature on past molecular studies of *M. volkensii* utilizing microsatellite markers, results of the current study has indicated existence of adequate gene flow within the orchard probably due to predominant out-crossing breeding nature and reproductive synchrony among trees within the orchard. Additionally, the seed orchard design and flowering synchrony with the background population could have contributed to the pollen introgression in the orchard. Review of the current isolation distance between the orchard and the background population should be considered to ensure integrity of the seed source.

5.2 Conclusions

i. There is high genetic diversity in *M. volkensii* trees growing in Kibwezi seed orchard block one.
ii. There exists adequate gene flow within the seed orchard for *Melia volkensii*, trees exhibit a mixed mating system with a predominant outcrossing breeding nature that is favoured by reproductive synchrony.

iii. There is considerable level of pollen contamination from the unimproved background population of *M. volkensii* in the Kibwezi seed orchard.

5.3 Recommendations

To maintain high genetic diversity of planting materials obtained from seed orchard and existence of adequate gene flow within the orchard it is vital to consider the following:

i. The panmictic design of the seed orchard and flowering synchrony with the background population could contribute to the pollen introgression in the orchard therefore regular studies should be done across the other blocks of the orchard to find out the extent of contamination and then come up with suitable mitigation which might include thinning.
ii. A review of the current isolation distance of 200 metres between the orchard and the background population should be considered to ensure the integrity of the seed source
iii. Incorporate study of morphological traits to molecular work when studying the genetic diversity of Melia for detailed comparisons among the trees from all over the *M. volkensii* growing belt.
iv. A study on gene flow patterns in other blocks of the seed orchard and progeny test sites for comparison of results.
v. Study on effect of insect population on intensity of mating system and pollen flow among trees within the orchard.

REFERENCES

Asudi, G.O., Ombwara, F.K., Rimberia, F.K., Nyende, A.B., Ateka, E.M., Wamocho, L.S., Shitanda, D., and Onyango, A. (2010). Morphological diversity of Kenyan papaya germplasm. *African Journal of Biotechnology*, 9(51), 8754-8762

Asudi, G. O., Ombwara, F. K., Rimberia, F. K., Nyende, A. B., Ateka, E. M., and Wamocho, L. S. (2013). Evaluating diversity among Kenyan papaya germplasm using simple sequence repeat markers. *African Journal of Food, Agriculture, Nutrition and Development*, **13**(1), 7307-7324.

Anderson, J.T. (2016). Plant fitness in a rapidly changing world. *New Phytologist*, 210(1), 81-87.

Almazroui, M., Saeed, F., Saeed, S., Islam, M.N., Ismail, M., Klutse, N.A.B., Siddiqui, M.H. (2020). Projected change in temperature and precipitation over Africa from CMIP6. *Earth Systems and Environment*, 4, 455-475. https://doi.org/10.1007/s41748-020-00161-x

Al-Sharook, Z., Byline, K., Jiang, Y., and Rembold, H. (1991). Insect growth inhibitors from two tropical Meliaceae: effects of crude seed extracts on mosquito larvae. *Journal of Applied Entomology*, 111, 425-430.

Avval, S. E. (2017). Assessing polymorphism information content (PIC) using SSR molecular markers on local species of Citrullus colocynthis. Case study: Iran, Sistan-Balouchestan province. *Journal of Molecular Biology Research*, **7**(1), 42-49.

Bahadur, B., Rajam, M.V., Sahijram, L., and Krishnamurthy, K.V. (2015). Plant biology and biotechnology: Volume II. *Plant Genomics and Biotechnology*, II, 1-768.

Bai, Y., and Lindhout, P. (2007). Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? *Annals of botany*, **100**(5), 1085-1094.

Barboza, K., Beretta, V., Kozub, P.C., Salinas, C., Morgenfeld, M.M., Galmarini, C.R., and Cavagnaro, P.F. (2018). Microsatellite analysis and marker development in garlic: distribution in EST sequence, genetic diversity analysis, and marker transferability across Alliaceae. *Molecular Genetics and Genomics*, 293(5), 1091–1106.

Barrett, S.C.H. (1998). The evolution of mating strategies in flowering plants. *Trends in Plant Science*, 3(9), 335-341. https://doi.org/10.1016/S1360-1385(98)01299-0

Barrett, S.C., and Harder, L.D. (2017). The ecology of mating and its evolutionary consequences in seed plants. *Annual Review of Ecology, Evolution and Systematics*, **48**, 135-157.

Botstein, 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(3), 314.

Broadhead, J.S., Black, C.K., and Ong, C.K. (2003). Tree leafing phenology and crop productivity in semi-arid agroforestry systems in Kenya. *Agroforestry Systems*, 58, 137-148.

Campbell, S.A. (2015). Ecological mechanisms for the coevolution of mating systems and defence. *New Phytologist*, 205(3), 1047-1053. https://doi.org/10.1111/nph.13212

Chandrawati, Singh, N., Kumar, R., Kumar, S., Singh, P.K., Yadav, V.K., and Yadav, H.K. (2017). Genetic diversity, population structure and association analysis in linseed (*Linum usitatissimum* L.). *Physiology and Molecular Biology of Plants*, 23(1), 207-219.

Dale, I.R., and Greenway, P.J. (1961). Kenya trees and shrubs. Nairobi. Buchanan's Kenya Estates Ltd.

Dieringer D., Schlötterer C. (2003) microsatellite analyser (msa) — a platform independent analysis tool for large microsatellite data sets. Molecular Ecology Notes, 3, 167–169.

Earl, D. A., and VonHoldt, B. M. (2012). STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation genetics resources*, *4*, 359-361.

El-Kassaby, Y.A., Fayed, M., Klápště, J., and Lstibůrek, M. (2014). Randomized, replicated, staggered clonal-row (R2SCR) seed orchard design. *Tree Genetics and Genomes*, 10(3), 555-563. https://doi.org/10.1007/s11295-014-0703-7

Evanno, G., Regnaut, S., and Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, **14**(8), 2611-2620.

Excoffier, L., and Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources*, **10**(3), 564-567.

FAO. (2020). Global forest resources assessment (FRA) 2020 Kenya – Report. Rome. Retrieved from https://www.fao.org/3/cb0019en/cb0019en.pdf on 27th March 2024.

Farooq, S., Shah T.M., Askari. E., Zaidi, A.A., Arif, M., and Iqbal, N. (1994). Identification of different wheat genotypes through polymorphism based on random amplified polymorphic DNA (RAPD). *Pakistan Journal of Botany*, 26, 373-382.

Farooq, S., Arif, M., and Iqbal, N. (1995). Utilization of RAPD markers for identification of cultivated and wild rice species. *Pakistan Journal of Botany*, 27, 127-138

Farooq, S., and Azam, F. (2002). Molecular markers in plant breeding-I: Concepts and characterization. *Pakistan Journal of Biological Sciences*, 5(10), 1135–1140.

Fowler, C., and Mooney, P.R. (1990). Shattering: Food, politics, and the loss of genetic diversity. University of Arizona Press.

Frankham, R., Briscoe, D. A., and Ballou, J. D. (2002). *Introduction to conservation genetics*. Cambridge university press.

Frankham, R., Ballou, J. D., Eldridge, M. D., Lacy, R. C., Ralls, K., Dudash, M. R., and Fenster, C. B. (2011). Predicting the probability of outbreeding depression. *Conservation Biology*, **25**(3), 465-475.

Frankham, R. (2015). Genetic rescue of small inbred populations: meta-analysis reveals large and consistent benefits of gene flow. *Molecular Ecology*, 24(11), 2610-2618.

Gaiotto, F. A., Grattapaglia, D., and Vencovsky, R. (2003). Genetic structure, mating system, and long-distance gene flow in heart of palm (*Euterpe edulis Mart*.). *Journal of Heredity*, 94(5), 399-406.

Gniech-Karasawa, M.M. (2016). Importance of plant mating systems for conservation and plant breeding. *Revista RG News*, 2(2), 110–118.

Goudet, J. (1995). FSTAT (Version 1.2): a computer program to calculate F-statistics. *Journal of Heredity*, 86, 485–486.

Goudet, J. (2001). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Electronic file available from http://www.unil.ch/ izea/softwares/fstat.html.

Goudet, J. (2002). FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9. 3.2). *http://www. unil. ch/izea/softwares/fstat. html*.

Govindaraj, M., Vetriventhan, M., and Srinivasan, M. (2015). Importance of genetic diversity assessment in crop plants and its recent advances: An overview of its analytical perspectives. In *Genetics Research International*, 1, p. 14. https://doi.org/10.1155/2015/431487

Guasmi, F., Elfalleh, W., Hannachi, H., dia, Res, K., Ferchichi, A. (2012). The Use of ISSR and RAPD markers for Genetic diversity among South Tunisian barley. *International Scholarly Research Notices*, e952196.

Guo, D.L., Guo, M.X., Hou, X.G., and Zhang, G.H. (2014). Molecular diversity analysis of grape varieties based on iPBS markers. *Biochemical Systematics and Ecology*, 52, 27-32.

Gwarinda, H.B., Tessema, S,K., Raman, J., Greenhouse, B., Birkholtz, L.M. (2021). Parasite genetic diversity reflects continued residual malaria transmission in Vhembe District, a hotspot in the Limpopo Province of South Africa. *Malaria Journal*. 20(1):96.

Hanaoka, S., Muturi, G. M., and Watanabe, A. (2012). Isolation and characterization of microsatellite markers in *Melia volkensii Gurke*. *Conservation Genetics Resources*, **4**(2), 395–398.

Hanaoka, S., Ohira, M., Matsushita, M., and Kariuki, J. (2016). Optimizing the size of root cutting in Melia volkensii Grke for improving clonal propagation and production of quality planting stock. *African Journal of Biotechnology*, 15(29), 1551-1558.

Hayward, A.C., Tollenaere, R., Dalton-Morgan, J., and Batley, J. (2015). Molecular marker applications in plants. *Methods in Molecular Biology*, 1245, 13-27.

Hodge, G. R., and White, T.L. (1993). Advanced-generation wind-pollinated seed orchard design. *New Forests*, *7*(3), 213-236. https://doi.org/10.1007/BF00127387

Hodgins, K.A., and Yeaman, S. (2019). Mating system impacts the genetic architecture of adaptation to heterogeneous environments. *New Phytologist*, ***224***(3), 1201-1214.

Horecky, C., Horecka, E., Futas, J., Janova, E., Horin, P., and Knoll, A. (2018). Microsatellite markers for evaluating the diversity of the natural killer complex and major histocompatibility complex genomic regions in domestic horses. *Hla*, *91*(4), 271–279.

Ivetić, V., Devetaković, J., Nonić, M., Stanković, D., and Šijačić-Nikolić, M. (2016). Genetic diversity and forest reproductive material - from seed source selection to planting. *IForest*, **9**(5), 801–812. https://doi.org/10.3832/ifor1577-009

Jaetzold, R., and Schmidt, H. (1983). Natural conditions and farm management: Farm management handbook of Kenya. Ministry of Livestock Development, Nairobi, Vol. 2.

Jayusman, J., Na'iem, M., Indrioko, S., Hardiyanto, E.B., and Nurcahyaningsih, I.L.G. (2018). Assessment of genetic diversity among surian *Toona sinensis* Roem in progenies test using random amplified polymorphic DNA markers. *Indonesian Journal of Biotechnology*, 22(1), 22-30. https://doi.org/10.22146/ijbiotech.25798

Kalinowski, S. T., Taper, M. L., and Marshall, T. C. (2007). Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular ecology*, *16*(5), 1099-1106.

Kamondo, B. M., Kimondo, J. M., Mulatya, J. M., and Muturi, G. M. (2004). Recent Mukau (*Melia volkensii Gurke*) research and development. In *Proceedings of the First National Workshop, KEFRI Kitui Regional Research Center. November* (Vol. 16).

Kamondo, B.M., Kariuki J.G., Luvanda, A.M., Muturi, G.M., and Ochieng, D. (2016). (eds). Guideline on production, distribution and use of improved Melia seed and seedlings in the drylands of Kenya. KEFRI, Kenya. retrieved from https://www.kefri.org/assets/publications/extension/Guideline%20on%20Production,%20Distribution%20and%20Use%20of%20Improved%20Melia%20Seed%20and%20Seedlings%20in%20the%20Drylands%20of%20Kenya.pdf on the 27th March 2024.

Kariuki, J., Hanaoka, S., Miyashita, B., and Muchiri, D. (2016). Breeding for improved Melia seed and seedlings. In: Kamondo B.M., Kariuki J.G., Luvanda A.M., Muturi G.M. and Ochieng D. 2016.(eds). Guideline on production, distribution and use of improved Melia seed and seedlings in the drylands of Kenya. KEFRI, Kenya retrieved from https://www.kefri.org/assets/publications/extension/Guideline%20on%20Production,%20Distribution%20and%20Use%20of%20Improved%20Melia%20Seed%20and%20Seedlings%20in%20the%20Drylands%20of%20Kenya.pdf on the 27th March 2024.

KEFRI. (2013). Republic of Kenya project for development of drought tolerant trees for adaptation to climate change in drylands of Kenya Annual Report (FY 2012).

Kenya, Ministry of Environment and Natural Resources (MENR). (2016). National Forest Programme of Kenya. MENR, Nairobi, Kenya. Retrieved from on the 27th March 2024.

Kidundo, M. (1997). Participatory technology development and nursery propagation of *Melia volkensii* Gürke: A potential agroforestry tree species in semi-arid Kenya. MPhil Thesis, University of Wales, Bangor.

Kim, S.K., Nair, R.M., Lee, J., and Lee, S.-H. (2015). Genomic resources in mungbean for future breeding programs. *Frontiers in Plant Science*, 6, 626.

Lavanya, G.R., Srivastava, J., and Ranade, S.A. (2008). Molecular assessment of genetic diversity in mung bean germplasm. *Journal of Genetics*, 87(1), 65–74.

Liengsiri, C., Boyle, T.J.B., and Yeh, F.C. (1998). Mating system in *Pterocarpus macroscarpus* Kurz in Thailand. *The American Genetic Association*, 82: 216-221

Li, M., Zhao, Z., Miao, X., and Zhou, J. (2013). Genetic diversity and population structure of Siberian apricot (*Prunus sibirica* L.) in China. *International Journal of Molecular Sciences*, 15(1), 377-400.

Liu, P., Zhu, X., Tan, Z., Zhang, X., and Ma, Y. (2016). Construction of *Escherichia coli* cell factories for production of organic acids and alcohols. *Bioreactor Engineering Research and Industrial Applications I: Cell Factories*, 107-140.

Lorenz, T. C. (2012). Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. *Journal of Visualized Experiments*, (63), e3998.

Lugadiru, J. 2004: *Melia volkensii* seed extractor. In: **Kamondo B.M., J.M Kimondo., J.M. Mulatya and G. M. Muturi (2006).** Eds. Proceedings of the First National Workshop on recent Mukau (Melia volkensii Gűrke). Research and Development held at KEFRI Kitui Regional Research Centre 16 to 19th November 2004. 51pp.

Luvanda, A. M., Musyoki, J., Cheboiwo, J., Wekesa, L., and Ozawa, M. (2015). An assessment of the socio-economic importance of *Melia volkensii* based enterprises in Kenya. 66.

Mabberley, D.J. (1997). The Plant-book: A portable dictionary of the vascular plants. 2nd edition. Cambridge University Press, 40 W. 20th St., New York, NY 10011-4211. 858 pp. ISBN 0-521-41421-0.

Markam, N. K., Nair, S. K., Saxena, R. R., and Nanda, H. C. (2018). Assessment of genetic diversity using SSR marker in moonbean [*Vigna radiata (L.)* Wilczek] genotypes. *Journal of Pharmacognosy and Phytochemistry*, *7*(2), 2750-2754.

Marshall, T.C, Slate J, Kruuk LEB, and Pemberton J.M. (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Molecular Ecology, **7**, 639–655.

Matheri, F., Mwangi, M., Runo, S., Ngugi, M., Kirubi, D. T., Teya, F., and Kamau, D. N. (2016). Phenotypic characterization of selected kenyan purple and yellow passion fruit genotypes based on morpho-agronomic descriptors. *Adv Crop Sci Tech*, ***4*(**226), 2.

Martins-Lopes, P., Lima-Brito, J., Gomes, S., Meirinhos, J., Santos, L. and Guedes Pinto, H. (2007). RAPD and ISSR molecular markers in Olea europaea L.: Genetic variability and molecular cultivar identification. *Genetic Resources and Crop Evolution*, 54(1), 117-128.

Matsushita, M., Omondi, S.F., Hanaoka, S., Kariuki, J.G., Cherotich, L., and Ndufa, J.K. (2021). Breeding strategies, mating systems and future perspective of indigenous tree species improvement in Kenya : a case study of *Melia Volkensii*. https://www.researchgate.net/publication/359369510

Mganga, K.Z., Musimba, N.K.R., and Nyariki, D.M. (2015). Combining sustainable land management technologies to combat land degradation and improve rural livelihoods in Semi-arid Lands in Kenya. *Environmental Management*, 56(6), 1538-1548.

Moeller, D.A., Briscoe Runquist, R.D., Moe, A.M., Geber, M.A., Goodwillie, C., Cheptou, P.O., Eckert, C.G., Elle, E., Johnston, M.O., Kalisz, S., Ree, R.H., Sargent, R.D., Vallejo-Marin, M., Winn, A.A. (2017). Global biogeography of mating system variation in seed plants. *Ecology Letters*, 20(3), 375-384. doi: 10.1111/ele.12738. Epub Jan 24. PMID: 28116770.

Mulatya, J.M. (2000). Tree root development and interactions in drylands: Focusing on *Melia volkensii* with socio-economic evaluations. PhD. Thesis, University of Dundee.

Muturi, G.M., Mutua G.K. and Maingi F.M. 2003. Inter-specific variation of biomass production and root morphology among 20 drylands species after 15 months of growth in an arid site in Kitui district, Kenya: Implication to dry land silviculture. In: Odera, J.A. and Kamondo, B.M. (Eds). Management of drylands biodiversity in Eastern Africa. Proceedings of the Regional Workshop held at the National Museums of Kenya, Nairobi, 30th July to 1st August, 1997. at KEFRI Kitui Regional Research Centre 16 to 19th November 2004. 51pp.

Mwangi, R.W., and Mukiama, T.K. (1989). Studies of insecticidal and growth-regulating activity in extracts of *Melia Volkensii* (Gurke), an indigenous tree in Kenya . *East African Agricultural and Forestry Journal*, 54(3), 165-171. https://doi.org/10.1080/00128325.1989.11663564

Nadeem, M.A., Nawaz, M.A., Shahid, M.Q., Doğan, Y., Comertpay, G., Yıldız, M., Hatipoğlu, R., Ahmad, F., Alsaleh, A., Labhane, N., Özkan, H., Chung, G., and Baloch, F.S. (2018). DNA molecular markers in plant breeding: current status and recent advancements in genomic selection and genome editing. *Biotechnology and Biotechnological Equipment*, 32(2), 261–285. https://doi.org/10.1080/13102818.2017.1400401

Nayanakantha, N. M. C., Singh, B. R., and Gupta, A. K. (2010). Assessment of genetic diversity in Aloe germplasm accessions from India using RAPD and morphological markers. *Journal of Cell Science* 39: 1-9.

Novick, R.R., Dick, C.W., Lemes, M.R., Navarro, C., Caccone, A., and Bermingham, E. (2003). Genetic structure of Mesoamerican populations of big-leaf mahogany (*Swietenia macrophylla*) inferred from microsatellite analysis. *Molecular Ecology*, 12, 2885–2893.

Ochieng D., Kamondo B. and Muturi G.M. 2018. (eds). Proceedings of international conference on 'Project on development of drought tolerant trees for adaptation to climate change in drylands of Kenya' held at KEFRI Muguga 13 -16 February 2017. KEFRI, Kenya. retrieved from https://www.researchgate.net/profile/Muturi-M/publication/353315442_Proceedings_of_International_Conference_on_the_Project_on_Development_of_Drought_Tolerant_Trees_for_Adaptation_to_Climate_Change_in_Drylands_of_Kenya_at_KEFRI_Headqurters_Muguga/links/60f2fa7bfb568a7098b92f6d/Proceedings-of-International-Conference-on-the-Project-on-Development-of-Drought-Tolerant-Trees-for-Adaptation-to-Climate-Change-in-Drylands-of-Kenya-at-KEFRI-Headqurters-Muguga.pdf on the 27th March 2024.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., and Anthony, S. (2009). Agroforestree Database:a tree reference and selection guide version 4.0. retrieved from http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp on the 27th March 2024.

Omondi, F.S., Kireger, E., Dangasuk, G.O., Chikamai, B., Odee, D.W., Cavers, S., and Khasa, D.P. (2010). Genetic diversity and population structure of *Acacia senegal* (L) Willd. in Kenya. *Journal of Tropical Plant Biology*, ***3***, 59–70.

Omondi, S.F., Machua, J., Gicheru, J., and Hanaoka, S. (2015). Isolation and characterization of microsatellite markers for *Acacia tortilis* (Forsk.) Hayne. *Conservation Genetics Resources*, 7(2), 529-531. https://doi.org/10.1007/s12686-014-0413-3

Omondi, E.O., Debener, T., Linde, M., Abukutsa-onyango, M., Dinssa, F.F., and Winkelmann, T. (2016). Molecular markers for genetic diversity studies in African leafy vegetables, *Advances in Bioscience and Biotechnology*, 7(3), 188-197.

Omondi, S. F., Odee, D. W., Ongamo, G. O., Kanya, J. I., and Khasa, D. P. (2016). Synchrony in leafing, flowering, and fruiting phenology of senegalia senegal within lake baringo woodland, kenya: implication for conservation and tree improvement. *International Journal of Forestry Research*, *2016*.

Omondi, S. F., Odee, D. W., Ongamo, G. O., Kanya, J. I., and Khasa, D. P. (2018). Mating patterns of the gum arabic tree (*Acacia senegal* synonym *Senegalia senegal*) in two different habitats. *New forests*, *49*, 53-65.

Omondi, S.F., Machua, J., Muturi, G.M., Gicheru, J., Hanaoka, S. (2019). Evidence of high genetic diversity and significant population structuring in *Vachellia tortilis* (Forsk.) Galasso & Banfi population in Kenya. *Annals of Forest Science*, 76(47), 1–14.

Palma-Silva, C., Cozzolino, S., Paggi, G.M., Lexer, C., and Wendt, T. (2015). Mating system variation and assortative mating of sympatric bromeliads (*Pitcairnia* spp.) endemic to neotropical inselbergs. *American Journal of Botany*, 102(5), 758-764.

Pannell, J.R. (2015). Evolution of the mating system in colonizing plants. *Molecular Ecology*, 24, 2018–2027. https://doi.org/10.1111/ mec.13087

Peakall, R., and Smouse, P.E. (2012). GenAlEx6.5: genetic analysis in Excel. Population genetic software for teaching and research–An update. *Bioinformatics*, 28, 2537-2539.

Peakall, R., and Smouse, P.E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, 6, 288-295

Pierre-Olivier C. (2012). The evolution of plant mating system: Is it time for a synthesis? *Studies in Population Genetics*. InTech. Available at: http://dx.doi.org/10.5772/35137

Poltri, S. M., Zelener, N., Traverso, J. R., Gelid, P. A. N. D., and Hopp, H. E. (2003). Selection of a seed orchard of *Eucalyptus dunnii* based on genetic diversity criteria calculated using molecular markers. *Tree physiology*, *23*(9), 625-632.

Pritchard, J. K., Stephens, M., and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, *155*(2), 945-959.

Pruett, C.L., and Winker, K. (2008). The effects of sample size on population genetic diversity estimates in song sparrows *Melospiza melodia*. *Journal of Avian Biology*, *39*(2), 252–256.

Raymond, M., and Rousset, F. (1995). An exact test for population differentiation. *Evolution*, 1280-1283.

Ritland, K. (1989). Correlated matings in the partial selfer Mimulus guttatus. *Evolution*, *43*(4), 848-859.

Ritland, K. (1996). Estimators for pairwise relatedness and individual inbreeding coefficients. *Genetics Research*, *67*(2), 175-185.

Ritland, K. (2002). Extensions of models for the estimation of mating systems using n independent loci. *Heredity*, *88*(4), 221-228.**Rousset, F. (1997).** Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, *145*(4), 1219–1228.

Rousset, F. (1997). Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, *145*(4), 1219–1228.

Runo, M.S., Muluvi G.M., and Odee D.W. (2004). Analysis of genetic structure in *Melia volkensii* (Gurke.) populations using random amplified polymorphic DNA. *African Journal of Biotechnology*, 3(8), 421-425.

Savolainen, O., Pyhäjärvi, T., and Knürr, T. (2007). Gene flow and local adaptation in trees. *Annu. Rev. Ecol. Evol. Syst.*, *38*, 595-619.

Serrote, C. M. L., Reiniger, L. R. S., Silva, K. B., dos Santos Rabaiolli, S. M., and Stefanel, C. M. (2020). Determining the polymorphism information content of a molecular marker. *Gene*, *726*, 144175.

Shuster, S.M. (2009). Sexual selection and mating systems. *Proceedings of the National Academy of Sciences* (PNAS), U S A. 16, 106. doi: 10.1073/pnas.0901132106.

Stewart, M., and Blomley, T. (1994). Use of *Melia volkensii* in semi agroforestry systems in Kenya. *Commonwealth Forestry Review*, 73(2), 128-131.

Takezaki, N., and Nei, M. (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*, *144*(1), 389-399.

Tambarussi, E.V., Pereira, F.B., da Silva, P.H.M., Lee, D., and Bush, D. (2018). Are tree breeders properly predicting genetic gain? A case study involving *Corymbia* species. *Euphytica*, 214**,** 150. https://doi.org/10.1007/s10681-018-2229-9

Tedd, J. 1997. Perception, Management and Use of *Melia volkensii* by farmers. A case study from the Kibwezi division, Kenya. MSc. Thesis, University of Nottingham.

Todorovska, E., Abumhadi, N., Kamenarova, K., Zheleva, D., Kostova, A., Christov, N., Alexandrova, Jacquemin, J-M., Anzai, H., Nakamura, C., and Atanassov, A. (2005). Biotechnological approaches for cereal crops improvement: Part II: Use of molecular markers in cereal breeding. *Biotechnology & Biotechnological Equipment*, *19*(sup3), 91-104.

Thakur, S., Choudhary, S., Singh, A., Ahmad, K., Sharma, G., Majeed, A., and Bhardwaj, P. (2016). Genetic diversity and population structure of Melia azedarach in North-Western Plains of India. *Trees - Structure and Function*, *30*(5), 1483–1494.

Van Oosterhout, C., Hutchinson, W. F., Wills, D. P., and Shipley, P. (2004). MICRO ‐ CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular ecology notes*, *4*(3), 535-538.

Vir, R., Lakhanpaul, S., Malik, S., Umdale, S., Green, Á., Mung, Á., and Wild, Á. (2016). Utilization of germplasm for the genetic improvement of Mung bean [*Vigna radiata* (L.) Wilczek]: The constraints and the opportunities. *Gene Pool Diversity and Crop Improvement*, 10, 367–391.

Wang, Z., Kang, M., Liu, H., Gao, J., Zhang, Z., Li, Y., Wu, R., and Pang, X. (2014). HighLevel Genetic diversity and complex population structure of Siberian apricot (*Prunus sibirica* L.) in China as revealed by nuclear SSR markers. *PLoS One*, 9(2):e87381.

Wang, G.T., Wang, Z.F., Wang, R.J., Liang, D., and Jiang, G.B. (2019). Development of microsatellite markers for a monotypic and globally endangered species, *Glyptostrobus pensilis* (Cupressaceae). *Applications in Plant Sciences, 7*(2). https://doi.org/10.1002/aps3.1217

Wekesa L., Muturi G., Mulatya J., Esilaba A.O., Keya G.A., and Ihure, S. (2012). Economic viability of *Melia volkensii* (Gurkii) production on smallholdings in drylands of Kenya. *International Research Journal of Agricultural Science and Soil Science*. 2(8), 364-369.

Wekesa L., Mwalewa, S., Muema, K., Muthini, J., Riziki, P., Mukirae, P., and Menya, C. (2019). An economic assessment of woodlots for smallholder farms in the Coast region of Kenya. *East African Agricultural and Forestry Journal*, *83*(1), 1–12. https://doi.org/10.1080/00128325.2018.1532694

Yan, P., Xie, Z., Feng, K., Qiu, X., Zhang, L., and Zhang, H. (2023). Genetic diversity analysis and fingerprint construction of Korean pine (*Pinus koraiensis*) clonal seed orchard. *Frontiers in Plant Science*, *13*, 1079571.

Yeh, F. C., Yang, R. C., and Boyle, T. (1999). POPGENE version 1.32: Microsoft Windows–based freeware for population genetic analysis, quick user guide. *Center for International Forestry Research, University of Alberta, Edmonton, Alberta, Canada*, 1-29.

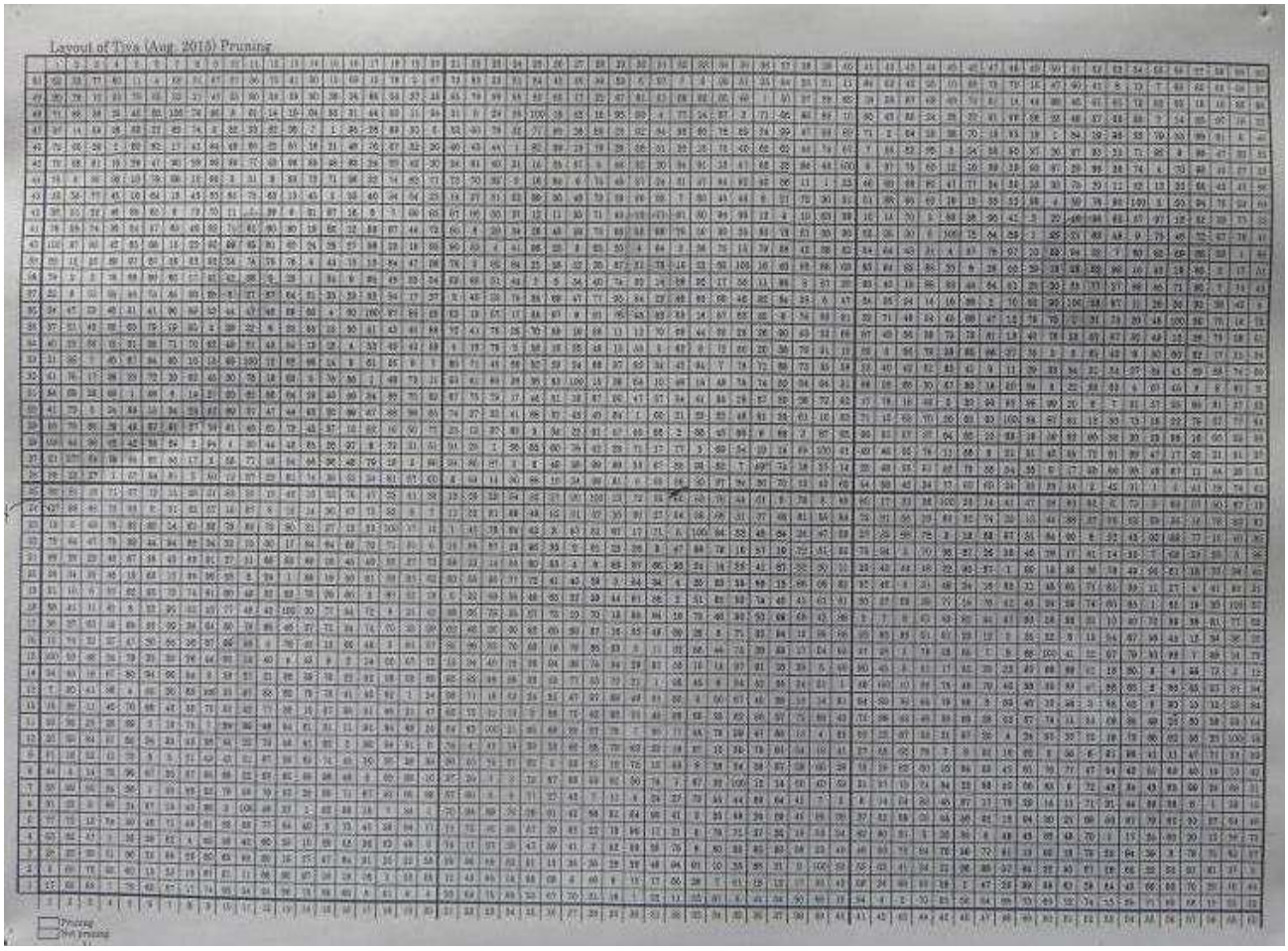
Zhou, H., Zhao, P., Woeste, K., and Zhang, S. (2021). Gene flow among wild and cultivated common walnut (**Juglans regia**) trees in the Qinling Mountains revealed by microsatellite markers. *Journal of Forestry Research*, *32*(5), 2189-2201.

APPENDICES

Appendix 1: Final number of Melia CPTs selected in various transects

S/No.	TRANSECT	No. of CPTs	Code
1	Voi – Mwatate	10	VM
2	Mutha – Inyali	12	MTA
3	Katulani – Kavisuni	10	KT
4	Mwingi – Tseikuru	8	TSK
5	Mwingi – Nuu	4	NUU
6	Mwea Special	2	MWA
7	Embu – Dams	7	EmbD
8	Embu – Ishiara – Gatunga	13	EmbIG
9	Meru – Isiolo	4	MI
10	Voi-Galana	10	VG
11	Garissa-Bangale	4	GB
12	Garbatulla-Wamba	6	GW
13	Wamba-Marsabit	10	WM
TOTAL		**100**	

Appendix 2: Layout plan of Kibwezi seed orchard for Melia volkensii



Appendix 3: Approval letter for research proposal from Kenyatta University

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke
P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School DATE: 9th September, 2021

TO: **Mr. Rotich Kipkoech Japhet** REF: I56/CE/34136/2016
C/o Department of Biochemistry, Microbiology & Biotechnology

SUBJECT: **APPROVAL OF RESEARCH PROPOSAL**

This is to inform you that Graduate School Board, at its meeting on **25th August, 2021**, approved your Research Proposal for the M.Sc. Degree entitled, **"Determining the Mating System and Gene Flow Patterns of Melia Volkensii within Clonal Seed Orchard in Kibwezi Using Microsatellites."**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking and Progress Report Forms per semester. The forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

CC. Chairman, Department of Biochemistry, Microbiology & Biotechnology

Supervisors:

1. Dr. George Ochieng Asudi
C/o Biochemistry, Microbiology & Biotechnology Dept.
Kenyatta University

2. Dr. Stephen Omondi
Forest Genetics and Tree Improvement Department, Kenya Forestry Research Institute.
C/o Biochemistry, Microbiology & Biotechnology Dept.
Kenyatta University

Appendix 4: Research Permit from NACOSTI

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

P O Box 30623 - 00100 Nairobi
Tel.: 020-4007000,0713788787
INVOICING DATE-26/Oct/2023

INVOICE: **32515**

INVOICE TO:
Rotich Kipkoech Japhet
Kenya

ITEM DESCRIPTION	CATEGORY OF RESEARCH	PROCESSING FEE	TOTAL AMOUNT (KES)
Application Fees For - Research (Masters) Biological Sciences - Kenyan Citizens	Research (Masters)	1,000	1,000
		Total Amount Payable (KES)	1,000

Issued By : -

Payment to be made to our account as detailed below:

East African Citizens - Kenya Shillings Account

Mobile money: Online Mpesa Express
or
Account Name: National Commission for Science, Technology and Innovation
Account No.: 1104162547 **Swift Code: KCBLKENX**
Bank: KCB Bank, Kipande House Branch, NAIROBI **Transaction Description.**: Research Licence Fee

Non-Kenyans - US Dollar Account

Account Name: National Commission for Science, Technology and Innovation
Account No.: 2904970067 **Swift Code: CBAFKENX**
Bank: NCBA Bank, City Centre Branch, NAIROBI **Transaction Description.**: Research Licence Fee

National Commission for Science, Technology and Innovation is ISO 9001:2015 Certified