REPRODUCTIVE BIOLOGY, PHENOLOGY AND GENETIC DIVERSITY OF

Osyris lanceolata (HOSCHT AND STEUDEL) IN KENYA

BY

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A thesis submitted to the school of Natural Resources Management in partial fulfillment of the requirement for the degree of Master of Science in Forestry (Tropical Forest Biology and Silviculture) of the University of Eldoret.

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DECLARATION

DECLARATION BY THE CANDIDATE

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DEDICATION

To my dear parents: Johnstone Naika and Jane Akeyo; loving and supportive wife

Lydiah Harriet Wandera and dear son Excellence Seth Esengo.

God bless you all. Amen!

"...For I know the plans I have for you, 'declares the Lord', plans to prosper you and

not to harm you, plans to give you hope and a future"

...Jeremiah 29:11...

ABSTRACT

Osyris lanceolata (Hoscht and Steudel) is an evergreen, drought resistant, African tropical tree species of *Santalaceae* family. It's facing extinction due to over-exploitation for its essential oil used for making expensive cosmetics and drugs. The study aimed at: (1) describing reproductive biology and phenology of *O. lanceolata*; (2) exploring the extent of its genetic diversity using morphological and Inter-simple sequence repeat (ISSR) molecular markers. Observations, treatments and samples collection were done in Gwasii, Meru, Baringo, Kabarnet, Margat, Kitui, Wundanyi, Mt. Elgon and Mau populations. Variations in phenology, reproductive biology and morphological traits were analyzed using ANOVA in SPSS package 12.0. The *F*-test for the parameters was run at 95% level, Duncan Multiple Range Test (DMRT) performed to partition means. Molecular data was analyzed using GenAlex 6.1 and Popgene 1.31 soft-wares. Results showed that *O. lanceolata* is a biannual outcrossing species flowering from January to June and September to December for first and second phase respectively with each phase taking about 109±16 days. Assisted pollination increased its reproductive success by 40%. Most morphological traits clustered Kitui population in the first cluster, Gwasii in the second and, Kabarnet and Marigat in the third cluster. Six primers generated 96 fragments, ranging from 150 to 1000 base pairs (bp). Percentage polymorphic loci *(P)* ranged from 51.04% (Wundanyi) to 82.29% (Gwasii) with a mean of 64.73%. Unbiased gene diversity index *(UHe)* was highest in Gwasii (0.321) and lowest in Wundanyi (0.175) with overall mean of 0.253. Mean number of effective alleles *(Ne)* was 1.430 while Shannon Information Index *(I)* mean was 0.365. Gwasii was the most genetically diverse followed by Mt. Elgon and least was Wundanyi. Overall coefficient of differentiation *Gst* was 0.3429. Analysis of molecular variance (AMOVA) indicated within population variation of 62%. Principle Coordinate Analysis *(PCoA)* grouped six populations in a distinct cluster with allelic overlap except Baringo. Dendrogram partitioned the populations into two clusters, based on breeding systems, population sizes, gene flow, genetic drift and natural selection but not on geographic proximity. The study concluded that, *O. lanceolata* experiences low reproductive success which was attributed to limited pollen transfer. The moderate high genetic diversity observed indicate small restricted populations with limited gene flow. It was therefore recommended that assisted pollination strategy and introduction of genotypes from Gwasii, Mt. Elgon Mau and Baringo populations should be adopted to enhance gene flow and male/female ratio in *in-situ* conservation and allow for increase in their regeneration capacity, which will eventually increase the population sizes as well as their genetic diversities.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
ASALs	Arid and Semi-arid Lands
Bp	Base pairs
CIA	Chloroform : Isoamyl alcohol
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CTAB	Cetyltrimethylammonium bromide
Dbh	Diameter at Breast Height
dH$_2$O	Distilled water
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FAA	Formalin Acetylic Alcohol
FAO	Food and Agricultural Organization of United Nation
IAEA	International Atomic Energy Agency
IBA	Indole-3 butyric acid
ISSR	Inter-simple sequence repeat
IUCN	International Union for Conservation of Nature
KEFRI	Kenya Forestry Research Institute
NEMA	National Environment Management Authority
MEGA	Molecular Evolutionary Genetic Analysis
PCR	Polymerase Chain Reaction
PVC	Polyvinylpyrollidone
PVP	Polyvinylpolypyrollidone
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
SPSS	Statistical Package for Social Science
SSR	Simple Sequences Repeats
TAE	Tris- Acetate EDTA
TBE	Tris-Borate EDTA
TE	Tris- EDTA
UNEP	United Nations Environmental Programme
US & WS	United States Forest and Wildlife Service

ACKNOWLEDGMENT

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Forests have provided vast products needed by large human populations especially in Arid and Semi-Arid Lands (ASAL) (Sutherland *et al.,* 1991). Africa's ASALs form about 55% of the total land area (Fitzgibbon, 2012). The ASALs form up to 80% of the total land area in Kenya and is supporting about 25% to 30% of the total population (Fitzgibbon, 2012).

In Kenya, the human populations living in the asals largely depend on forest for such products as grass for livestock, fuel wood for lighting and heating, poles for construction and other non-timber forest products like honey, cosmetic products, and medicine among others (UNEP and GoK, 2000). High demand for forest products from the surrounding urban areas has also exacerbated the exploitation of forests and forest tree species (UNEP and GoK, 2000). *Osyris lanceolata* (Hoscht and Steudel), commonly known as East African sandalwood (Walker, 1966; Mbuya *et al.,* 1994) is one of the forest tree species exploited for its valuable and diverse products that include essential oils and herbal medicines (Mengich, 2010). Exploitation of this valuable species from the forests is unsustainable and hence causing its depletion (Mathenge *et al*., 2005). Continued illegal harvesting of *O. lanceolata* from the forests has reduced the treasured tree population in Kenya, Tanzania, Uganda and other East Africa countries despite various governments' restrictions (Mwang'ingo *et al.,* 2007; CITES, 2013). In Tanzania, a population of *O. lanceolata* in handeni has virtually disappeared due to continued illegal harvesting (Mwang'ingo *et al.,* 2007). This has

aroused the need for scientists to carry out studies for efficient planning, management and conservation of the species in their natural environment.

In Kenya, *O. lanceolata* populations are few and mostly distributed in Western, Eastern, Coastal and Rift Valley regions (Kamondo *et al.,* 2014; CITES, 2013). The populations have been decreasing since 2002 because of the heavy exploitation for international trade (CITES, 2013). The sharp rise in the extraction of *O. lanceolata* in Kenya and Tanzania is believed to be linked to a decline of the resource in the world's primary sources of sandalwood in Australia and India (Mwang'ingo *et al.,* 2003; Mwang'ingo *et al.,* 2007; CITES, 2013). As a result, conservationists are now concerned about its diminishing population areas. Kenya has made several applications for the species to be included in Appendix II of the IUCN red list and it is being debated for inclusion in the "threatened species" category of the IUCN red list to foster its conservation (IUCN, 2009; IUCN, 2013). The level of extraction of the species from the wild has not been documented and could be at a worrying level (CITES, 2013).

The Kenyan *O. lanceolata* is protected by Legal Notice No. 3176 of 2007 under the Forests Act, 2005 (GoK, 2007; CITES, 2013). The legal notice mandates Kenya Forest Research Institute (KEFRI) to propagate the species in the wild (GoK, 2005; Machua *et al*., 2009). This is to allow for the development of sustainable harvesting mechanisms and find ways to conserve the species in the country. However, current reports show continued illegal harvesting in spite of the ban (CITES, 2013).

Conservation of *O. lanceolata* germplasm can be well formulated and executed based on the information on its genetic diversity pattern, phenology and reproductive biology (Rhian and Michael, 2008). Genetic diversity is widely recognized as the key

component for the long term survival of the tree species hence an important component in the species conservation (Milligan *et al.,* 1994; Ramesha *et al.,* 2007; Rhian and Michael, 2008). The estimation of genetic diversity of plant population is a fundamental event to mark out *in-situ* and *ex-situ* conservation strategies (Holsinger and Gottlieb, 1991; Dangasuk*,* 1999; Rhian and Michael, 2008). This demands a particular design and extensive amount of sampling during seed collection which will represent the extent of the genetic diversity existing among the species populations (Nybom and Bartish, 2000; Rhian and Michael, 2008).

Genetic diversity of plant populations has been assessed using morphological characterization but later complemented by the use of Isozymes (Shweta *et al.,* 2008). However morphological markers and isozymes have proven to be slow and inadequate to determine the genetic diversity among various plants population (Xiao-ru and Alfred, 2001). Recently, molecular techniques have been introduced to detect genetic variability in various plant species (Kaundun and Park, 2002; Jones *et al.,* 2009; Porth and El-Kassaby, 2014). Some of these widely applied techniques include: Restriction Fragment Length Polymorphisms (RFLPs) (Botstein *et al.,* 1980), Amplified Fragment Length Polymorphism (AFLPs) (Vos *et al.,* 1995), Randomly Amplified Polymorphic Deoxyribonucleic acid (RAPD) (Williams *et al.,* 1990), Simple Sequences Repeats (SSR), Inter-Simple Sequences Repeats (ISSR) (Zietkiewicz *et al.,* 1994; Awashi *et al.,* 2004; Manish *et al.,* 2012) and Single Nucleotide Polymorphism (SNP) (Nasu *et al.,* 2002)

Allozymes are predominantly co-dominant and there use in analyzing genetic variations is applicable to large number of samples and has well established statistical methods for estimation of genetic variation (Wang and Szmidt*,* 2001). The technique is relatively inexpensive and technically straightforward (Hamrick and Godt, 1989;

Charlesworth and Yang, 1997). However allozyme studies investigate only a highly restricted part of the variation of enzymatic gene products and are few in number and normally reveal low levels of genetic polymorphism since they depend on only part of the genome products (Wang and Szmidt, 2001; Shweta *et al.,* 2008). Isoenzymes have not been used in *O. lanceolata* but have shown occurrence of low genetic diversity in population of *Acacia senegal* (L.) Willd (Chevellier *et al.,* 1994). They are few and normally reveal low levels of genetic polymorphism since they depend on only part of the genome products (Shweta *et al.,* 2008).

Restriction Fragment Length Polymorphisms (RFLP) is typically co-dominant and display simple Mendelian inheritance (Byrne *et al*., 1994). However, it requires large quantities of pure DNA for them to be used in a genetic study of a species. They are species-specific DNA probes and generally use short-lived radioisotopes in the detection system (Wang and Szmidt, 2001). Furthermore, RFLP analysis technique is laborious; making it impractical for many forest population based studies.

Amplified Fragment Length Polymorphism (AFLP) is a Polymerase Chain Reaction (PCR)-based technique that has emerged as powerful tools for DNA finger printing and genetic mapping (Zabeau and Vos, 1993). It uses selective amplification of a subset of digested DNA fragments to generate and compare unique fingerprints for genomes of interest (Vos *et al.,* 1995; Jonah *et al*., 2011). Its advantage over others is that it does not require prior information regarding the targeted genome; it has high reproducibility and sensitive for detecting polymorphism at the level of DNA sequence (Mueller and Wolfenbarger, 1999); it produces a higher number of markers per assay (Vos *et al.,* 1995); and require only small amounts of starting DNA template (Jonah, *et al*., 2011). The complexity of AFLP fingerprints can be manipulated by increasing or

decreasing the number of selective bases and changing base composition (Zabeau and Vos, 1993). However, AFLP method is a relatively labor-intensive method (Farooq and Azam, 2002; Jonah *et al*., 2011) and is dominant (Zabeau and Vos, 1993; Farooq and Azam, 2002).

Randomly Amplified Polymorphic DNA (RAPD) analysis does not require any prior DNA sequence information and it relies on single short, random oligonucleotides for amplification of unspecified target DNAs (Weatherhead and Montgomerie, 1991). However, RAPD reproducibility is low and it is inconsistent in its polymorphism (Shweta *et al.,* 2008, Gupta *et al*., 2008).

Microsatellites or simple sequence repeats (SSRs) are 1 to 6 base pairs (bp) tandem repeated DNA motifs which may vary in the number of repeats at a given locus. Simple sequence repeats are usually isolated from genomic libraries by screening with specific repeat motifs as probes (Powell *et al*., 1996). They are multiallelic, co-dominantly inherited, widely dispersed across the genome and relatively easily scored (Morgante and Olivieri, 1993; Devey *et al*., 1996; Powell *et al*., 1996; Barreneche *et al*., 1998). However, SSRs may take a long time to identify these regions from a genomic library. The large genome size and complexity of genome have rendered the identification of single-locus, reproducible SSR markers for many forest tree species a challenging task and hindered their application in population studies (Wang and Szmidt, 2001). Their utility as genetic markers for tree species has also been limited owing to a paucity of available sequence data. Furthermore, SSR markers can lead to underestimation of heterozygosity in case of the presence of null alleles (Wang and Szmidt, 2001).

Inter-Simple Sequence Repeat (ISSR) markers have been widely used to investigate clonal diversity and population genetic structure (Zietkiewicz *et al.*, 1994; Tani *et al.,* 1998; Wolfe and Liston, 1998; Esselman *et al*., 1999; Rossetto *et al.,* 1999; Ramesha *et al.,* 2007). As a PCR-based marker, ISSR is applicable to organisms from diverse genera and because of the large number of primers available for analyses; it provides good overall genome coverage (Williams *et al.,* 1990; Zietkiewicz *et al.*, 1994; Gupta *et al*., 2008; Ge *et al.,* 2005). ISSR show dominant inheritance and are useful in detecting genetic polymorphisms among accessions by generating a large number of markers that target multiple microsatellite loci distributed across the genome and evolve rapidly (Fang and Roose, 1997; Esselman *et al.,* 1999; Gupta *et al.,* 2008; Shweta *et al.,* 2008; Trindade *et al.*, 2012; Ansari *et al.,* 2012). Further, they are environmentally independent and are easier to analyze (Godwin *et al.,* 1997; Ramesha *et al.,* 2007; Patel *et al.,* 2016) and unlike SSR markers, ISSR markers do not need prior knowledge of the target sequences flanking the repeat regions (Yang *et al.,* 2012; Gaafar *et al.,* 2014; Žukauskienė *et al.,* 2014; Panda *et al.,* 2015). They are neutral, produce reliable and reproducible bands because of the higher annealing temperature and longer sequence of ISSR primers and hence are accurate and able to deduce variations in a species with wide geographical habitat variation (Tsumura *et al.,* 1996; Nagaoka and Ogihara, 1997; Qian *et al.,* 2001; Reddy *et al.,* 1999; Deshpande *et al.,* 2001; Reddy *et al.,* 2002; Ramesha *et al.,* 2007). These advantages of ISSR over the other DNA molecular markers are the reasons why the technology was preferred to study the genetic diversity in seven provenances of *O. lanceolata* in Kenya. This is therefore the first study on phenology, reproductive biology and genetic diversity of *O. lanceolata* species in Kenya.

1.2 Statement of the problem

Overexploitation of many sandalwood species for their prized heartwood and medicine has caused its decline in natural stand and hence raising concerns about their sustainability across the world (Mwang'ingo *et al.,* 2007; Tshiisikhawe *et al.,* 2012). Currently, African Sandalwood is listed as threatened species under United Sates Forest (USF) and Wildlife Service (WS) (2013). In Kenya, *O. lanceolata* is endangered and its survival threatened. It is debated on for inclusion in the CITES appendix to foster its conservation status (CITES, 2013). The sandalwood trade sustainability and the species products availability are in jeopardy (Kamondo *et al.,* 2014). The high demand for the species products due to high international prices has imposed profound pressure on it locally and in other regional countries (Mwang'ingo *et al.,* 2007). The harvesting has now gone to the extent of uprooting the whole tree for its trunk and roots which are believed to contain more oils per unit weight (Ruffo *et al.,* 2002). More so, harvesters are said to prefer the female tree which is believed to have high oil content compared to the male trees (Ruffo *et al.,* 2002; Mwang'ingo *et al.,* 2007). This threatens the species reproductive success, genetic pattern and hence may cause its extinction from the wild.

Research reports indicate that the natural regeneration, growth and development of *O. lanceolata* is generally low, and this is made worse by the destructive methods used in harvesting the species (Mwang'ingo *et al*., 2007; CITES, 2013). In Kenya the species has low recruitment in the natural range but some populations do well when managed *in-situ* (Kamondo *et al.,* 2014; Mothogoane, 2011). The species experiences reproductive failure and is mainly outcrossed as revealed in the study conducted in Southern Australia populations of *Santulum lanceolatum* R.Br and *O. lanceolata* of Tanzania (Warburton *et al.,* 2000; Mwang'ingo *et al*., 2007). Kamondo *et al.* (2014)

also observed reproductive failure among some Kenyan populations and seeds have recorded very low levels of germination probably due to various seed dormancies or few viable seeds formation (Mwang'ingo *et al*., 2004; Kamondo *et al.,* 2014). However, propagation methods such as marcoting, when done at the right age and season, have proven to root successfully (Mwang'ingo *et al*., 2007). This means that there is a significant commercial opportunity for the establishment of *O. lanceolata* if planting materials are availed to farmers to incorporate them in the local farming practices. However, lack of basic biological knowledge of the species in Kenya is holding back such a development. The implication of such a practice on the species adaptability to the changing environment is not known and thus impeding the formulation of an informed conservation programme.

The flowering phenology of the species in Tanzania was observed to commence in the mid-February/August and to fruit in April/September (Mwang'ingo *et al*., 2007). Mwang'ingo *et al*. (2007) observed that the phenology events were varying among trees within a given population and also among populations. In Kenya the flowering calendar appears to be poorly documented with different authors giving different flowering periods: from March to August or even later (Coates, 1977), October to February (Pooley, 1993) and September to February (Schmidt *et al*., 2002).

The genetic diversity of Australian populations species like *S. lanceolatum, Santalum album* (Linn.)*, Santalum spicatum* (R.Br.) using various molecular markers like RAPDs and allozymes, has been studied (Trueman *et al*., 2001). However, the genetic diversity of *O. lanceolata* in Kenya using quantitative traits, biochemical characteristics or molecular markers has not been studied hence making it difficult to formulate conservation measures and resource management programme.

1.3 Justification of the study

Sandalwood has for thousands of years been valued for its essential oils that are highly priced in the world market, contributing positively to the Gross Domestic Product (GDP) of many countries (Mwang'ingo *et al.,* 2007). The emergence of global demand for the resource in sandalwood industry has become increasingly entrepreneurial thus successfully marketing its products into the global economy (Tonts and Selwood, 2002). This has led to excessive harvesting of the species from the forests. Overexploitation of *O. lanceolata* has resulted in its decline (Githae *et al.,* 2011). There is therefore a need to check the current status of *O. lanceolata* population in Kenya. The Kenyan government imposed a harvesting ban in 2007 but reports show that illegal harvesting is still going on. Therefore, further measures to stop overexploitation should be set followed by comprehensive sustainable conservation strategies. Detailed assessments need to be carried out to understand the species phenology and determine the cause of its reproductive failure. Knowledge of *O. lanceolata* reproductive biology will help in understanding how well to maximize on the yield and quality of the viable seed produced, the range of germplasm utilization and its conservation (Jackeline and Freitas, 2008). The knowledge is also vital in tree improvement and breeding programme (Bawa and Krugman, 1991). Furthermore, such knowledge enable conservationists understand the species recruitment, the causes of its reproductive failure, its gene flow mechanisms and consequently, its genetic diversity (Janick *et al.,* 1982). There is also need to study the genetic diversity within and among natural populations of the *O. lanceolata* to determine the extent of their genetic variability in Kenya to enable development of strategies desirable for conservation, breeding and tree improvement programmes (Changtragoon and Schmidt, 1997). A population that is genetically diverse merits to be given a high priority for breeding

because of its high adaptability potential in a rapidly changing environment (National Research Council, 1991; Runo *et al.,* 2004). Conversely, a population with low genetic variability will demand a lot of effort for conversation so that the important but rare genetic traits are not lost in the event the species is extinct.

1.4 Objectives of the study

Broad objective

To investigate phenology, reproductive biology of *O. lanceolata* in Kenya; and its genetic diversity using morphological traits and ISSR-PCR makers for designing a comprehensive conservation and sustainable management programme of the species genetic resource.

Specific objectives

1. To characterize the phenology and reproductive biology of *O. lanceolata* in Kenya.

2. To determine the genetic diversity in *O. lanceolata* based on morphological and PCR-ISSR markers, in Kenya.

1.5 Hypothesis

The hypotheses of the study were:

1. H_o: There is no reproductive biology failure in *O. lanceolata* in Kenyan population.

2. H_A: There are reproductive biology failures in *O. lanceolata* in Kenyan population.

3. H_o: There is no genetic variability within and among *O. lanceolata* in Kenya.

4. H_A: There is significant genetic variability within and among *O. lanceolata* in Kenyan.

CHAPTER TWO

LITERATURE REVIEW

2.1 General overview

Forests comprise of trees and other plants that have been a source of medicine for preventive and curative measures against diseases for human beings and livestock , source of firewood, fodder for livestock, construction materials, food and weapon handles since civilization of mankind started (Bawa, 1974; Beentje, 1994; World Bank, 2008). Undeniably, more than 1.6 billion people worldwide depend on forests and trees in and outside forests for livelihood (World Bank, 2008).

The increasing human population in the world has led to high demand of land for cultivation and shelter from the forests (Gibson *et al*., 2011; Nageswara-Rao *et al*., 2012). This has impacted detrimentally on the forest resources in the world hence the loss and consequently draining of forest genetic diversity (Dick, 2001; Gibson *et al*., 2011; Nageswara-Rao *et al*., 2012). Furthermore, forest overexploitation has resulted in the extinction of local forest species due to forest fragmentation that has reduced forest population sizes, disrupted forest pollinators' foraging cycles and seed dispersal agents in the forest ecosystem (Herrera *et al.,* 1998; Dick, 2001; Dharani *et al.,* 2010; Ganzhorn *et al.,* 2015). Reduced forest areas in the tropics have led to poor soil management thus low land yields. Bear land left after forest harvesting has encouraged emergence and growth of shrubs and grasses that are less palatable to the existing animal population (Chikamai and Odera, 2002; Dangasuk, 2009). The impact is worse in ASALs of Africa (Chikamai and Odera, 2002). The woodlands in the ASALs are essentially supplying various products including timber for construction and making domestic implements handles among other (GoK, 2005). The woodlands also provide non-wood forest products such as gums and resins, aloe gel, indigenous fruits, honey,

essential oils, frankincense, silk, commercial juices and edible herbs to the populations living in the areas (Shackleton *et al.,* 2002; GoK, 2005). These products have also entered the market and are now highly demanded. Most traded are trees with medicinal and cosmetic values (Dick, 2001; Chikamai and Odera, 2002). The increased trade in herbal medicine and natural cosmetic products from forests without proper legal framework is the main factor causing decline in the wild supply of forest products (Dharani *et al.,* 2010). The worst of all is that, most of the tree species exploited are specific to their habitats, take a long time to reach maturity (2 to 3 decades) and yet are usually harvested in an indiscriminately destructive manner for their bark, roots or trunk products (Kokwaro, 1991; Beentje, 1994; Kamondo *et al.,* 2014). This poses a challenge of extinction of some of these tree species, unless urgent measures are taken to arrest the trend of trees overexploitation (Kokwaro, 1991).

Records clearly indicate that some predominantly large trees have been ring barked leading to their drying up (Kokwaro, 1991). Most affected species are those that belong to Saussureaceae, Rosaceae and Santalaceae families found in East and West Africa (Mwangi, 2001; Ruffo *et al.,* 2002). Such species, especially those that grow in both tropical forests and in the ASAL areas need to be efficiently conserved given their economic importance. The genetic variation and structure of these forests need to be studied to ensure effective species improvement programs and gene conservation efforts (Ramesha *et al.,* 2007).

ASALs form about 55% of Africa's total land area with characteristic short season rainfalls of ranging from 100 to 800 mm annually (Jama and Zeila, 2005). Kenya's ASALs cover about 80% of total land with unpredictable and poorly distributed rainfall, holding 25% of the total population (Mengich, 2010). The annual rainfall ranges from 250 to 800 mm (Dangasuk, 1999). The harsh climatic conditions in these

areas favor drought resistant trees and shrubs like acacia and *O. lanceolata.* They also play a key role in ecological regulation, shelter and nursing of other dependent tree species, recycling water and controlling soil erosion (FAO, 1985).

2.2 General botanical description of *O. lanceolata*

The East African sandalwood (*O. lanceolata*) is a member of the Order Santalales and Family Santalaceae (Teklehaimanot *et al*., 2003; Mwang'ingo *et al.,* 2007; Machua *et al*., 2009; Githae *et al.,* 2011). It is dioecious, hemi-parasitic, evergreen shrub with average height of 6 m, but often reaching maximum height of 10 m at maturity in favourable forest conditions (Beentje, 1994; Mwang'ingo *et al.,* 2007; Machua *et al*., 2009). The diameter at breast height (dbh) when mature ranges from 3.5 cm to 6 cm with some trees reaching large diameters of 11.5 cm, though quite rare (Teklehaimanot *et al*., 2003, Mwang'ingo *et al.,* 2007). The leader stems produced per tree may be up to 7, but most frequently ranging from 2 to 3 stems in both sexes (Teklehaimanot *et al*., 2003; Mwang'ingo *et al.,* 2007).

PLATE 1: Female and male *O. lanceolata* growing in Gwasii (Source: Author 2013).

Male *O. lanceolata* trees have open crown with observed bluish–green leaves and dropping branches while the female trees have yellowish-green leaves and straight branches (Plate 1) (Teklehaimanot *et al*., 2003; Mwang'ingo *et al.,* 2007).

2.3 Geographical distribution and ecology of *O. lanceolata*

Records reveal that species of Santalaceae family of various genera are found naturally in Australia, Asia (India and China), Pacific Islands, Europe (Iberian Peninsula and Balearic Island) and Socotra. *Osyris lanceolata* grows in Europe (Iberian Peninsula and Balearic Island), Asia (India to China), Socotra (Brand, 1999; Hudson, 2008; CITES, 2013) and Pacific Islands (*S. insulare*) (Butaud *et al.,* 2005).

Osyris lanceolata is documented to occur in South Sudan, Rwanda, Burundi, Eritrea, Algeria and Somalia. It is also found in Zambia, Malawi, Mozambique, Zimbabwe and South Africa (Mwang'ingo *et al.,* 2007; CITES, 2013). It grows well in the East African countries and most common in Kenya, Uganda and Tanzania (Teklehaimanot, *et al.,* 2003; Mwang'ingo *et al.,* 2007; Machua *et al*., 2009) (Figure 2.1).

In Kenya, the species has a wide distribution ranging from the hilly areas of Amboseli, Loitoktok, Kibwezi (Chyullu Hills), Taita Hills, Narok, Mbeere, Kitui, Mau, Kajiado, West Pokot, Makueni, Gwasii Hills, Ngong Hills, Turkana, Laikipia, Marsabit, Koibatek and Meru North Districts (Mathenge *et al.,* 2005; Kamondo *et al.,* 2014; CITES, 2013).
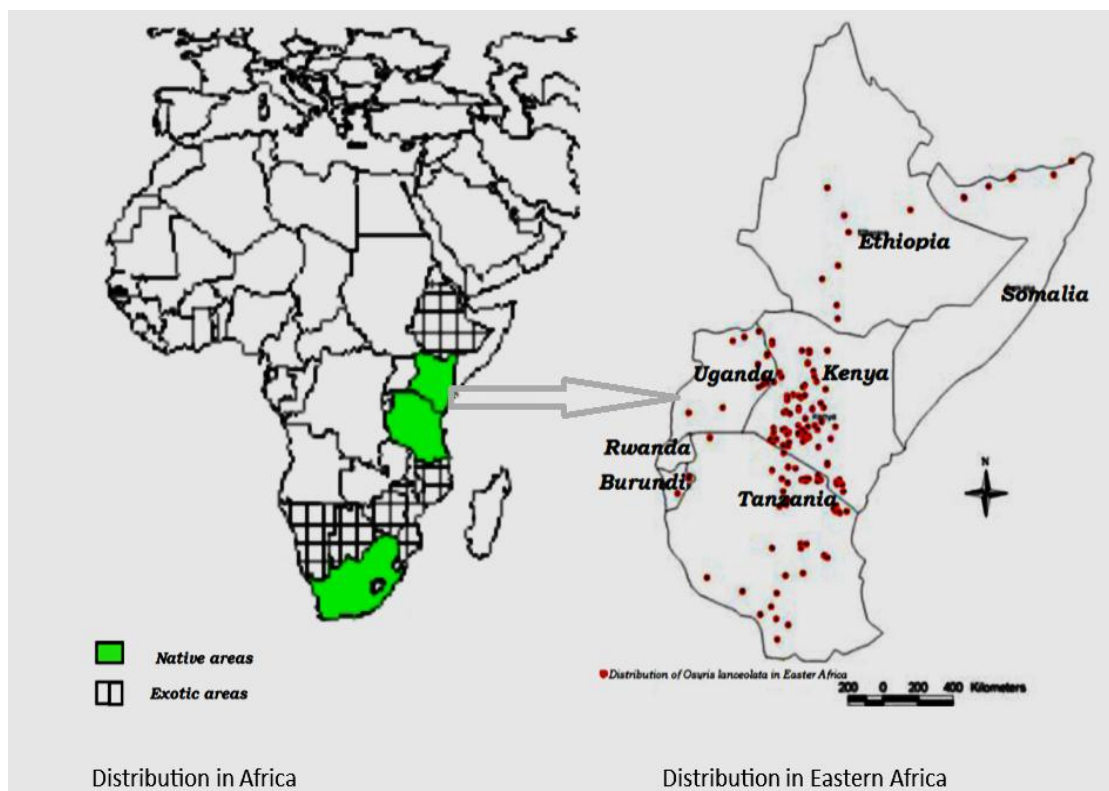
Figure 2. 1: Distribution of *O. lanceolata* (Courtesy of Agroforestry database).

The *O. lanceolata* species is drought resistant growing on poor sites with rock outcrop (Plate 2). The trees occur mostly on rocky ridges, mountain slopes, ASAL forests, margins of evergreen bushland, grasslands and thickets (Machua *et al*., 2009; CITES, 2013). ASAL areas usually experiences 5 to 8 months of dry spell and wide temperature range from 20 to 38 °C. Again, the annual rainfalls in the species range of distribution vary greatly ranging from 100 to 1200 mm (Mathenge *et al.,* 2005). The altitude range of the populations is 900-2700 masl (Mathenge *et al.,* 2005; Machua *et al*., 2009; CITES, 2013). It is adapted to coarse textured soils like slightly loamy sands, nitisols and also heavy clay soils (Orwa *et al.,* 2009; Mwang'ingo *et al*., 2010). The wide geographic range of *O. lanceolata* grows may have resulted in development of locally adapted populations. This may explain the evident distinctive variation in the morphological traits in these populations. The environmental variations contribute to a species evolutionary trend (Dangasuk, 2009).

PLATE 2: *O. lanceolata* **growing on poor, rocky sites- Marigat (Source: Author 2013).**

Like other sandalwood trees species, *O. lanceolata* show preference for certain tree species as hosts compared to the others for better establishment and growth in the field (Hewson and George, 1984; Ma *et al*., 2005; Machua *et al*., 2009; Mwang'ingo *et al.,* 2007). Studies revealed that the host is preferred largely to promote early growth to achieve better heights, diameter and overall root/shoot biomass ratio of *O. lanceolata* (Mwang'ingo *et al.,* 2007). The preferred hosts were noted to be *Rhus natalensis* (Krauss), *Dodonaea viscose* (L) Jacq*, Tecomaria capensis* (Thunb) Lindl*, Catha edulis* (Vahl.) Endl, *Apodytes dimidiate (*E.Mey. ex Arn*), Brachystegia spiciformis* (Benth)*, Pongamia pinnata* (L.) Pierre, *Casuarina equisetifolia* (L.) and *Aphloia theiformis* (Vahl) Benn. (Teklehaimanot *et al.,* 2003; Mwang'ingo *et al.,* 2007). *Brachystegia spiciformis* and *R. natalensis* were the most common hosts in most natural populations of *O. lanceolata* in Tanzania (Mwang'ingo *et al.,* 2010; Githae *et al.,* 2011) but its growth was hindered by *Swietenia mahogany* (King.) in India (Ruffo *et al.,* 2002).

2.4 Exploitation and economic use of *O. lanceolata*

Exploitation of the sandalwood trees for essential oils used in perfumery and the pharmaceutical industry to make expensive cosmetics and drugs, has been done for centuries especially in India and Australia (Rai and Sarma, 1990; Ruffo *et al.,* 2002; Mwang'ingo *et al*., 2003; Mwang'ingo *et al*., 2007). The demand for sandalwood is so high that the current supply cannot meet the international demand from perfumery and pharmaceutical industries causing overexploitation of the species to rise alarmingly (Teklehaimanot, *et al.,* 2003; Mwang'ingo *et al.,* 2007). This is as a result of the major decline in the Indian *(S. album*) and Australian (*Santalum spicatum* R.Br) sandalwood populations which are now unable to support the world market (Mwang'ingo *et al*., 2007). This has turned the focus of the international traders and businessmen of sandalwood products to *O. lanceolata* (East African sandalwood), hence threatening its growth and sustainability (Ruffo *et al.,* 2002).

Like other sandalwood trees, East African sandalwood (*O. lanceolata*) is known for fragrant-scented wood from which essential oils are extracted (Mwang'ingo *et al*., 2003; Mwang'ingo *et al*., 2007; Tshiisikhawe *et al*., 2012, Subasinghe, 2013). The wood is yellowish-red, fine grained and of high density (Mwang'ingo *et al*., 2003; Mwang'ingo *et al*., 2007).

In Kenya, the species has been highly valued for centuries by the people of East Africa, because of its valuable red dyes, wood for royal walking sticks and beautiful carvings (Rai, 1990; Mwai, 2005), and wood for making chests and jewelry boxes (Kamau, 2006). Sandalwood species in other genera are also highly priced globally for their oils (Mwang'ingo *et al*., 2010) and are planted for soil conservation, as a source of edible fruits, for firewood and fodder for browsers especially in the ASALs of Australia (Beentje, 1994).

Since the 1990s, *O. lanceolata* has been overharvested indiscriminately by uprooting the whole tree including its roots (Plate 3) (Teklehaimanot, *et al.,* 2003; Mwang'ingo *et al.,* 2007; Machua *et al*., 2009). The harvesting is done by the communities from East African countries especially in Kenya, Uganda and Tanzania, and sold to middlemen to earn a living (Kamondo *et al.,* 2014).

PLATE 3: *Osyris lanceolata* **stump harvested (Source: Author 2013).**

It is reported that the middlemen prefer mature trunks and roots which is believed to contain a higher concentration of essential oil (Mwang'ingo *et al*., 2007; Machua *et al*., 2009). The harvested products infiltrate into Tanzania through the porous Kenya-Tanzania borders which are then transported to Zanzibar (Mwang'ingo, 2002; Ruffo *et al.,* 2002; Mwang'ingo *et* al., 2007; Machua *et al*., 2009) where the tree parts harvested are chopped into small pieces, packed into containers and shipped to Middle East, Europe and other Far East countries where it is used in making expensive perfumes (CITES, 2013).

Initially, exploitation of the species was going on in areas close to the Kenya-Tanzanian border, for example Loitoktok, Amboseli and in area like Chyulu Hills, but

this has since spread to the other parts of the country that hitherto were untouched, such as Mbeere and Baringo (Mathenge *et al.,* 2005). In 2007, about 7 tonnes of sandalwood was impounded in Baringo District, where it was reported that dealers bought it from the villagers in Baringo and East Pokot districts who had harvested it to earn a living (Kamondo *et al.,* 2014). The most recent recorded confiscation of *O. lanceolata* products in Kenya was on 18th September 2012 in Nairobi (CITES, 2013). It is reported that during the period between 2007 and 2011, 276 tonnes of sandalwood material have been seized and confiscated in Kenya (CITES, 2013).

The species in this family have also wide product range. For instance in Australia the Aborigines make a nutritive delicious food from the kernel, nuts and fruits of *S. spicatum* (Murphy and Garkaklis, 2003; Murphy *et al.*, 2005). The products of the species in the family are also useful in religious ceremonies for its sweet fragrance by the Hindu, Budhists, Mulsims among other religious faithful's internationally (Teklehaimanot, *et al.,* 2003; Mwang'ingo *et al.,* 2007). The Indian sandalwood (*Santalum album* L.) has been part of Indian culture and heritage for thousands of years, and was one of the many items traded by Indians with other countries (Rai, 1990).

In Indonesia, finely ground sandalwood mixed with water is rubbed on the body for its cooling effect (Rai, 1990). In India and East Asian countries, sandalwood oil is also used to treat acne, coughs, depression, diarrhea, insomnia, restlessness, and nervous system problems (Rai, 1990; Beentje, 1994). Other ailments that are treated by the tree oil products are: stress, anxiety, varicose veins, eczema, sore throat, scabies, nausea, abdominal cramps, bronchitis, dermatitis, catarrh, laryngitis, dandruff, and even lice killing (Beentje, 1994; Kamondo *et al.,* 2014). The oil is also said to have antiviral activity against Herpes and treats bladder infection and gonorrhea (Rai, 1990). The oil

is a popular sedative in oriental medicine, has narcoleptic effect and chemo-preventive effects, treating inflammatory eruptive skin diseases, and is also used in treating urinary infections among other diseases (Okasaki and Oshima, 1953; Mbuya *et al*., 1994; Okugawa *et al.,* 1995; Dwivedi and Zhang, 1999).

In Kenya, *O. lanceolata* has different uses among different peoples in Kenya. The Pokot who reside in the North Rift region of Kenya take a concoction of roots to treat diarrhea and other stomach ailments, while the Kamba use the burned roots for treating snake bites, and root fibers in basketry (Beentje, 1994; Machua *et al*., 2009; Mukonyi *et al.,* 2011). The Maasai and Tugens use a strong red dye from the roots in beverage as herbal tea substitute and the Meru use burned stems as milk preservative (Beentje, 1994; Machua *et al*., 2009).

The trunk and roots from female *O. lanceolata* trees are most preferred in the world market and hence most harvested (Teklehaimanot, *et al.,* 2003; Mwang'ingo *et al.,* 2007; CITES, 2013). It is reported that the female trees have better quality heartwood that yields high quality essential oils than male trees (Teklehaimanot *et al.,* 2003). The wood from the female tree has a very pleasant smell and with the highest concentration of santalols and santalyl acetate which are the major components of the essential oils (Burfield and Widwood, 2004). Essential oil from *O. lanceolata* provides perfumes and when used in small proportions in a perfume, it is an excellent fixative to enhance the head space of other fragrances (Teklehaimanot *et al.,* 2003; Mwang'ingo *et al.,* 2007). In cosmetic industry, the essential oil is used to manufacture quality lotions, rare soaps, scented candles and the oil also has flavour ingredient used in food industry (Burdock and Carabin, 2008).

2.5 Illegal harvesting of *O. lanceolata*

The increased unjustifiable destruction of *O. lanceolata* and other genera in the family by the people who live around forests has drastically increased worldwide (CITES, 2013). This is caused by the increasing economic demand of the people and poor legal institutional framework and management of natural resources (Lethbridge, 2001). As the international market demand for supply of sandalwood increases, *O. lanceolata* has become one other targeted species from Africa to meet the demand (Mwang'ingo *et al*., 2010). As a result, overexploitation has drastically lowered the population size of the species causing it to be listed as an endangered species in Africa (USF and WS, 2013).

Initially *O. lanceolata* species had a wide range of distribution in Kenya. This has greatly shrunk due to overexploitation (Beentje, 1994; Machua *et al*., 2009; CITES, 2013). For instance, records at the East African Herbarium and National museums of Kenya indicate that collections of *O. lanceolata* was done from Kiambu and in the 1930's from Nairobi, Nyandarua, West Pokot, Samburu, Machakos and other parts of the country (Beentje, 1994; CITES, 2013). It is believed that the populations that initially occupied arable land and near cities have disappeared due to agriculture and urbanization (Beentje, 1994; CITES, 2013).

Illegal harvesting of the species has been noted to be smuggled out of gazetted forests in West Pokot, Baringo, Nakuru, Gwasii and Kitui, from game parks and nature reserves especially those near Kenya's border to Uganda and Tanzania (Mathenge *et al.,* 2005; Kamondo *et al.,* 2014; Machua *et al*., 2009). The illegal harvesters from neighbouring countries have taken advantage of the porous borders of Kenya and hence caused great damage on the species population area in Kenya (Kamondo *et al.,* 2014; CITES, 2013).

2.6 Natural regeneration of *O. lanceolata*

Spontaneous recruitment rates for all the species in these genera are considered to be significantly low as evidenced by a preliminary survey in which no single seedling was found growing in the natural range (Mathenge *et al*., 2005). The survey revealed that sandalwood populations are declining at a high rate due to human related forces (Mwai, 2005). This speed of decline cannot be countered by the species' regeneration rate in the wild. According to Brand (1999), the Australian populations of *S. spicatum* at Paynes, contained only mature trees indicating that there has been no successful recruitment of the trees for more than 30 years. However, in Western Australia, Murphy and Garkaklis (2003) indicated that human activities that cause habitat fragmentation, grazing, the species parasitic nature that inclines it to specific host for successive establishment and poor seed dispersal were the major causes of its poor recruitment. Furthermore, they noted that in habitats that were less disturbed and in which woylies (*Bettongia pencillata* (Gray-brush tailed bettong) were still present, many new sandalwood seedlings were regenerating across the road from the main plantation (Murphy and Garkaklis, 2003; Murphy *et al.,* 2005). There are no equivalent known animal dispersal agents for *O. lanceol*ata species.

Osyris lanceolata grows much slower compared to other indigenous trees species in the East Africa (Mwang'ingo *et al*., 2007; Kamondo *et al*., 2014). According to Mwang'ingo *et al*. (2007) the tree matures at the age of at least 10 to 20 years. A study in Tanzania shows that, for viable seed collection, the tree needs to be at least 10 - 20 years old. At this age, the tree can also be harvested for good quality essential oils production (Mwang'ingo *et al*., 2003). The tree species produces few seeds that generally achieve very low germination percentage (Mwang'ingo *et al*., 2004; Kamondo *et al*., 2014). Among the Kenyan population, a mature sandalwood tree

could produce up to about 14 kg of seed annually with half of the seeds being damaged in most cases due to pest attacks. These predicaments hamper its propagation (Beentje, 1994; Kamondo *et al*., 2014).

The species characteristic for host preference also deters propagation in the wild. For instance, Mwang'ingo *et al*. (2007) reported that in Tanzania, only few seeds could germinate and when transplanted to the field, they soon dried up in less than a year due to lack of an appropriate host. Mwang'ingo *et al*. (2007) further reported that the seeds exhibit a form of chemical dormancy and that they have recalcitrant characteristics. In this case therefore, propagation of the species by seed is slower (Anon, 2006) and hence the cause of low natural regeneration in the natural range.

PLATE 4: Air layering of *O. lanceolata* at KEFRI (Source: Author 2013).

Researches reveal that vegetative propagation is successful. Air layering (marcotting) showed success in Kenya and Tanzania (Plate 4) but this has to be done on an appropriate nurse plant (Mwang'ingo *et al*., 2007; Kamondo *et al.,* 2014). Mwang'ingo *et al*. (2007) reported that the success is influenced by both season of collection and

application of rooting hormones Indole-3 butyric acid (IBA) with the optimal rooting achieved in June and September when the dry season is setting in. Other modes of regeneration of *O. lanceolata* are mainly from exposed root suckers, or rootstocks and re-sprouting from coppices/ stumps (Mathenge *et al*., 2005; Mwang'ingo *et al*., 2007; Orwa *et al*. 2009). Grafting has also showed great promise as means for propagating this species (Telkehaimanot *et al*., 2003; Mathenge *et al*., 2005).

2.7 Phenology and reproductive biology of *O. lanceolata*

The species is dioecious and often runs into the risk of limited reproductive success due to either low levels of pollen grain production or limited pollen grain movement (Mwang'ingo *et al*., 2007). The species is known to produce seeds of low germination capacity. According to Mbuya *et al. (*1994) and Mwang'ingo *et al* (2007) the low germination may be because by reproductive phase failure during its reproductive cycle. Factors attributed to the species reproductive failure include the over harvesting of the female trees (CITES, 2013), the un-synchronized budding, flowering and pollination periods between the male and female, and the poor mechanism of pollination of the species (Herrera *et al.,* 1998; CITES, 2013). However, a study by Mwang'ingo *et al*. (2007) showed that assisted pollination significantly increased the species reproductive success, hence increasing the seed viability. In addition, seed germination of *O. lanceolata* could be enhanced by applying suitable, pre- sowing treatments to break dormancies (Mwang'ingo *et al*., 2003). Furthermore, establishment of the species using seeds at the nursery level require a primary host such as the grain legume *Cajanus cajan* (L) Millsp. Other studies showed that, indigenous wild fruit trees such as *R. natalensis* and *Carissa spinarum* Linn hosted *O. lanceolata* resulting in higher survival over an 18 months period (Kamondo *et al.,* 2014).

The species mating system is not well studied but other studies in the family reveal outcrossing nature of sandalwood (Murphy *et al.,* 2005). However, mixed mating system of outcrossing and selfing is observed in *S. album* in China (Baskorowati, 2011). The low rate (2%) of self-pollination observed shows that this species is preferentially outcrossing although the flower structure of the species was designed for self-pollination (Charlesworth *et al.,* 1979; Ma *et al.,* 2005; Baskorowati, 2011). Pollination mechanism and seed dispersal of *O. lanceolata* is not fully studied although some studies depict that it is pollinated by insects and flies (Mwang'ingo *et al*., 2007; Kamondi *et al.,* 2009). Seed dispersers have not been fully understood.

Tropical trees are known to observe sub-annual flowering patterns (Sanford *et al*., 1994). *Osyris lanceolata* trees flower more than once a year and often varies slightly from population to population. Flowering phenology of tropical forests is a conservative trait within evolutionary lineages (Kochmer and Handel, 1986; Wright and Calderon, 1995). Therefore flowering pattern of trees in the tropics play an important role in the species pollination success through regulation of pollen flow and foraging behavior of pollinators (Sakai, 2001). This pattern could be the reason for the presence of few or no pollinators during some flowering seasons in *O. lanceolata* species in Kenya.

Knowledge on phenology is important in seed collection programme with the aim of formulating a conservation strategy for the species (Jackeline and Freitas, 2008). Understanding species phenology will ensure timely collection of quality and quantity seed that guarantees high seed viability for planting material and hence achieving the set conservation goals. Knowledge on the plants reproductive biology helps us understand and solve any potential reproductive problems in tree species to help check any future growth failures i.e. optimizing on fertilization, seed formation and maturity

(Jackeline and Freitas, 2008). In order to conserve and develop a better management strategies and utilize this important naturally occurring tree resource, there is need to maximize on the quantity and quality of the seeds produced. Information on the phenology and reproductive biology of the species will help enhance the conservation of its germplasm.

2.8 Genetic diversity of *O. lanceolata*

Genetic diversity in sandalwood is generally jeopardized owing to the unjustifiable felling of the trees perpetrated by smugglers and poachers. The current genetic erosion of the *O. lanceolata* is linked to the declining area of sandalwood among the world sandalwood suppliers, due to an ever increasing demand for its products (Mwang'ingo *et al*., 2007; CITES, 2013). The genetic structure and clonality assessment within five southern Australia populations of *S. lanceolatum* using allozymes and RAPD analysis showed that asexual reproduction by root suckers was responsible for the increase in the population size (Trueman *et al*., 2001) hence inferring to its low genetic diversity. The Western Australian Sandalwood (*S. spicatum*) showed moderate levels of genetic diversity and genetic differentiation between populations in the semi-arid versus arid regions (Byrne *et al.,* 2003). The genetic diversity of *S. album,* a South Indian species, has also been documented using the allozymes (Suma and Balasundaran, 2003), which revealed that tropical and temperate deciduous forests of the Deccan plateau contain an extensive sandalwood tree population that has high genetic diversity (Rao *et al.,* 2007).

Primarily, the East African sandalwood (*O. lanceolata*) distinct populations in Kenya are yet to be studied based on the quantitative traits and DNA molecular markers. Therefore there is need for such a study to generate information on the genetic variation within and among populations and the genetic differentiation among

populations to necessitate formulation of management strategies for conservation of genetic resources of *O. lanceolata* (Muona, 1990; Milligan *et al.,* 1994).

2.9 Conservation of *O. lanceolata*

In India, Japan and China, sandalwood is under state protection and is often referred to as a sacred tree as it is used in worship and various ceremonies (Rai, 1990). Indian and Chinese authorities imposed a ban on the harvesting of sandalwood trees and now only authorize sustainable harvesting where there are large plantations (Rai, 1990). The stringent regulations in China have discouraged poachers, who now take advantage of the legal and policy loopholes in Kenya to import the species (Murphy *et al.,* 2005). Australian and Indians has greatly conserved their resource sustainably in plantations (Murphy *et al.,* 2005). Establishment of *O. lanceolata* plantations has not been done anywhere in Africa though research on its propagations underway (Kamondo *et al.,* 2014). The Kenya's sandalwood tree protection policy is not thoroughly enforced and also some populations are found on private land, hence making them highly vulnerable (Mwai, 2005). Nevertheless, the government of Kenya has since 2007 imposed a ban on trade on sandalwood and its products, though there are allegations of continued illegal harvesting going on (GoK, 2007; Kamondo *et al.,* 2014).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study area

The populations of *O. lanceolata* naturally occurring in Gwasii, Baringo, Mt Elgon, Kitui, Wundanyi, Meru, Mau and Kabarnet were sampled for the entire study (Figure 3.1).
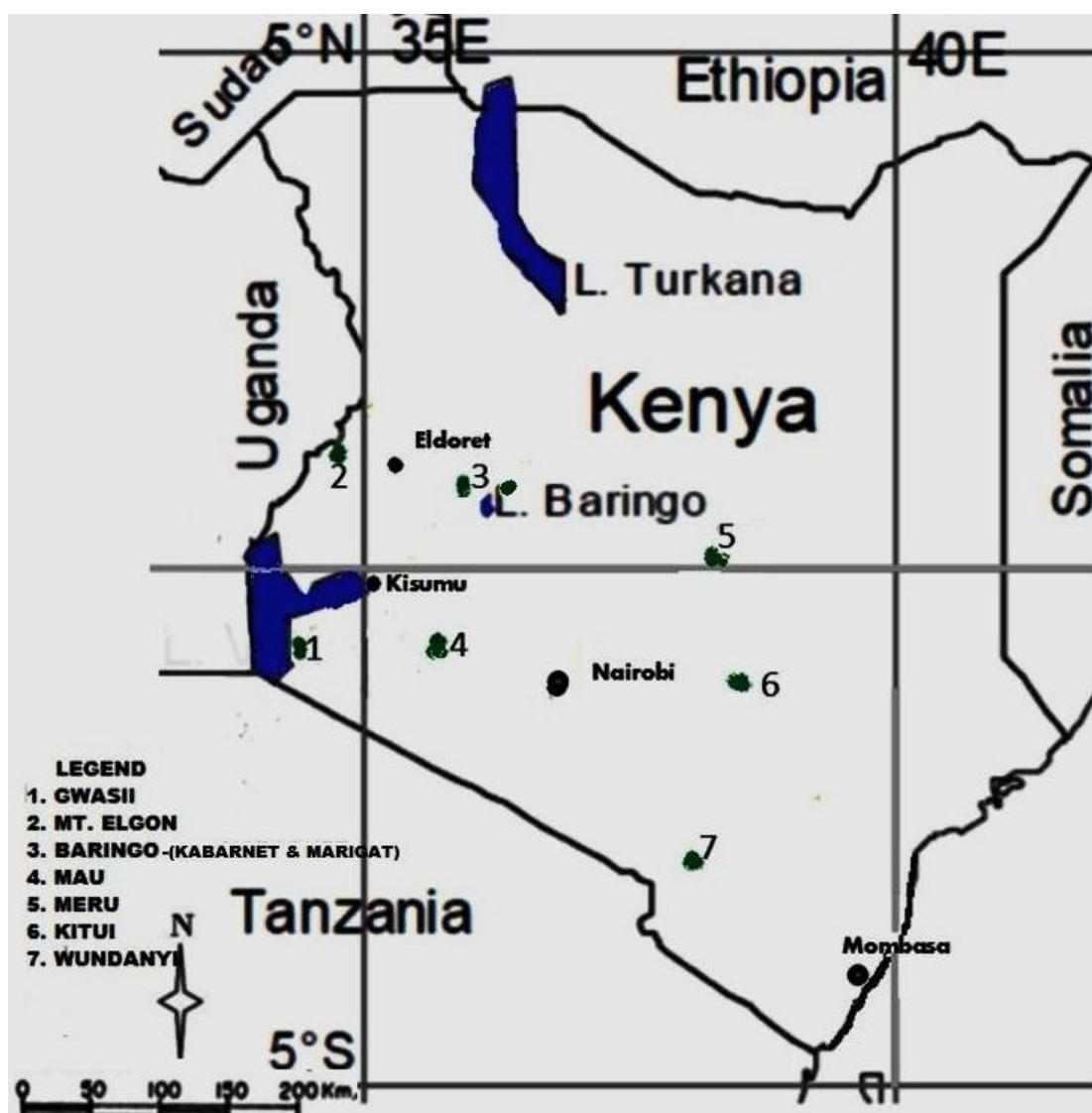
Figure 3. 1: Location of sampled populations of *O. lanceolata* in Kenya

Two representative populations (Kabarnet and Kitui) near KEFRI research centres were chosen for continues and timely observation and study of phenology and reproductive biology. Four populations in Kitui, Gwasii, Kabarnet and Marigat were

sampled for morphological diversity and leaf samples for genetic diversity studies based on molecular DNA analysis using ISSR primers were collected from seven distinct geographical locations i.e. Gwasii, Baringo, Mt Elgon, Kitui, Wundanyi, Meru and Mau (Figure 3.1). Geographical description of the study areas are as summarized in Table 3.1.

Table 3. 1: Geographical profiles of the study areas.

Population	Longitude	Latitude	Altitude (m)	Mean annual Rainfall (mm)	Mean annual Temperatures ($^\circ$C)
Gwasii	34° 10'E	00° 30' S	1600.7	1100.0	26.0
Mt Elgon	34°43'E	00° 10' N	2007.4	1280.0	18.5
Baringo, (Marigat, Kabarnet)	35° 68'E	00° 50' N	2040.0	635.0	25.0
Kitui	38° 00'E	01° 21' S	1185.4	775.0	25.0
Meru	37° 34'E	00° 00' N	1427.7	1440.0	20.7
Mau	35° 16'E	00° 36' S	2288.0	1025.0	21.0
Wundanyi	37° 39'E	01° 10' S	1319.9	650.0	23.0

3.2 Data collection

3.2.1 Overview

Data collection on phenology, reproductive biology and the species genetic diversity based on the morphological traits and molecular analysis (DNA) using PCR-ISSR markers was done from November 2011 to June 2013.

3.2.2 Phenology of *O. lanceolata*

The study on the selected populations started on the onset of the flowering season. Five mature trees showing evidence of budding were identified in each sex (male and female trees), tagged and monitored for the full flowering season. The duration of each stage, the onset and end dates of flowering, fruiting and fruit ripening were monitored on each marked tree and recorded.

3.2.3 Reproductive biology of *O. lanceolata*

A total of 5 male and 5 female healthy, large and mature trees of at least 3.0 cm dbh and with evidence of flower budding were randomly selected from each natural population. Each tree selected was large enough to accommodate various treatments for the study of reproductive biology. Three healthy branches on each tree were selected randomly and marked *A* (branch for anther removal treatment), *P* (branch for assisted pollination) and *C* (control branch) then covered with muslin cloth before the beginning of flower bud initiation period.

On the initiation of flower buds, 10 flower buds on the first branch marked *C*, on each tree were marked and monitored entirely until fruit maturation stage was complete (all newly emerging flower buds were removed using razor blade). On the second branch marked *P*, 10 flower buds were covered using muslin cloth and allowed to open into flowers (Plate 5). Pollen grains from male trees were applied on the receptive stigmas of female flowers for three consecutive days using a fine brush according to guidelines by Ngulube (1996). Each time the pollen grains were applied, the flowers were covered and then monitored over the entire stages of flower development till mature ripe fruits were formed (all newly emerging flower buds were removed using razor blade). On the third branch marked *A*, 10 flower buds were marked and covered with muslin cloth and allowed to open into flowers (female trees only). When they had opened into flowers, all anthers from each flower were removed using scalpel and covered again. The entire fruit development stages were then monitored (all newly emerging flower buds were removed using razor blade).

PLATE 5: *Osyris lanceolata* **branches baged with muslin cloth (Source: Author 2013).**

The total number of flower buds that developed into flowers, to fruits and then to mature and ripe fruits on each tree and in each treatment were counted and recorded. The total length of time each stage of fruit development took was recorded. The mature ripe fruits that developed on each branch were collected and processed to extract seeds. The seeds were cross-sectioned and inspected for viability by determining whether the embryo was fully developed or empty. The seeds were scored as full embryo developed or empty and the number of seeds in each category recorded.

3.3 Measurement of field quantitative traits of *O. lanceolata*

3.3.1 Tree morphology data collection

Twenty four (24) mature trees in each sex with evidence of flower budding activity and 50 m apart to check on the genetic biasness due to inbreeding, were systematically selected within each population and marked for the study.
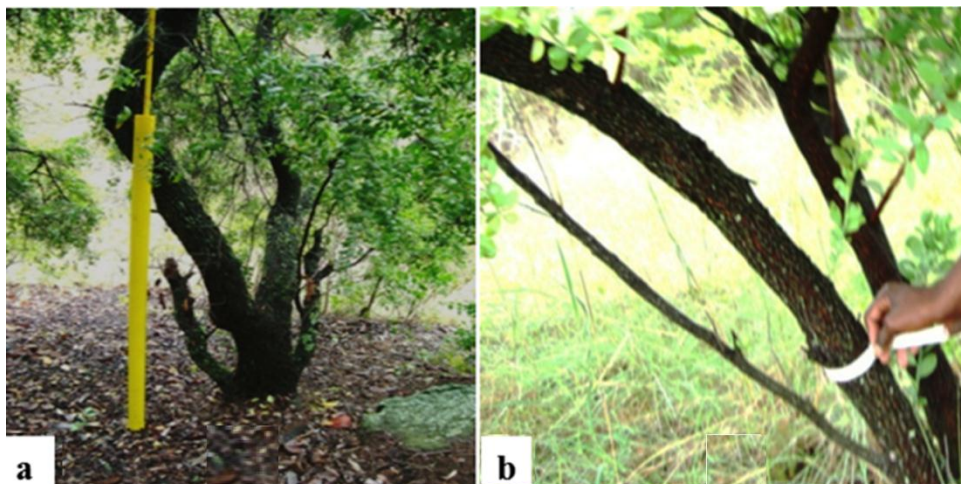
PLATE 6: Measuring tree height -(a), and Dbh -(b) (Source: Author 2013).

The following tree quantitative parameters were measured from each tree: tree height in meters using a tree height bole; trees diameter at breast height (dbh) in centimeters using a tree diameter tape (Plate 6); tree crown diameter in meters measured using linear tape for two crosswise diameters and averaged; and the number of leader stems were physically counted and recorded.

3.3.2 Floral morphology data collection

Three small branches with mature flowers in each sex were randomly selected from the 24 previously marked trees in each of the four populations namely Gwasii, Kitui, Marigat and Kabarnet. The flowers were collected and stored in labeled universal bottles containing Formalin Acetylic Alcohol (FAA) preservative, and taken to the laboratory for measurements.

Thirty flowers from each tree sex in the four populations were randomly picked and measured for lengths and diameter using a millimeter ruler as shown in Plate 7 and recorded. Diameters was measured crosswise and averaged. All the measurements for length and diameter were recorded.
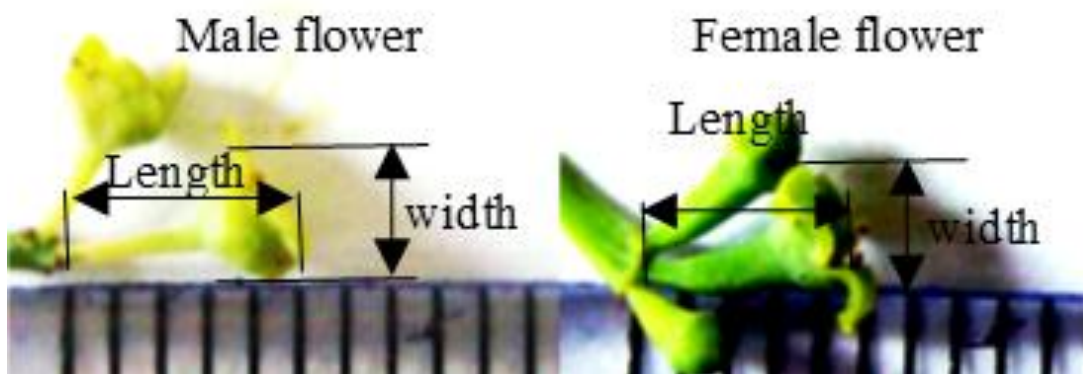
PLATE 7: Measuring flowers dimensions of *O. lanceolata* (Source: Author 2013).

3.3.3 Fruit morphology data collection

Thirty mature fruits were randomly collected from 24 previously marked trees from the four populations namely Gwasii, Kitui, Marigat and Kabarnet. The fruits were stored in labeled universal bottles with Formalin Acetylic Alcohol (FAA) preservative, and taken to the laboratory for measurements.
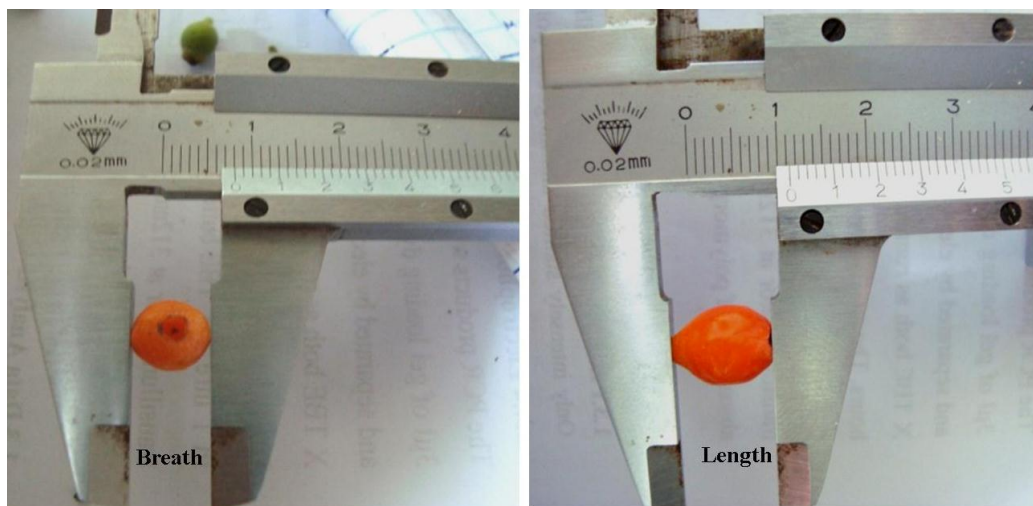
PLATE 8: Measuring length and diameter of a fruit (Source: Author 2013).

Thirty fruits from each population were randomly measured for length and diameter using a Vernier caliper and recorded (Plate 8).

One hundred fruits were randomly collected from trees in each population and taken to the laboratory for seed extraction. The seeds were extracted and then oven-dried at 36 °C until constant dry weight was attained and the results recorded.

3.4 Generation of molecular data of *O. lanceolata*

3.4.1 Samples collection

Mature trees that occurred at least 50 m apart were marked from each population for genetic material collection. Young and healthy apical leaves from the marked *O. lanceolata* trees were collected and stored in zip bags with silica gel and taken to the laboratory for DNA extraction.

3.4.2 DNA extraction

Plant genomic DNA was extracted following the guidelines of CTAB (cetyltri-methyl ammonium bromide) protocol of FAO/IAEA (2002) and modified to suit the species under study. Fresh leaf samples were put in silica gel and stored in KEFRI laboratory (Chase and Hillis, 1991). Five grams (5 g) of dry leaf samples of *O. lanceolata* was weighed and put into 1.5 ml eppendorf micro-centrifuge tubes in the extraction room. Two dry sterilized steel balls were put in each tube using sterilized pincers and the micro-centrifuge tube closed tightly and loaded in a steel ball mill grinder (model Retsch MM400) and ground at at 60 Hz for five minutes to fine powder for 5 minutes.

Six hundred micro-liters of 1.5% freshly prepared and preheated (65 °C) CTAB extraction buffer (CTAB 1ml, PVP 2 ml, NaCl 2.8 ml, EDTA 0.4 ml, Tris 1 ml, Mecarpto-ethanol 0.5 ml, Water 2.75 ml) was added to the leaf powder in the 1.5 ml eppendorf micro-centrifuge tubes and closed tightly. The mixture was vortexed gently in a vortexer (model: Fisons Whirl Mixer) for 1 minute, and then incubated in a water bath at 65 °C for 1 hour with interval gentle inversion of the tubes after every 20

minutes for the entire period of incubation to allow complete denaturation of the proteins in the suspension. The mixture was left for 4 minutes to cool to room temperature, and 600 µl of chloroform: isoamyl alcohol (CIA) added to it at ratio 24:1.

The mixture was centrifuged using a Hermle-microcentrifuge (model Eppendorf centrifuge 5804R, Hamburg Germany) at 13,000 revolutions per minute (rpm) at 5 °C for 10 minutes. Five hundred µl of the upper, supernatant phase was transferred into fresh sterile eppendorf tubes marked accordingly using 1000-µl micropipette, and 2/3 volume of isopropanol added and mixed gently by inversion to precipitate the total nucleic acids in the blend. The mixture was then refrigerated at -20 °C for 30 minutes to allow the DNA extracted to precipitate.

The precipitated DNA was centrifuged at 13,000 rpm for 10 minutes to separate it and the top liquid drained carefully leaving DNA pellets at the bottom of the tube. The pellets were washed with 70% ethanol and centrifuging at 13000 rpm at 5 °C for 2 minutes. This was repeated twice to maximize on cleaning. The ethanol was decanted and the tubes inverted on an absorbent paper on the extraction bench for 3 hours to allow the pellets to dry well. One hundred µl of NaCl TE was added and incubated overnight in a water bath at 55 °C to dissolve the dried DNA pellets.

3.4.3 DNA standardization and quantification

The DNA extracted was quantified using Bio-photometer (cuvets) dsDNA (Appendix 4). The DNA was diluted with TE at the ratio of 2:100 µl, with reference of blank cuvet 0.00 µl at 260 nm. The working samples of DNA were then diluted with PCR water at a volume ratio of 1:9 (DNA: H_2O_{PCR}). Five µl of diluted DNA were pipetted into Elisa plate wells. Two µl of the loading dye (bromophenol blue) was added for staining DNA and also to assist in tracking DNA movement in the gel during

electrophoresis. A total of 7μl of Elisa content was then pipetted into the wells of 2%

agarose gel (2 grams of agarose dissolved in 100 ml 0.5X TBE buffer). A reference

dye (standard low DNA mass ladder-invitrogen) was also pipetted into the remaining

three wells for reference in the order of 2, 4 and 6 μl per well on each side of the gel.

The electrophoresis system was set at 100 V (50 A) and run for 1 hour.

After electrophoresis, the gel was stained in ethidium bromide for 30 minutes and then

rinsed in dH_2O for 5 minute. It was then loaded into the trans-illuminator imaging

system (Kodak MI [MT]). The image was captured using a computer. The visible bands

were marked, compared to the standard low DNA mass ladder-invitrogen and the

bands values scored.

A total of fifteen ISSR primers were available for screening (Table 3.2).

Table 3. 2: ISSR primer oligonucleotide sequences and annealing temperature (°C).

	Primer code	Primer sequence 5'→3'	T_m °C
1	802	ATATATATATATATATG	28
2	806	TATATATATATATATAC	28
3	808*	AGAGAGAGAGAGAGAGC	47
4	809*	AGAGAGAGAGAGAGAGG	47
5	810*	GAGAGAGAGAGAGAGAT	45
6	811*	ACACACACACACACACC	47
7	813	CTCTCTCTCTCTCTCTT	45
8	817	CACACACACACACACAA	45
9	818*	CACACACACACACACAG	47
10	820	GTGTGTGTGTGTGTGTC	47
11	822	TCTCTCTCTCTCTCTCA	45
12	824	TCTCTCTCTCTCTCTCG	47
13	825*	ACACACACACACACACT	45
14	829	TGTGTGTGTGTGTGTGC	47
15	849	GTGTGTGTGTGTGTGCA	47

= primers selected for use

The primers were reconstituted using formulae (1) below:

$$C_1V_1 = C_2V_2; \qquad \text{where,} \qquad\qquad\qquad\qquad (1)$$

$$C_1 = \text{Initial primer concentration,}$$

$$V_1 = \text{Desired volume } H_2O_{PCR},$$

$$C_2 = \text{Set concentration of primer,}$$

$$V_2 = \text{Set } H_2O_{PCR} \text{ volume.}$$

The annealing temperature T_m ($^\circ$C) of each primer was calculated as in the formulae below,

$$T_{M-} = 64.9\ ^\circ C + 41\ ^\circ C \times (\text{number of G's and C's in primer-16.4})/N. \qquad (2)$$

Where; Tm=annealing temperature, N= the length of the primer (longer oligos >14 bases) (BioMath.Tm Calculation for Oligos) (Yoon *et al.,* 2002).

The PCR thermo-cycler was programmed as follows: one cycle of 94 °C for five minutes (Hot start step); 40 cycles at 94 °C for 30 seconds (denaturation step); a range between 45- 47 °C (depending on the specific primer's annealing temperature) for 45 seconds (template annealing temperature step); 72 °C for 2 minutes (polymerization step); and a final extension phase of 10 minutes at 72 °C (Williams *et al.,* 1990). Then 2 µl of loading dye was added into tubes of PCR products and mixed gently by vortexing for 10 seconds.

Four DNA samples from each population were selected for ISSR-PCR amplification using all the 15 primers available. The samples were labeled and then varying volume ratios of PCR water pipetted into each tube. The final content was: 25 ng of DNA template; puRe Taq Ready-to-go PCR Beads, containing BSA, dATP, dCTP, dGTP, dTTP and ⁓2.5 µl units of puRe Taq DNA polymerase invitrogen. Several runs were repeatedly done to test on the reproducibility of each primer. The primers that showed

high reproducibility, good polymorphism with clear and scorable bands were chosen for PCR-ISSR amplification for the seven populations as shown in Table 3.2.

ISSR amplification working stock was set taking the standard ratios of 21 µl PCR water, 3µl primer and 1µl DNA set in group A. The groups B, C, D and E were then set with varying ratios of PCR water, Primer and DNA as shown in Table 3.3. The final tube volume had concentration of each dNTP200 µM in 10 mMTris-HCL, (pH 9.0 at room temperature), 50 mMKCl and 1.5 mMMgCl$_2$; 2.5 mM each of dATP, dCTP, dTTP, dGTP; primer; DNA and 0.3 U Taqpolymerase. The tubes were then closed tightly, mixed gently by vortexing for 10 seconds and amplified in a programmed PCR thermocycler (Appendix 3).

The stained PCR products were then loaded to a 2.5% agarose gel for electrophoresis. Seven microliters (7 µl) of low mass ladder was pipetted in volumes of 2, 4, and 6 µl in three consecutive gel wells besides the stained PCR products and run for one and a half hours at 100 Volts. The electrophoresis gel was stained in ethidium bromide for 30 minutes and rinsed in distilled water for 15 minutes. Samples were then visualized in the UV light and imaged by Kodak camera (Kodak MI [MT]). The presence of DNA bands were compared to the standard DNA mass ladder. The ratios of PCR water to Primer to sample DNA (H$_2$O$_{PCR}$: P: DNA) for PCR amplifications of group E (Table 3.3) was chosen for the seven population DNA analysis.

Table 3.3 shows the results for the ratios of stock that produced the best bands for the

ISSR-PCR *O. lanceolata* genetic study work.

Table 3. 3: ISSR amplification working stock

Stock for ISSR –PCR Amplification					
	A	B	C	D	E
H$_2$O	21 µl	20 µl	19 µl	18 µl	17 µl
Primer	3µl	3 µl	3 µl	3 µl	3 µl
DNA	1 µl	2 µl	3 µl	4 µl	5 µl

3.4.4 Final ISSR-PCR amplification

Based on the PCR amplification ratios and primers selected, the ISSR amplification

was performed on all the samples from the seven populations. Amplified products

were subjected to electrophoresis for one and a half hours on 2.5% agarose gel run in

0.5× TBE buffer (100 mMTris-HCl, pH 8.3; 83 mM acetic acid; 1 mM EDTA) at 100

V. The gels were then stained with 0.8 mg ethidium bromide for 30 minutes. They

were then rinsed in distilled water for 15 minutes and photographed under ultraviolet

(UV) light illuminated chamber. The size of the amplified products was compared to

100 bp (2µg/µl) DNA ladder, loaded on both ends of the gel wells to help determine

bands. Only well stained visible bands were scored as binary data for analysis.

3.5 Data analysis

3.5.1 Phenology and reproductive biology data analysis

The total duration of the populations' onset and end dates of flowering cycle was

observed and recorded in days. The ranges, means and standard deviation of days in

phenology in each stage were determined using MS Excel. The reproductive variations

in the flowers that formed and developed into a viable fruit were determined in each

treatment and fed on MS Excel spreadsheet. The data set obtained was subjected to

ANOVA in SPSS 12.0 version 2010. Whenever *F*-test for significance of variation for the reproductive success among the treatments in the two populations was significant, Duncan's Multiple Range Tests (DMRT) was performed in order to compare differences between means.

3.5.2 Morphological data analysis

The differences in the quantitative traits of the trees among the populations were tested for normal distribution and then analyzed using one-way ANOVA in SPSS 12.0 version statistical package (Bewick *et al.,* 2004). Whenever the *F*-test for the parameters was significant at 95% level, Duncan's Multiple Range Tests (DMRT) was performed in order to compare differences between means.

3.5.3 Molecular data analysis

Only well stained visible polymorphic bands were scored for presence (1) or absence (0) (Wendel and Weeden, 1989). The binary matrix data file created was configured as an input for the data analysis. Nei's (1973) unbiased gene diversity indices (*UHe)* assuming Hardy-Weinberg equilibrium, percent of polymorphic loci *(P),* analysis of molecular variance (AMOVA), Shannon information index *(I)*, number of effective allele *(Ne),* and coefficient of differentiation (*Gst)* were derived using PopGen version 1.32 (Yeh *et al.,* 2000). The genetic distance *(D)*, Principle Coordinate Analysis (PCoA) were derived using GenALEx software 1.61 (Peakall and Smouse, 2012). The pair-wise population matrix of Nei's unbiased genetic distance generated by GenAlex with 1000 permutations was used with Molecular Evolutionary Genetic Analysis (MEGA) 4 software (Tamura *et al.,* 2007) to produce an UPGMA dendrogram (Nei's, 1973).

CHAPTER FOUR

RESULTS

4.1 Phenology and reproductive biology

4.1.1 Phenology of Kenya's *O. lanceolata* species

Table 4.1 shows results of observed phenological events *O. lanceolata* and the cycle of

occurrence in the two populations.

Table 4. 1: Periods of phenological events in *O. lanceolata*.

Events	1st Cycle	2nd Cycle	Remarks
Flower buds initiation	Jan/Feb	Sept/Oct	Budding continues till early June for 1st Cycle and early November in 2nd cycle.
Flower buds opening	Jan/Feb	Sept/Oct	Buds opening continues till early June for 1st Cycle and early November in 2nd cycle.
Fruit initiation	Feb/Mar	Oct/Nov	Fruit initiation continues till mid-June for 1st Cycle and late November in 2nd cycle.
Fruit maturation	April/May	Nov /Dec	Fruit initiation continues till Mid July for 1st Cycle and early December in 2nd cycle.

Generally, flower bud initiation started in January and September respectively in the

two flowering cycles. The maturation of fruits started in late March in the first cycle

and November during the second cycle. Bud initiation in Kitui was 3 to 4 days earlier

than the Kabarnet population. New bud initiation continued up to late June though

fruits initiated and their development into viable fruits reduced (Table 4.1).

Table 4.2 shows the results of the duration in days taken in each flowering stage in the

flowering cycles from bud initiation to fruit ripening. The flowering cycle of male

trees ended with flower buds withering while the female trees cycle came to an end

when the fruits had fully ripened.

Table 4. 2: Phenology of both male and female *O. lanceolata* trees.

Events	Sex	Range Duration in days	Variance	Standard Deviation	Mean days	Cumulative days
Flower budding to flower bud opening	F	4 – 6	1	1	5	5
	M	5 – 9	2.5	1.58	7	7
Flower bud opening to flower withering	F	12 – 17	3.5	1.87	14.5	19.5
	M	14 – 20	4.67	2.16	17	24
Fruit initiation to fruit maturation	F	29 - 36	6	2.45	32.5	52
Fruit maturation to fruit ripening initiation	F	13 - 17	2.5	1.58	15	67
Ripening initiation to active fruit ripening	F	16 – 33	28.9	5.3	26	83
Total		74-109		16	109	

To complete one flowering cycle, *O. lanceolata* took 109±16 days in both Kitui and Kabarnet populations. It took 14 and 17 days for the flower buds initiated to form active flowers in male and female trees respectively. The floral parts of active flowers that had formed withered within 12 to 17 days in female flowers and 14 to 20 days in male flowers. This marked the beginning of fruit initiation in female trees and the end of flowering activity in males. This took place 12 to 20 days after flower bud initiation in both sexes. Fruits that formed achieved maturity (unripe fruits) after 29-36±5 days, which were 52±6 days since bud initiation. Twelve to sixteen days later, fruit ripening phase started. This was 67 days since flower bud initiation. Fruits under study started ripening within 16 days and achieve active ripening after 33 days (Table 4.2).

4.1.2 Reproductive biology of Kenya's *O. lanceolata* populations

Male and female *O. lanceolata* trees had loose inflorescence with 2 to 5 flower buds in female while male had 6 to 11 flower buds per every inflorescence (Plate 9). Male and female flowers were distinguished by their physical structure. Male flowers had shallow receptacle that were relatively larger in diameter as compared to female

flowers. In most cases, 1 to 2 fruits matured on every female inflorescence while all the male flower buds withered (Plate 10).
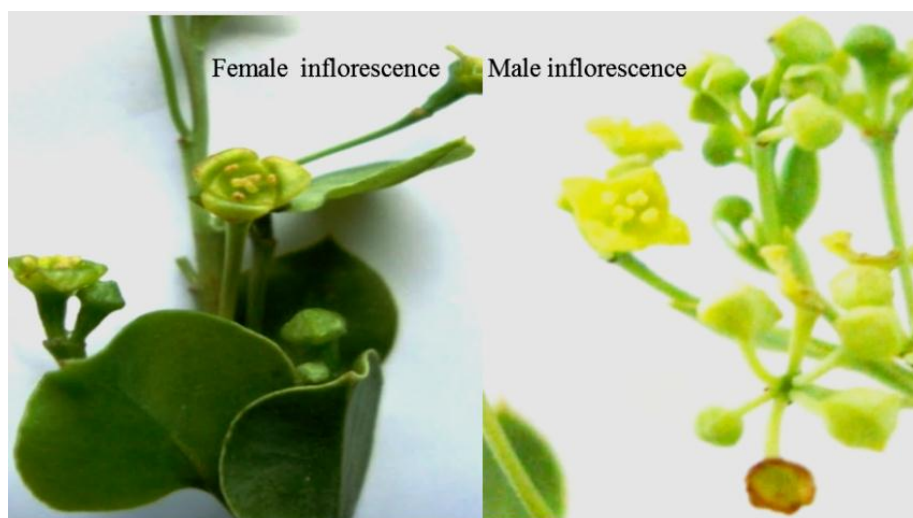
PLATE 9: Male and female *O. lanceolata* inflorescence (Source: Author 2013).

PLATE 10: Physical appearance of male and female flowers (Source: Author 2013).

The reproductive success of the species based on treatment namely; natural pollination-*C*, assisted pollination-*P* and anther emasculation-*A*, are as showed in Table 4.3. In Kabarnet, 40% of the flower in the control developed into fruits while in Kitui, 30% of the flowers developed into fruits. In assisted pollination treatment (*P)*, 50% of the flowers developed into fruits in both Kabarnet and Kitui population. A mean of less than 5% of the flowers that had their anthers removed (A), developed into

fruits. The 58.67% of the fruits matured in Kabarnet population while 63.33% matured in Kitui population when naturally pollinated (*C*). In the assisted pollination, 65.38% and 77.43% of the fruits matured in Kabarnet and Kitui respectively (Table 4.3).

Table 4. 3: Relative success rates in female reproductive cycle in Kitui and Kabarnet.

Treatment	Population	Number of flower buds	% flowers to fruits	% of raw fruits to ripe fruits	% of ripe viable fruits	Average success (%)	% of ripe empty fruits
C	Kabarnet	50	40.0	58.67	20.0		80.0
	Kitui	50	30.0	63.33	33.3	26.65	66.67
P	Kabarnet	50	50.0	65.38	62.94		48.0
	Kitui	50	50.0	77.43	69.84	39.74	46.0
A	Kabarnet	50	5.0	2.0	0	0	2.0
	Kitui	50	3.0	1.0	0	0	1.0

Key: C=control / natural pollination treatment, P=assisted pollination treatment, A=anther emasculated.

Mean percentage of viable fruits that matured in natural pollination was 20% in Kabarnet and 33.3% in Kitui populations. In the assisted pollination, 62.94% and 69.84% of the mature fruits in Kabarnet and Kitui had full embryos (Plate 11).

Plate 11 shows *O. lanceolata* seeds that were viable or had empty embryo after cross sectioning of the seeds extracted.
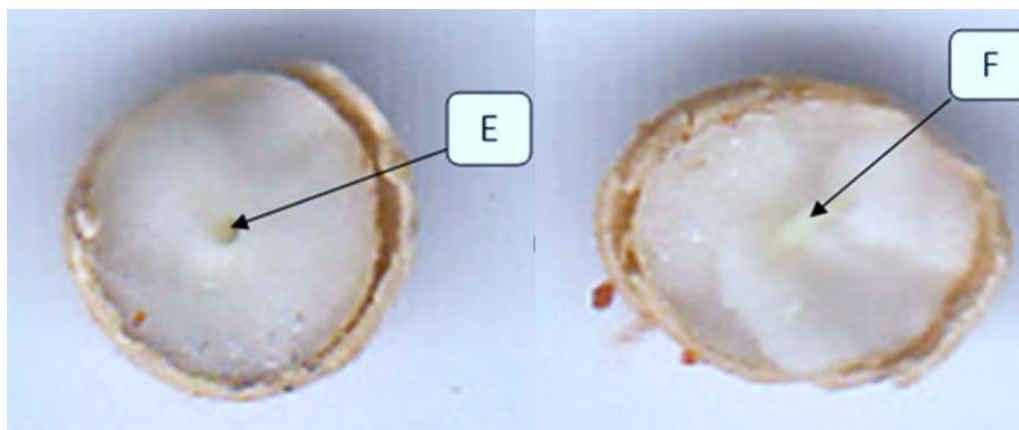
PLATE 11: A full embryo seed (F), and a embty seed (E) (Source: Author 2013).

A mean of 1.5% in the anther removed flowers setup formed fruits that ripened but did not have embryo. The assisted pollination treatment (*P*), produced viable fruits significantly higher at 95% level (*p=0.034*) than in control treatment (*C*) in both populations. Therefore, artificial pollination (*P*), significantly increased the number of viable seeds that formed (Table 4.4).

Table 4. 4: ANOVA for reproductive biology treatments in *O. lanceolata* populations.

SOURCE OF VARIATION	SS	Df	MS	F	P-value
Between Treatments	1578.076	1	1578.076	28.017	0.034
Within Treatments (Error)	112.649	2	56.325		
Total	**1690.725**	**3**			

Table 4.5 shows the summary of the results of an ANOVA for the fruiting, fruit ripening and viable seeds that formed in Kabarnet and Kitui populations. The ANOVA for reproductve stages revealed that there was no significant variation among reproductive stages in Kitui and Kabarnet populations.

Table 4. 5: ANOVA for reproductive stages of *O. lanceolata* within the populations.

Traits	Mean squares	F	sig (*p<0.05*)
Fruiting	0.300	0.109	0.744 [ns]
Fruit ripening	0.833	0.283	0.599 [ns]
Viable seed	2.700	1.246	0.274 [ns]

ns=Not significant at p<0.05 confidence level

Figure 4.1 shows graphical results of the proportion of fruits that developed into mature ripe fruits for each treatment in Kitui and Kabarnet populations.

C= Control, P= Pollination assisted, A= Anther removed.
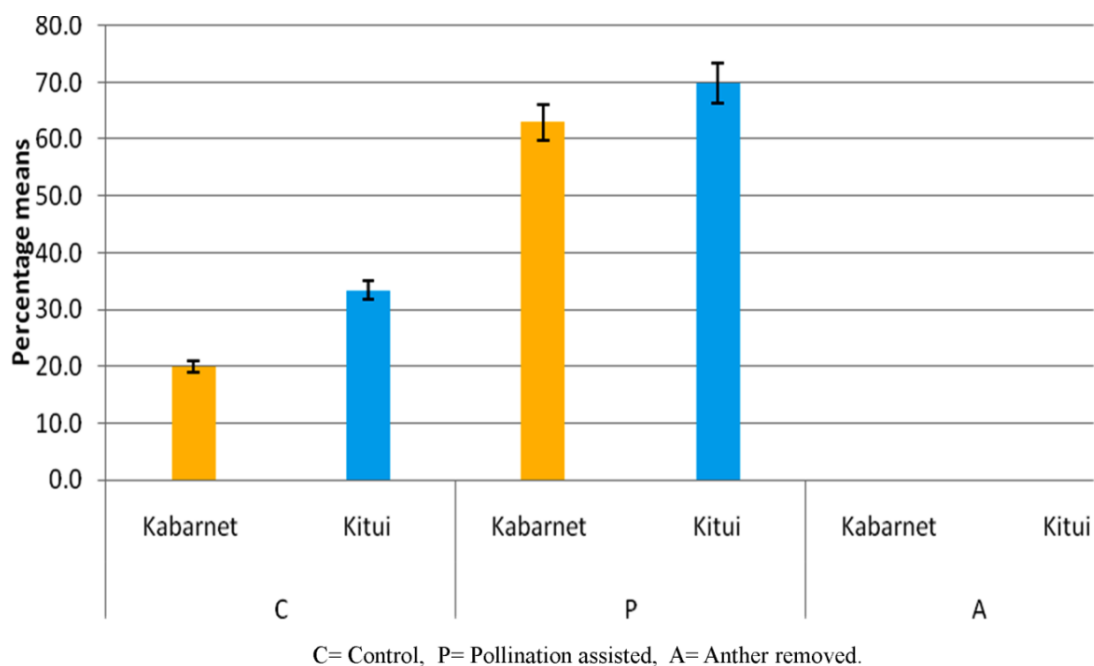
Figure 4. 1: Success variations in fruit development within Kabarnet and Kitui.

4.2 Morphological trait variations in *O. lanceolata* populations

The analysis of nine parameters measured on *O. lanceolata* trees in the four

populations are as showed in the Table 4.6.

Table 4. 6: Quantitative traits of *O. lanceolata* assessed in four populations.

Parameters	Means	Range
Height (m)	3.86	2.00- 6.30
Dbh (cm)	4.67	2.50- 9.34
Crown diameter (m)	2.39	1.00- 4.90
Stem count	2.44	1.00- 5.00
Flower length (mm)	4.69	4.00- 5.20
Flower width (mm)	3.33	2.90- 3.90
Fruit length (mm)	10.4	10.10- 10.90
Fruit width (mm)	9.9	9.20- 10.80
Seed weight (1000 air-dried)(g)	116.51	
Seed weight (1000 oven-dried)(g	91.84	

The trees were observed to be multi-stemmed (Plate 1) with the mean height of populations was 3.86 m ranging from 2.0 to 6.3 m. The mean diameter at breast height (Dbh), crown diameter and stem count were 4.67 cm, 2.39 m and 2.44 m respectively, with ranges 2.1-9.34 cm, 1- 4.9 m and 1- 5 respectively (Table 4.6).

Gwasii had the highest percentage means in tree height, Dbh, crown diameter; flower length, flower diameter, fruit length, fruit diameter and seed weight (Figure 4.2).
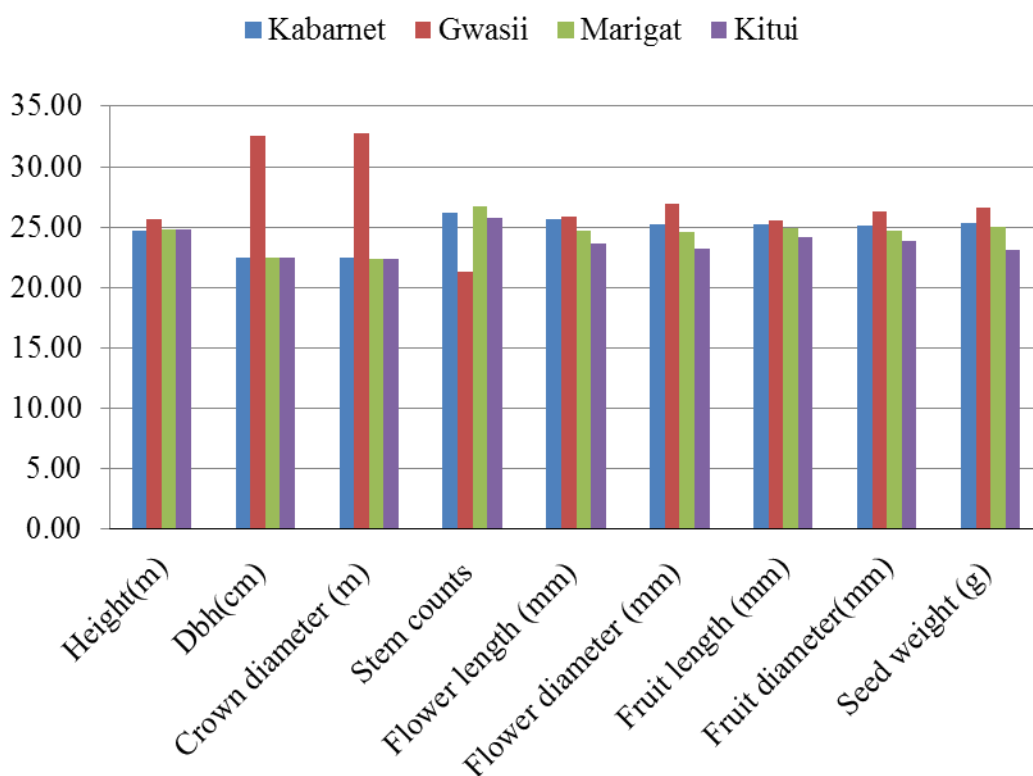
Figure 4. 2: Comparative means of morphological parameters of *O. lanceolata*.

The trees were multi-stemmed and Gwasii had the lowest percentage mean in stem count. Fruit traits were not included in this graphical comparison because male trees do not bear fruit (Figure 4.3).
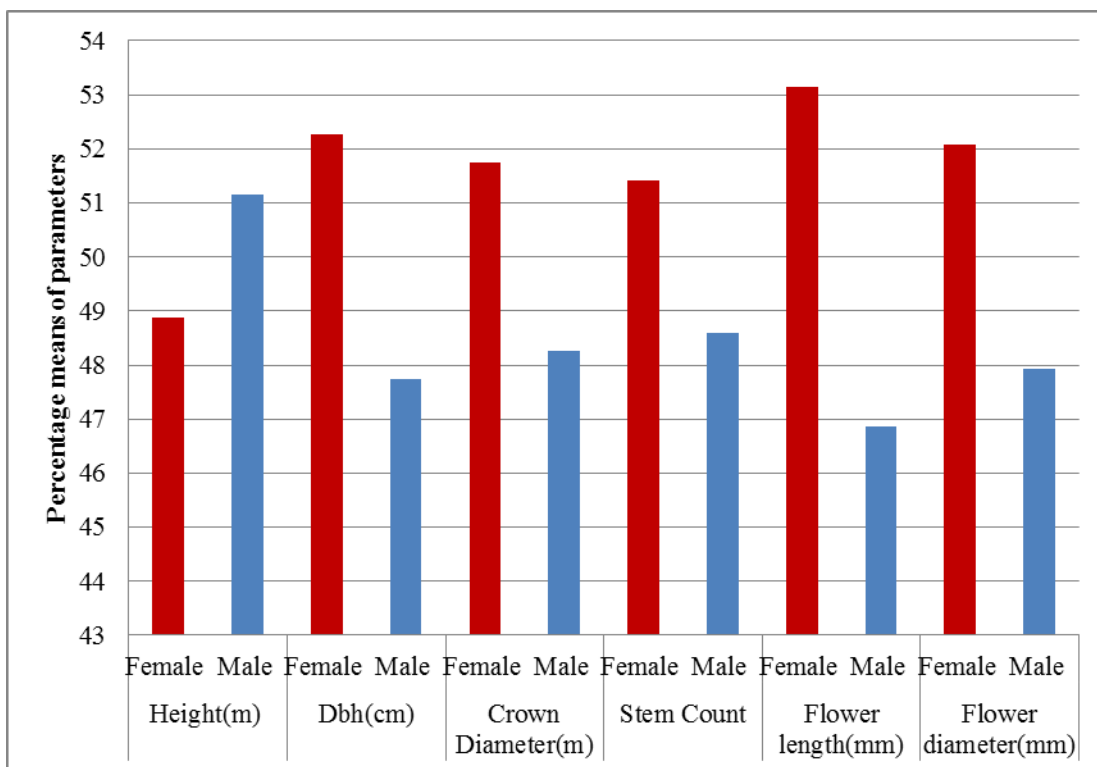
Figure 4. 3: Morphological traits variations among tree sexes.

Table 4.7 shows the ANOVA results for the nine morphological traits of *O. lanceolata* in the four populations in Kenya. The results revealed that *p-values* obtained were significant (95% level) except for height and stem count traits thus, null hypothesis that there is no significant difference in the morphological traits of the *O. lanceolata* trees in Kabarnet, Gwasii, Marigat and Kitui populations was rejected. The alternative hypothesis that there is morphological variability in the morphological traits among the four populations was considered. Height and stem count variations among the four population were not statistically significantly at 95% level (*p=0.900*, and *p=0.605*) and hence not used in DMRT to partition means.

Table 4. 7: ANOVA for quantitative traits in *O. lanceolata* for the four population.

	Traits	Mean squares	F	sig (*p<0.05*)
1	Heights (m)	0.027	0.192	0.900 [ns]
2	Dbh (cm)	5.378	6.327	0.003**
3	Crown diameter (m)	1.481	7.860	0.001**
4	Stem counts	1.158	5.848	0.605 [ns]
5	Flower length (mm)	0.223	1.651	0.010*
6	Flower diameter (mm)	0.255	5.682	0.016*
7	Fruit length (mm)	0.185	7.661	0.010**
8	Fruit diameter (mm)-female only	0.485	5.376	0.025*
9	Fruit weight (g)-female only	0.764	6.544	0.015*

[ns] =not significant at *p=0.05* confidence level, *=significant at *p=0.05* level,

**= significant at *p=0.01* level

When significance tests for each parameter was run based on the trees sex, the stem counts and flowers sizes varied significantly in both trees sexes among the four populations at *p<0.05* and *p<0.01* (Table 4.8).

Table 4. 8: Summary ANOVA for quantitative traits in female and male *O. lanceolata*.

	Sexes traits	Mean squares	f	sig (p<0.05)
1	Heights female(m)	0.006	0.035	0.991 [ns]
2	Heights male (m)	0.026	0.150	0.927 [ns]
3	Dbh female (cm)	2.616	2.247	0.160 [ns]
4	Dbh male (cm)	2.763	3.347	0.076 [ns]
5	Female crown diameter (m)	0.859	3.845	0.057 [ns]
6	Male crown diameter (m)	0.630	2.826	0.107 [ns]
7	Female Stem counts	0.161	13.741	0.002**
8	Male Stem counts	1.470	05.040	0.030*
9	Female Flower length (mm)	0.037	11.736	0.003**
10	Male Flower length (mm)	0.323	14.029	0.001**
11	Female Flower diameter (mm)	0.144	04.681	0.036*
12	Male Flower diameter (mm)	0.113	04.945	0.031*
13	Fruit length(mm)	0.185	07.661	0.010
14	Fruit diameter(mm)	0.485	05.376	0.025
15	Seed weight (g)	0.764	06.544	0.015

[ns] =not significant at *p=0.05* level, *=significant at *p=0.05*, **=significant at *p=0.01*

According to Duncan Multiple Range Test (DMRT), all the morphological parameters discretely separated Kitui in cluster one and Gwasii in cluster two. Generally, most of the morphological parameters i.e. flower and fruit dimensions and seed weight grouped Kitui population in cluster one, Gwasii populations in cluster two and Kabarnet and Marigat population in cluster three. Only the flower length that grouped Kitui in cluster one, Kabarnet and Gwasii into cluster two and Marigat in cluster three. Crown diameter and dbh parameters grouped Kitui, Kabarnet and Marigat in cluster one and Gwasii population in cluster two (Table 4.9).

Table 4. 9: DMRT between parameter means for partitioning populations.

Quantitative Traits	Kabarnet	Marigat	Gwasii	Kitui	SE
Dbh (cm)	4.2006a	4.1965a	6.0938b	4.2038a	2.592
Crown diameter (m)	2.1510a	2.1365a	3.1354b	2.1385a	0.643
Flower length (mm)	5.1271b	4.6125c	4.9917b	4.4396a	0.136
Flower diameter (mm)	3.2458c	3.2229c	3.4750b	3.0021a	0.050
Fruit length (mm)	10.2106c	10.2767c	10.7042b	9.9696a	0.044
Fruit diameter (mm)	9.9454c	9.8817c	10.0850b	9.7273a	0.021
100 Seed weight (g)	8.6333c	8.5333c	9.1000b	7.8750a	0.116

Populations with same letter are grouped in one set basing on the parameter. The parameter discriminated the populations into three clusters.

Table 4.10 show the phenotypic correlation coefficient (*r*) of the morphological traits of *O. lanceolata* trees in Kabarnet, Gwasii, Marigat and Kitui populations. At the level of significance of *p<0.01*, height correlated positively with both dbh and crown diameter (r=0.510 and r=0.533 respectively). The dbh correlated with crown diameter of the trees (r=0.678) at *p <0.01*. Flower length and flower diameter positively correlated with fruit length and fruit diameter respectively (r=0.619, 0.522) and again,

fruit length showed correlation with fruit diameter (r=0.732) at *p*-value 0.01. Crown diameter and stem count did not correlate with any of the trees morphological parameters.

Table 4. 10: Correlation co-efficient (r) values for nine parameters among population

Traits	Dbh	Crown diameter	Stem count	Flower length	Flower diameter	Fruit length	Fruit diameter	Seed weight
Heights	0.510[a]	0.533	-0.173	0.036	0.019	-0.022	-0.031	-0.07
Dbh		0.678[a]	-0.200	0.221	0.340	0.035	0.031	-0.13
Crown diam			0.128	0.203	0.396	0.051	0.061	0.123
Stem counts				0.047	-0.058	0.117	0.076	0.199
Flower leng					0.183	0.619[a]	0.291	0.092
Flower diam						0.220	0.522[a]	-0.04
Fruit length							0.732[a]	0.178
Fruit diam								0.105

[a] *Significance at 0.01 level*

The Table 4.11 shows the results of Shannon-Weaver analysis (*Hs*) for *O. lanceolata* tree traits in the four populations in Kenya. The most variable morphological parameter across the four populations were the fruit dimensions (Fruit length *Hs*=24.050 and Fruit diameter *Hs*=22.647). The same traits varied highly in Kabarnet (Fruit length *Hs*=25.397 and fruit diameter *Hs*=23.176) and least in Kitui population (Fruit length *Hs*=22.926 and fruit diameter *Hs*=22.129). The second highest trait was flower length (*Hs*=7.519), followed by Dbh (*Hs*=7.274), height (*Hs*=5.202), flower diameter (*Hs*=3.519), crown diameter (*Hs*=2.120), stem count (*Hs*=1.841) and the least being the seed weight (*Hs*=0.219). The mean Shannon diversity index (*Hs*) for all the nine morphological traits measured across the four populations under this study

was *Hs*= 8.225. Based on the Shannon diversity index (*Hs*), Kabarnet and Marigat

populations were grouped together in cluster one, Gwasii in the second and Kitui in the

third cluster.

Table 4. 11: Shannonn's diversity index (Hs) based on morphological traits.

Traits	Kabarnet	Gwasii	Kitui	Marigat	Mean *Hs*
Heights (m)	5.090	5.436	5.144	5.139	5.202
DBH (cm)	6.029	11.013	6.037	6.019	7.274
Crown Diameter (m)	1.648	3.583	1.626	1.622	2.120
Stem Counts	1.971	1.489	1.934	1.971	1.841
Flower Length (mm)	8.381	8.026	6.618	7.052	7.519
Flower Width (mm)	3.392	4.328	3.300	3.344	3.591
Fruit Length(mm)	25.397	23.935	22.926	23.944	24.050
Fruit Width (mm)	23.176	22.647	22.129	22.636	22.647
Seed Weight (g)	0.226	0.220	0.212	0.220	0.219
Mean *Hs*	**8.317**	**8.915**	**7.722**	**7.945**	**8.225**

Figure 4.4 below shows the phylogenetic relationship of the four populations based on

the nine morphological traits results. The dendrogram delineated Kabarnet, Marigat as

one related group geographically. Gwasii and Kitui population were depicted as being

distinct and far from each other geographically.
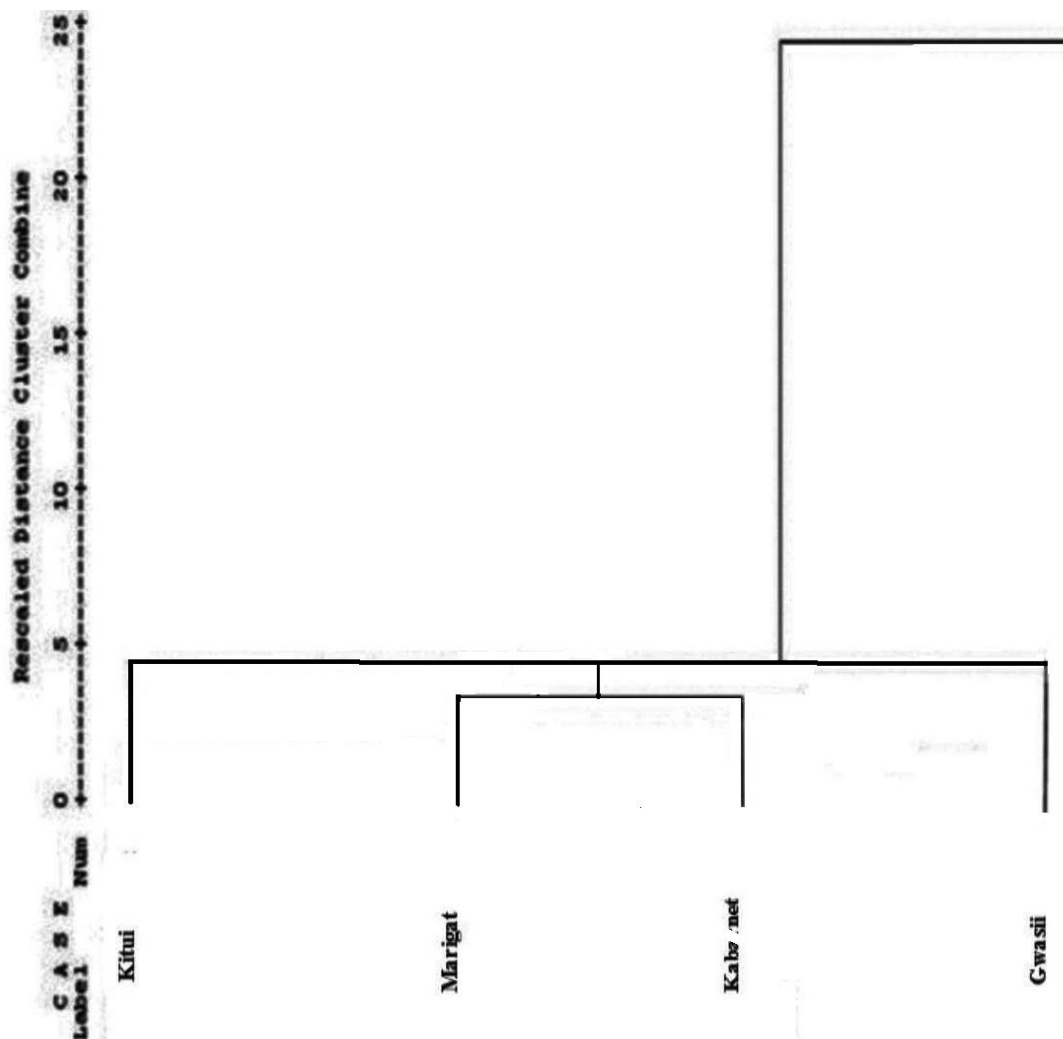
Figure 4. 4: Hierarchical cluster analysis of 9 morphological parameters.

4.3 Genetic diversity in *O. lanceolata* based on ISSR markers

4.4.1 ISSR primers screened

The Inter-Simple Sequence Repeat (ISSR) amplification was performed on all the 178 samples with six primers selected based on their clear banding pattern and high reproducibility out of 15 screened primers. Primers with (AG), (GA), (AC), and (CA) repeats showed higher polymorphism in this species than primers with other di-, tri- or tetra-nucleotide repeats (Table 4.12 and Figure 4.5).

Table 4. 12: ISSR primers percentage polymorphic fragments.

Primer Code	Primer sequence 5'→3'	No of fragments amplified	No of polymorphic fragments	Percentage of polymorphic fragments (%)
808	AGAGAGAGAGAGAGAGC	13	3	23.1
809	AGAGAGAGAGAGAGAGG	16	6	37.5
810	GAGAGAGAGAGAGAGAT	15	5	33.3
811	ACACACACACACACACC	15	5	33.3
818	CACACACACACACACAG	20	10	50.0
825	ACACACACACACACACT	17	7	41.2
Mean		16	6	36.4
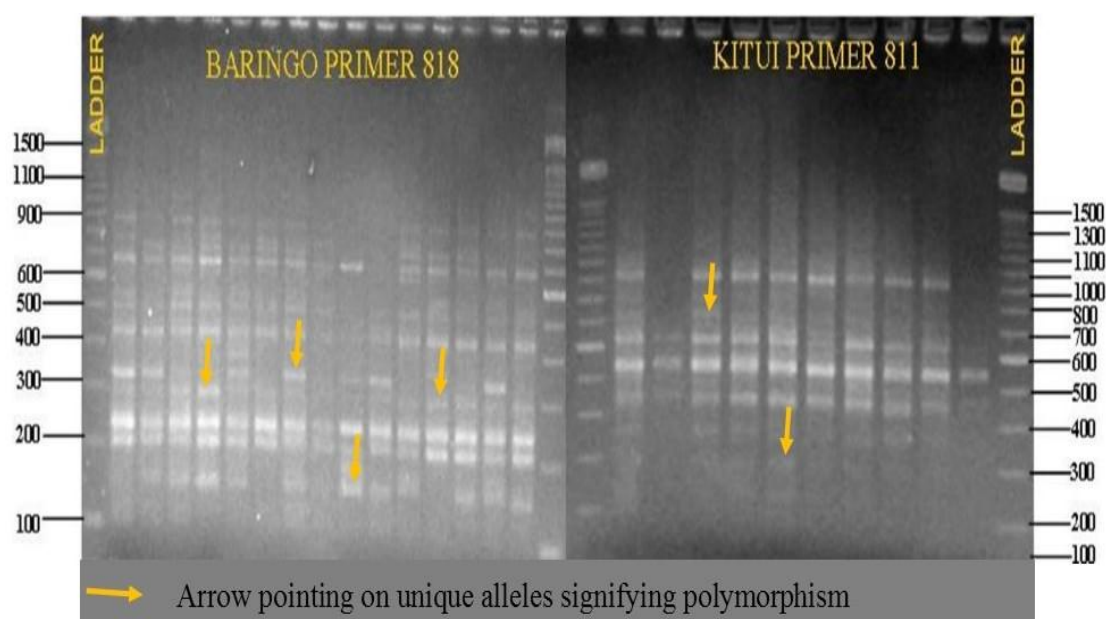
Figure 4. 5: 2.5 % gel images of differentially amplified loci using ISSR primers.

4.3.2 ISSR markers fragments

The six ISSR primers used produced 96 fragments. The product size ranged from 150 to 1000 base pairs (Figure 4.5). A total of 36 polymorphic bands were produced with an average of the 6 polymorphic loci. The largest number of polymorphic bands were amplified by primer 818 (10 bands) followed by primer 825 (7 bands). The least (3 bands) were amplified by primer 808 (Table 4.12).

4.3.3 Genetic diversity parameters in *O. lanceolata* based on ISSR markers

The percentage polymorphic loci (*P* %) ranged from 82.29% for the Gwasii population, to 51.04% for the Wundanyi population, with a mean percentage polymorphism of 64.73%. The unbiased diversity index *(UHe)* was highest in Gwasii (0.321), followed by Mt. Elgon (0.308), Kitui (0.240) and the lowest being Wundanyi with *UHe* of 0.175. The mean *UHe* for the seven populations was 0.253. The seven populations had Shannon Information Index *(I)* ranging from 0.232 for Kitui population to 0.446 for Gwasii population, with mean Shannon Information Index *(I)* of 0.365. Number of effective alleles (*Ne*) value ranged from 1.229 (Wundanyi) to 1.575 (Gwasii) with means of 1.430 (Table 4.13).

Table 4. 13: Genetic diversity indices of *O. lanceolata* populations studied.

County	Population	*N*	*Na*	*Ne*	*I*	*UHe*	*P*
Homa Bay	Gwasii	30	1.667	1.575	0.446	0.321	82.29
Bungoma	Mt Elgon	30	1.635	1.552	0.372	0.308	78.13
Nakuru	Mau	30	1.375	1.405	0.307	0.234	62.50
Baringo	Baringo	30	1.417	1.364	0.441	0.211	60.42
Kitui	Kitui	20	1.302	1.427	0.232	0.240	59.38
Meru	Meru	20	1.375	1.401	0.307	0.227	59.38
TaitaTaveta	Wundanyi	18	1.229	1.229	0.338	0.175	51.04
Mean			**1.429**	**1.430**	**0.365**	**0.253**	**64.73**

Key: N=sample size, Na=Observed number of alleles, Ne=number of effective alleles, I=Shannon information index, UHe=unbiased diversity index, P=per cent polymorphic.

4.3.4 Genetic differentiation and phylogenetic relationships among populations

The Aanalysis of Molecular Variance (AMOVA) performed on 178 samples for the seven populations revealed total estimated variations of 20.3% with 37.9% residing among populations and majority of the variations (62.1%) residing in individuals within populations (Table 4.14). Comparable results were obtained with estimated G_{st} (0.3429) (*p=0.001*), indicating that 34.29% of the total variation existed among the population while the majority of the variation (65.71%) resided within population (Table 4.15).

Table 4. 14: AMOVA for *O. lanceolata*, based on variation in ISSR loci.

Source of variations	Df	SS	MS	Est. Var.	%
Among Populations	6	1241.291	206.882	7.698	37.9
Within Populations	171	2146.889	12.555	12.555	62.1
Total	177	3388.180		20.253	100

Table 4. 15: Coefficient of gene differentiation across different loci of *O. lanceolata*.

Primer	No. of loci	Ht	Hs	Gst
Primer 808	13	0.3911	0.2039	0.4714
Primer 809	16	0.3559	0.2713	0.2384
Primer 810	15	0.3605	0.2230	0.3680
Primer 811	15	0.4523	0.2884	0.3636
Primer 818	20	0.3369	0.2431	0.2696
Primer 825	17	0.3788	0.2487	0.3283
Mean	**96**	**0.3765**	**0.2474**	**0.3429**

Ht- gene diversity, Hs- average gene diversity within subpopulations, Gst-
Coefficient of gene differentiation

The dendrogram discriminated the seven populations into two broad groups (i.e. A and B) mainly according to genetic similarities as opposed to geographic grouping. Cluster A consisted of Wundanyi, Kitui, Gwasii, Mau, Mt. Elgon and Meru populations and cluster B consisted of Baringo population only (Figure 4.6).
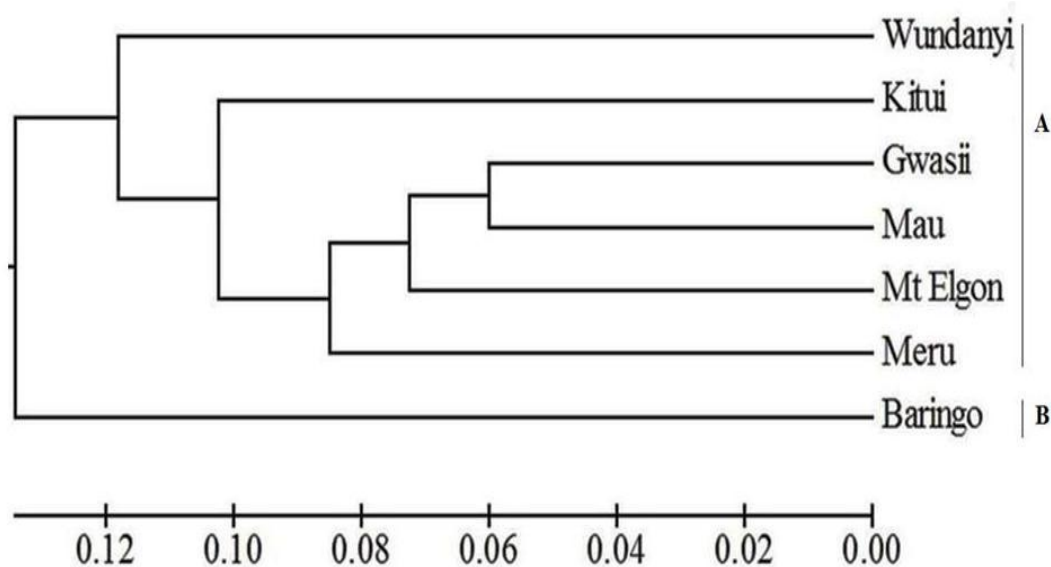
Figure 4. 6: Dendrogram based on Nei unbiased genetic distance from 178 individuals.

The relationship among individuals and populations was as summarized in PCoA Figure 4.7. This PCoA indicated that there are two clusters with overlap among six populations. Baringo population is distinctively separate from the rest. Wundanyi population is restricted on one side of x-axis in the PCoA but overlaps with Meru, Mt. Elgon and Gwasii. Kitui population is restricted to the other side of the X-axis of the PCoA but overlaps with Mau, Mt. Elgon and Gwasii populations. Wundanyi, Kitui and Baringo populations were distinct from one another and did not overlap among themselves.
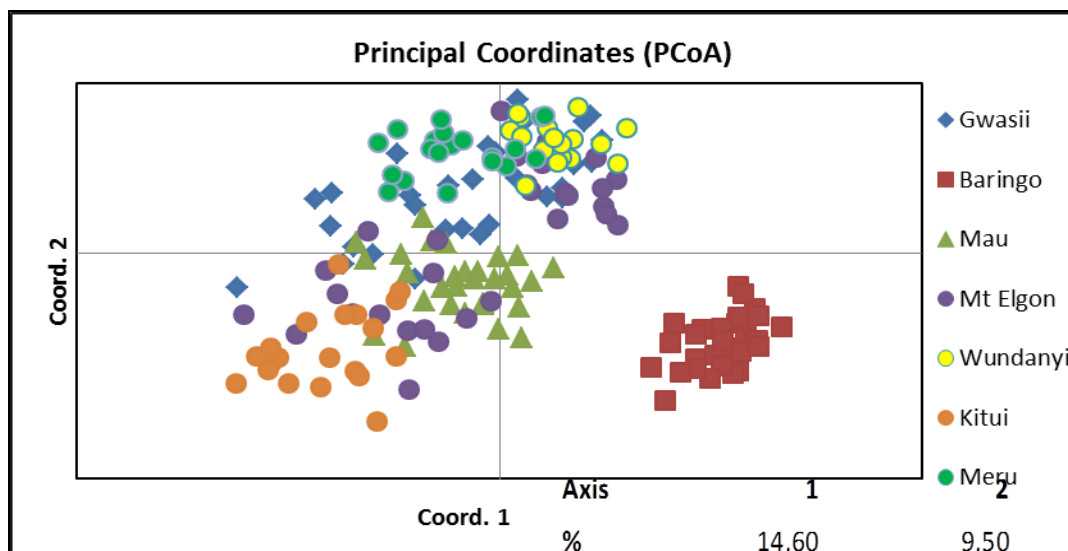
Figure 4. 7: The principle coordinates analysis of 178 *O. lanceolata* **individuals.**

The pairwise matrix of Nei's 1973 unbiased measure of genetic distance among the seven populations of *O. lanceolata* is shown in Table 4.16. Genetic distance between the 7 populations ranged from 0.124 between Gwasii and Mau populations to 0.310 between Baringo and Kitui populations with overall populations mean genetic distance of 0.2241. Mau, Mt. Elgon and Gwasii populations appeared to be closer to each other with genetic distance ranging from 0.124 to 0.129. Baringo population emerged as being the most differentiated from the rest of the populations with genetic distance ranging from 0.234 to 0.310. It was followed with Wundanyi with distance ranging from 0.216 to 0.310.

Table 4. 16: Nei's unbiased genetic distance of *O. lanceolata* **populations in Kenya**

Population	Gwasii	Baringo	Mt Elgon	Mau	Wundanyi	Kitui	Meru
Gwasii	0						
Baringo	0.254	0					
Mt Elgon	0.129	0.259	0				
Mau	0.124	0.234	0.158	0			
Wundanyi	0.216	0.310	0.241	0.222	0		
Kitui	0.189	0.295	0.212	0.175	0.294	0	
Meru	0.147	0.290	0.183	0.185	0.212	0.245	0
Mean	0.1765	0.2776	0.1985	0.194	0.253	0.245	**0.2241**

CHAPTER FIVE

DISCUSSION

5.1 Phenology of *O. lanceolata*

The first flowering cycle of *O. lanceolata* in some populations Kenyan populations began in late January while others were in early February. The second phase also varied from population to population as some begun in late September while other were in early October. Fruit formation stated in March while fruit maturation commenced in April/May and ripening was within April/May months. In the second cycle, fruiting stated in October/November and ripening within November/December. There was no distinct delineation of the reproductive stages from one to the other successive developmental stages of *O. lanceolata* in Kabarnet and Kitui populations. Observations by the local communities revealed that months in which flowering occurs tend to vary yearly depending on the prevailing weather conditions. Previous studies of flowering and fruiting of other species in Santalaceae family for instance *S. album* in Java China, and Indonesia revealed that flowering time occurs from June to October (Ma *et al.,* 2005). In India, flowering of *S. album* occurs from July to September with continuous sporadic flowering occurring in other months of the year (Baskorowati, 2011). This could be influenced by variation in ecological factors and also the genetic differences.

The *O. lanceolata* flowering period took 109±16 day to complete one cycle. The phenology of male and female trees differed significantly in both Kabarnet and Kitui populations. Female bud initiation and flower bud opening was about 2 to 3 days before the male, which is in agreement with similar results obtained in a study on Tanzanian populations of *O. lanceolata* by Mwang'ingo *et al.* (2007). In the Kenyan populations, male flowers opened late and withered 3 to 4 days later after female

flowers had withered. The withering of male flowers marked the end of flowering events in male trees, but the beginning of fruit initiation in female trees. The withering of male flowers days after the female the flowers may have been a reproductive strategy of *O. lanceolata* to ensure that there is successive maximum fertilization of the female flowers as also suggested by Ayasse and Arroyo (2011) in their study on pollination and reproductive biology of plants. However, *O. lanceolata* species efficiency in utilization of the pollen grains produced may have been affected by the fact that female trees flowered earlier than male trees. In addition, the Kenya population of *O. lanceolata* might be facing a challenge of few pollinators available for pollen grain transfer. This was also observed by Mwang'ingo, *et al.* (2007) in the Tanzanian populations. The dearth of pollinators observed in the populations of *O. lanceolata* during the study suggests that there are barriers to pollen transfer between trees and therefore low rate of cross-pollination in these populations. This could be contributing to the observed low regeneration.

Studies reveal that species in the same genera will tend to have similar flowering patterns especially if they are growing in environments with similar ecological conditions (Kochmer and Handel, 1986). A study on constraints and competition on flowering phenology in Japan and, North and South Carolina in the USA showed that most populations of the same genera growing in similar ecological conditions had similar flowering patterns (Kochmer and Handel, 1986). In this case, the ecological conditions in the Tanzanian populations (annual rainfall, temperatures, soil characteristics and species hosts) are similar to the prevailing ecological conditions in the Kenyan sites (Mwang'ingo *et al.,* 2007; Mukonyi *et al*., 2011). This therefore suggests that the flowering phenology of *O. lanceolata* in Kenya and Tanzania are similar. The populations therefore experience same reproductive processes. Slight

variations in the flowering phenology might be as a result of slight variations in prevailing climatic conditions and presence of pollinators (Sakai, 2001). This might affect flower initiation and end dates or length of phenological events.

The Kenyan *O. lanceolata* flower buds took 5 to 7 day to open into active flowers from initiation. The results on flower bud opening to active flowers did not deviate far from those observed from the study by Mwang'ingo *et al.* (2007) in Tanzanian populations. Studies of Australian species have reported variations in bud opening; for instance, *S. lanceolatum* flower opening takes 12 to 24 hour while the *S. austrocaledonicum* Vieill have flower buds that open and close within 24 to 48 hours (Page and Tate, 2010). These are shorter duration of flower buds opening as compared to the *O. lanceolata* species.

5.2 Reproductive biology of *O. lanceolata*

The flowers of *O. lanceolata* were found within axillary panicles consisting of not more than 5 flowers per inflorescence in female and about 5 to 11 flowers in male trees. A study by Page and Tate (2010) on the breeding behaviour self-, intra and inter-specific cross-compatibility of *S. lanceolatum*, a species in the Santalaceae family in Australia, observed that the number of flowers per inflorescence on *S. austrocaledonicum* and *S. album* trees ranged from 20 to 40 flowers. The floral morphology of *S. album*, *S. austrocaledonicum and S. lanceolatum* were observed to be similar (Page and Tate, 2010). In this study, flowers of *O. lanceolata* in this study were 4.6 mm long. However, Page and Tate (2010) observed that *S. lanceolatum and S. austrocaledonicum* flowers were approximately 3.0 mm long while *S. album* flowers were 2.5 mm long, which was comparable to *O. lanceolata* in this study. The stigma of the sandalwood trees as studied by Page and Tate (2010) had 3 to 4 lobes as recorded on the *O. lanceolata* in this study. *Osyris lanceolata* in this study had an inferior ovary

that swells to become a single seeded drupe after fertilization just like *S. austrocaledonicum, S. album* and *S. lanceolatum* (Page and Tate, 2010).

The results of reproductive biology showed that assisted pollination significantly increased the quantity of viable seeds formed to 39.7% compared to natural pollination that resulted to 26.7% full embryo seeds. This indicates that the female flowers were receiving low pollen grains in the natural pollination, hence reducing pollination success in the natural populations (Setsuko *et al.,* 2013). The pollen grains produced were either low in quantity, few pollinators were present or the pollinators were limited in the distance they can cover from tree to tree hence less flowers being fertilized. A study by Ma *et al.* (2006) on fragmented populations of *S. album* in the family Santalaceae in India found that 2 to 3% of open pollinated *S. album* flowers set seed compared to 26.7% in this study. The percentage of full embryo seed set in *S. album* increased to 14% during artificial pollinations (Ma *et al.,* 2005) while in this study, artificial pollination increased the percentage of seed set with full embryo to 40%. This suggests that the species is mainly outcrossing. *Santalum lanceolata* in Australia is also an outcrossing species (Page and Tate, 2010). This suggests that seed setting in members of Santalaceae family can be improved by using controlled pollination.

Few fruits formed (1.5 %) from flowers in which anthers had been removed. However, the seeds that formed in this case were not viable. This therefore suggests that this species has ability for the development of parthenocarpic fruits but not full embryo seeds. *Santalum album* and *S. lanceolatum* in China and Queensland had no seeds set on flowers isolated from open pollination by bags (Ma *et al.,* 2005). Most of the seeds formed in *O. lanceolata* natural pollinations in Kenya had poorly developed embryo or damaged embryo. A reproductive biology study by Sindhu and Ananthapadmanabho

(1996), on *S. album* shows that self-crosses yielded (1.3 %) viable seed on isolated trees whose flowers were restricted by pollination bags. In this study, pollen gains from the anthers on the female flowers were not applied on the female flower stigma.

During the study, pollinators of the species were not observed. This may indicate that the pollinators foraging pattern have changed due to reduction of the individuals in the populations or the timing was not in harmony with pollinators foraging time. In *S. lanceolatum*, foraging pattern of pollinators was not affected by change in the temperature during the day (Page and Tate, 2010). Page and Tate (2010) also reported that the amount of pollinators was high when the flower intensity was high. This was not observed in *O. lanceolata* population in Kenya. As observed in *S. lanceolatum,* most species in the family Santalaceae showed that though the species were mainly outcrossing, there is some degree of selfing (Page and Tate, 2010). This could be attributed to low number of pollinators or extent of the populations' fragmentation in the regions. White *et al.*, (1999) reported that the ease and diverse pollen grain movement within and among populations, favours gene flow and hence high genetic variability. This is not observed in *O. lanceolata* population in Kenya. The seeds of *O. lanceolata* have a hard seed coat, relatively large in size and heavy with average length of 10.43 mm, average diameter of 9.93 mm and weight per 1000 seed of 116.5 g. This might be the reason for the lack of dispersal agents in the wild. This is not the case with the Australian sandalwood (*S. spicatum*) populations. The presence of *Bettongia pencillata*-brush tailed bettong (small mammals) help in seed dispersal over long distances to check on dispersal restriction (Murphy and Garkaklis, 2003; Murphy *et al.,* 2005). This is why there is regeneration observed on the edges of the forested areas with *S. spicatum* populations (Murphy *et al., 2005*).

5.3 Morphological variability of *O. lanceolata*

The scored means of morphological traits indicated that all the traits differed significantly at 95% level in all the populations except for height and stem count (*p=0.900* and *p=0.605* respectively). The variations in these traits might have been due to wide geographical ranges that characterize the variation in substrate, soils moisture availability, nutrients among other edaphic factors in the natural populations. Variations in ecological factors, temporal heterogeneity in precipitation or wide variety of host species or the varying levels of haustorial connectivity due to its hemi-parasitism that influence accessibility to water and nutrients for the nourishment of the species compounds this environmental effect (Parker, 1991; Schuster *et al.,* 1994; Mahmood *et al.,* 2005; Chiveu *et al.,* 2009; Jones *et al*., 2009; Athena *et al.,* 2014). A wide geographical range causes plants to develop adaptive morphological modification to fit in their habitat which plays a key role in species evolution (Dangasuk and Panetsos, 2004). Adaptive differentiations significantly influence speciation, resulting to formation of locally adapted plant populations that are morphologically variable in their wide range of habitats (Elberse *et al.,* 2003). In such a case, the morphological traits variability may be genetically influenced or induced by the environment (Chiveu *et al.,* 2009). The natural distribution of *O. lanceolata* in Kenya shows that it occurs in a wide range of soil types (Maundu and Tengnas, 2005). *Osyris lanceolata* distribution in the humid highlands positively correlated with good moisture content, level of clay in soil and soil nitrogen, while in the dry lowlands, the species presence did not correlate with any of the edaphic variations (Mwang'ingo *et al*., 2010). This may suggest that genetic expression varies with variation in soil variables hence affecting the distribution of *O. lanceolata* in Kenya and that the morphological variability observed in *O. lanceolata* could be more of the genetic influence coupled with the

ecological factors. The low tree morphological variations could be because of the similarity in the ecological factors in the area the species in growing in Kenya.

Gwasii population was revealed as the most genetically diverse followed with Kabarnet and Marigat populations, and with Kitui population being the least. Duncan Multiple Range Test (DMRT) and Shannon-Weaver diversity index (*Hs*) grouped Kitui in the first cluster, Gwasii in the second, Kabarnet, and Marigat in the third cluster by all criteria, based on the most of the morphological parameters i.e. flower and fruit dimensions and seed weight. This was in agreement with the dendrogram derived from morphological traits that delineated Kabarnet and Marigat as one related group geographically. Gwasii and Kitui population were depicted as being distinct and far from each other geographically. The populations were grouped as western, Rift Valley and Eastern regions. A compareable study on morphological diversity of *Psidium guajava* L. trees in Kenya showed that the mean proportion of fruit weight was significantly higher (*p=0.003*) in Coast region (10.7%) and Western region (10.6 %) than from the Rift valley region (Chiveu *et al.,* 2016). Chiveu (2008) found similar regional clustering as observed in this study when analyzing the morphological diversity of *A. senegal* in Kenya. Studies based on nuclear microsatellites in *A. senegal*, reveal the similar grouping with Rift Valley remaining geographically distinct (Omondi *et al.,* 2010). This seems to indicate geographic gene flow barriers of tropical forest tree species in Rift Valley as was observed among Kabarnet and Marigat populations that reside in the Rift Valley, from the rest in this study.

Osyris lanceolata female trees were observed to be dimensionally larger than the male trees. It is argued that the female trees have developed mechanisms to compensate for extra energy that is required for reproduction and physiological activities, and one of these strategies is to have a large leaf area that assists in photosynthesis and

accumulation of extra energy for its reproductive processes (Wallace and Rundel, 1979; Popp and Reinartz, 1988; Mwang'ingo *et al*., 2010). With large morphological dimensions, female trees are able to accommodate extra load of flowers and fruits (Wallace and Rundel, 1979; Popp and Reinartz, 1988; Mwang'ingo *et al*., 2010).

Kenyan *O. lanceolata* was observed to be a multi-stemmed tree but the number of stems produced per tree did not vary significantly between populations. The number of stems per tree in each population ranged from 1 to 5 with an average number of stems of 2.4 produced per tree. A similar study in Tanzania revealed that *O. lanceolata* number of stems produced per tree varied significantly between populations at 99% level. The stems per tree in Tanzanian populations ranged from 2 to 6 with a mean number of stems per tree of 3 stems (Mwang'ingo *et al.,* 2003). Trees in Gwasii had the least stem count indicating that the trees concentrate on secondary growth rather than production of many stems hence reach large dbh as depicted in this study. The inaccessibility to *O. lanceolata* in Gwasii population and its favorable growing physical environment could be contributing to rapid secondary growth reaching to high heights and broad girth, which agrees with Murphy *et al.* (2005) report on forest accessibility and availability of large girth trees. Most trees in Kabarnet, Marigat and Kitui populations had their dbh contributed by more than 3 leader-stems, while trees in Gwasii had few leader stems per tree. *Osyris lanceolata* population in Gwasii grows on the windward side of Gwasii Hills (Westside slope facing L. Victoria). The hill side is generally wetter than Kabarnet, Marigat and Kitui; with a mean annual rainfall of about 1100 mm that is well distributed all year round (Kibwage, 2007). The availability of adequate moisture and nutrients in fertile gravely volcanic clay soils (Okalebo *et al.,* 2002) in the region could be the reason for the enormous *O. lanceolata* trees growth. The population is also on hilly areas which is inaccessible by many

poachers and so is not exposed to illegal harvesting. Therefore, many trees in Gwasii had been growing in their habitat for a long enough to achieve large sizes.

The *O. lanceolata* trees in Kitui were generally small compared to the other populations. This could be attributed to the environmental stress due to low annual rainfall and high temperatures; thence trees adapt a mechanism to reduce on detrimental effects of stress (NEMA, 2009). The soil type in the region is sandy loam volcanic soils with low organic matter (Okalebo *et al.,* 2002). This indicated that trees in Kitui were experiencing harsh environment thus having little resources to enable them grow into big trees with larger flowers and hence lower values of sessile traits like flower and fruits sizes (Athena *et al.,* 2014). This could be reason it was clustered alone based on flower length and fruit size traits. Male flowers were generally smaller than female flowers in Kitui.

As was observed, trees that grew in the light shade had big flowers compared to those growing in the open field. Kabarnet and Gwasii trees were growing on the edges of forested areas with large trees like *J. procera* and *Podocarpus latifolia* Thunb, while Marigat trees had slight shading due to predominantly large Acacia trees, but the Kitui trees were mainly in the open field, along roads and on farm boundaries. A lot of agricultural cultivation to feed the ever growing human population and long drought experienced in Kitui region has reduced the vegetation cover to very low levels (NEMA, 2009), thus exposing the trees to much light, although influence of shade on the growth behaviour of *O. lanceolata* was not the main focus in this study.

Chaisurisri *et al.* (1992) correlated the seed size with the extreme environmental drought and reported that the seed size increase with increase in level of drought. This increase was up to a certain level when seed sizes reduced with acute low moisture

conditions. The large seeds in Kabarnet, Marigat and Gwasii could offer enough food for germination and hence may yield high survival rate of seedlings, though it is also affected by the host preference. The viability and survival rate of *O. lanceolata* seeds from these populations has not yet been done. Kitui had the smallest seed sizes, indicating low levels of moisture content in the soil thus conferring an adaptive survival mechanism for high germination capacity. Kitui experiences high rainfall just as Baringo but it occurs very erratically and is poorly distributed all year round (Johansson and Svensson, 2002). Kitui is also dry most part of the year with few months experiencing rainfall.

Osyris lanceolata height correlated positively with dbh and crown diameter (r=0.510 and r=0.533) whereas dbh correlated with crown diameter at *p<0.01*. This study suggests that dbh and crown diameter of trees will increase as the trees achieve higher heights. This is common with most tropical tree species growing in their natural habitats (Wonn and O'Hara, 2001). A tree that grows tall without expanding in diameter and crown diameter is usually shaded and could be struggling to survive the pressure of suppression by higher crown trees (Sterck and Bongers, 1998). Flower traits significantly correlated positively with fruit traits. Big fruits therefore set from large size flowers. Stem counts and weights of the seeds were not associated with the *O. lanceolata* tree size in the four populations in Kenya.

5.4 Genetic variation of *O. lanceolata* among seven Kenyan populations

The number of effective alleles (*Ne*) observed in this study ranged from 1.229 in Wundanyi to 1.575 in Gwasii with a mean of 1.430 which was lower than the *Ne* (1.86) observed for *Acacia senegal* L. using ISSR markers, a tropical tree growing in the same habitat with *O. lanceolata* (Chiveu *et al.,* 2009). It was also less than *Ne* (1.95) obtained for *S. spicatum*, a species belonging to the same family as *O.*

lanceolata, using RFLP in Western Australia (Byrne *et al.,* 2003). However, the results in this study were comparable to other tropical tree species such as *Jatropha curcas* L. (*Ne*=1.407) using ISSR markers (Gupta *et al.,* 2008), *Michelia coriacea* (Magnoliaceae) (*Ne* =1.460) using ISSR markers (Zhao *et al.,* 2012) which showed high genetic diversity. The closeness of the number of observed alleles (*Na* =1.429) and the number of expected alleles (*Ne* =1.430) indicted that the populations are in a state of genetic equilibrium (Chamberlain *et al.,* 1996). This indicates the fact that the major factors that endanger the existence of *O. lanceolata* in Kenya are ecological and anthropogenic indicating habitat specialization and fragmentation and over-exploitation rather than genetic (Wu *et al.* 2014).

The Shannon Information Index *(I)* which is a measure of the degree of variation within populations in this study ranged from 0.232 (Kitui) to 0.446 (Gwasii) with a mean of 0.365 for the species reflecting a moderately high genetic diversity. This mean is comparable to the 0.374 obtained in *J. curcas* populations in India, based on ISSR markers, which was considered high (Gupta *et al.,* 2008). On the contrarily, it was less than the 0.436 for *M. coriacea* (Zhao *et al.,* 2012), 0.460 for *Argania spinosa* in Morocco (Naima *et al.,* 2015) and 0.430 for *Pongamia pinnata* a tropical tree species in Brazil (Rout *et al.,* 2009). The results of Shannon Information Index *(I)* in this study concurred with AMOVA results of 62% within population variation, which was moderately high.

The unbiased genetic diversity index (*UHe*) in this study ranged from 0.175 in Wundanyi to 0.321 in Gwasii with a mean *UHe* of 0.253, which means that there is was random mating among the populations. This was lower than 0.270 obtained in tropical tree species such as *A .senegal* in Kenya using ISSR markers (Chiveu *et al.,* 2009). It was comparable to the 0.245 found in *J. curcas* populations in India using

ISSR markers (Gupta *et al.,* 2008). Zhao *et al* (2012) obtained higher value (*UHe* = 0.283) in *M. coriacea* in China using ISSR markers, which showed high levels of genetic diversity. Playford *et al* (1993) found comparatively lower *UHe*= 0.208 in *Acacia melanoxylon* R. Br. I.

The percent polymorphic loci *(P)* obtained in this study ranged from Wundanyi (51.04%) to Gwasii (82.29%) with an overall mean of 64.73%, which is comparatively similar to 63.5% found in *Santalum spicatum* in Australia, a tree species of the same family (Byrne *et al.,* 2003), but lower than 87.4% for *A. senegal* in Kenya (Chiveu *et al.,* 2009), 96.36% for *M. coriacea* in China (Zhao *et al.,* 2012), 76.54% for *J. curcas* genotypes in India (Gupta *et al.,* 2008), 94.34% for *Pongamia pinnata* in Brazil (Rout *et al.,* 2009), and 79.54% for *Argania spinosa* in Morocco (Naima *et al.*, 2015) using ISSR markers. It could be concluded that comparatively, the level of polymorphism obtained in this study at species level is moderately high. This was supported by the fact that out of the seven populations only two had high polymorphism (Gwasii 82.29% and Mt. Elgon 78.13%) and the remaining five had moderate polymorphism ranging from 51.04% in Wundanyi to 62.50% in Mau.

The distribution of the genetic indices values among the populations does not show a clear relationship with the environmental parameters such as; altitude, mean annual rainfall and mean annual temperatures. This is in agreement with Hamrick and Godt (1989) that geographic range was not significantly associated with variation among populations or population differentiation (*Gst*). The pattern of genetic variation as portrayed by the genetic indices seems to be influenced by the population sizes. Gwasii and Mt. Elgon had large and high density populations of *O. lanceolata*, while Wundanyi had the smallest population with scattered individual trees to the extent that only 18 trees were sampled from the target of 30. A similar scenario as of Wundanyi

was observed in Kitui and Meru where only 20 trees were sampled from each population, short of the targeted 30 per population. Baringo and Mau populations were intermediate between Mt. Elgon and Kitui. This is in perfect agreement with Hamrick (1984) that tropical tree populations especially those that occur at high density maintain a high level of genetic diversity. Low effective population sizes allow the loss of genetic diversity due to genetic drift (Hamrick, 1984; Wu *et al.,* 2014).

5.5. Population differentiation of *O. lanceolata*

The genetic distance *(D)* of Nei (1978) showed Baringo population as being the furthest genetically from the rest of the population with a mean genetic distance of 0.278 followed by Wundanyi and Kitui (0.253 and 0.245 respectively) while Gwasii population was depicted as having the least mean genetic distance of 0.177. Overall, the genetic distance has a mean of 0.224 which is high indicative of a high level of geographic separation among the seven populations of *O. lanceolata*. A long term logging impact model showed that logging, including selective logging, still resulted into a loss of alleles and genotypes and an increased genetic distance (Sebbenn *et al.,* 2008). This is in agreement with the status of *O. lanceolata* in Kenya, which has been indiscriminately over-exploited over the years.

The principle coordinates PCoA of 178 *O. lanceolata* individuals sampled showed that the first two axes accounted for 24.1% of the total variance. This was less than 40.1% obtained in *A. senegal* in which the 95 individuals sampled showed complete overlap with no clear differentiation (Chiveu *et al.,* 2009) and also less than the 58.47% obtained in endangered *M. coriacea* in China which showed low differentiation (Zhao *et al.,* 2012). This therefore confirms the presence of high differentiation in *O. lanceolata* which also showed Baringo as a population completely isolated from the rest though with high genetic diversity. Baringo population could be genetically and

geographically isolated from the rest since it is located deep in the Rift Valley, hence it could be suffering from barriers which limits seed and pollen dispersals and impedes gene exchange for several plant species, for instance African acacias (Ruiz-Guajardo *et al.,* 2010) and *Prunus africana* (Kadu *et al.,* 2013). This coupled with the small population size and fragmentation indicates that Baringo could be affected by genetic drift, thus forming an ecotype (Burley *et al.,* 1986; Zang *et al.,* 2009). Wundanyi, Kitui and Mau occupies specific areas in the PCoA cluster diagram with minimal overlaps between them indicative of the high differentiation in the species, while only Gwasii and Mt. Elgon which showed highest genetic diversity were spread out extensively across the two axes and overlapped with the rest of the populations except Baringo.

Based on Analysis of molecular variance (AMOVA) most of the genetic variation in *O. lanceolata* resides within population (62%). This is consistent with findings of Loveless and Hamrick, (1984) for predominantly outcrossing and entomophilous plant species and with results obtained in other tropical tree species such as *A. senegal* 95% (Chiveu, *et al.,* 2009), *Acacia totilis* 76% (Olong'otie, 1992) and *J. curcas* 69% (Gupta *et al.,* 2008). However, the 62% observed for *O. lanceolata* is less than the 95% for *A. senegal*, 76% for *A. tortilis* (Olong'otie, 1992) and *J. curcas* 69% (Gupta *et al.*, 2008) and this could be attributed to the small population sizes which consist of scattered individual trees in *O. lanceolata*, which could result in plasticity of the mating system depicting inbreeding to a certain level hence detrimental to within population genetic diversity (Zhang *et al.,* 2009). Similarly, the coefficient of differentiation *Gst* of 0.3429 observed in this study suggests that 34% of the variation resides among populations and high variation resides within population (66%) which concurs very well with the AMOVA results.

The coefficient of differentiation *Gst* of 0.3429 from this study is considerably higher than 0.0573 observed for *Acacia senegal* (Chiveu *et al.,* 2009) and 0.187 for *M. cariacea* (Zhao *et al.,* 2012). Overall, the results showed high genetic differentiation in Kenyan *O. lanceolata*. The reasons for higher levels of population differentiation in tropical species includes; small population sizes with lower densities, more widely scattered populations that reduce gene flow and increase genetic drift and greater spatial variation in the natural selection pressure (Bawa, 1986, Liengsiri *et al.,* 1995). Other factors associated with high degree of genetic differentiation among populations are mating system, evolutionary and life history (Hogbin and Peakal, 1999, Zhao *et al.,* 2012). Currently, most of the *O. lanceolata* population in Kenya are of small sizes, and consist of scattered individual trees on rocky and/or mountainous sites, which constitute barriers for gene flow between populations in *O. lance*olata (Dai *et al.,* 2013). In addition, habitat loss and fragmentation, over-exploitation of the species and introduction of exotic species in the natural environment where *O. lanceolata* occur, has created subdivision of the populations into smaller units and imposes barrier to gene flow thus leading to greater differentiation, as supported by Ledig (1992) and Dai *et al*. (2013). Compared to widespread taxa, many endangered species may become genetically differentiated because of their small population sizes (Zhao *et al.,* 2012). The rocky, mountainous and valley type of habitats where *O. lanceolata* is found in Kenya could provide barriers which hinders gene flow among populations which leads to genetic isolation, coupled with the small population sizes, fragmentation, genetic drift and greater differentiation is the ultimate result. This is consistent with the finding of Bottin *et al.* (2005) for *S. austrocaledonicum*.

5.6. Phylogenetic relationships in Kenyan *O. lancelata* populations

The dendrogram derived from Nei's unbiased genetic distance (Nei, 1978) in this study discriminated the *O .lanceolata* into two broad clusters, which seems to have been influenced by several factors such as; breeding systems, population sizes, gene flow, genetic drift, evolutionary history and natural selection but not geographic proximity. This concurred with the findings of Hamrick and Godt (1990) and is further supported by the fact that natural selection preserves and results in an increase in the number of genotypes most suited to a specific environment (Wright, 1976; Zobel and Talbert, 1984; and Chiveu *et al.,* 2009). Cluster *A* consists of Gwasii, Mau, Mt. Elgon, Meru, Wundanyi and Kitui populations. In this cluster, Wundanyi and Kitui populations occur at comparatively lower altitudes (1185.4 - 1319.9 masl), in conditions of low mean annual rainfall (650 – 775 mm) and high mean annual temperatures (23 – 25 °C). They are located in semiarid areas with smallest effective population sizes and incidentally contain the least genetic polymorphism (51.04 - 59.38%). Gwasii, Mau, Mt. Elgon and Meru populations in the same cluster occurs at high altitudes (1427.7 – 2288 masl) where there is high mean annual rainfall (1025 – 1440 mm) and comparatively lower mean annual temperatures (18.5 – 26 °C). These populations are located in the mountainous areas of Kenya, have denser populations and incidentally have the highest genetic polymorphism (59.38 – 82.29%). Cluster *B* consists of Baringo population alone. This population occurs at a high altitude (2040 masl), with the lowest mean annual rainfall (635 mm) and high mean annual temperature (25 °C). It is located within the Rift Valley, apparently physically isolated from the rest of the populations and had a moderately high genetic polymorphism (60.42%). The uniqueness of Baringo population was also been reported for *A. senegal* by Chiveu *et al*. (2008 and 2009) using RAPD and ISSR markers. Moran (1992) said that species

that grow in wide natural ranges, exhibit patterns of genetic sub-structuring between geographic regions, particularly where there are barriers to pollen and seed dispersal such as mountains, valleys or lakes. Similarly, the environmental diversity encountered within a species natural range also causes a species geographic variability (Zobel and Talbert, 1984).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

6.1.1 Phenology and reproductive biology of *O. lanceolata*

This study has shown that *O. lanceolata* in Kenya takes about 109 days for one entire flowering cycle in Kabarnet and Kitui populations. The species in Kenya has two flowering phases starting in late January and September in the first and second cycles and ends in June and December respectively. The study also showed that assisted pollination significantly increased the quantity of viable seeds produced by 40% compared to natural pollination that had about 27 %.

6.1.2 Morphological variability of *O. lanceolata*

Most of the morphological traits revealed low diversity with Gwasii showing the highest among the rest. The populations were delineated into three clusters i.e. Kitui population in the first cluster, Gwasii in the second and, Kabarnet and Marigat in the third cluster. Height, dbh and crown diameter parameters had varied clustering, indicating that sessile floral and fruit traits are the best in determining the genetic diversity of a species in its natural habitat. Gwasii population had large and taller trees with fewer branches, big flowers and fruits while Kitui had the smallest trees with many leader stems. High variability was observed in fruits traits.

6.1.3 Genetic diversity of *O. lanceolata*

Osyris lanceolata in Kenya showed moderately high genetic diversity. Gwasii population had the highest genetic diversity by all criteria followed by Mt. Elgon and least was Wundanyi with the rest being intermediate between them. Most of the genetic variations resided within than among populations. Baringo population was

completely isolated from the rest of the six populations due to Rift Valley barrier that seems to inhibit gene flow. The seven Kenyan populations of *O. lanceolata* were highly differentiated and not clustered according to geographical proximity but according to their genetic relationships into two clusters. Meru, Kitui, Wundanyi and Baringo were small and restricted populations and do not share alleles between them indicating a certain level of population isolation, reduced gene flow, genetic drift and possibly some level of inbreeding.

6.2 Recommendations

1. *In-situ* conservation is considered as the most effective method to conserve endangered species (Shen *et al.,* 2009); therefore Gwasii, Mau, Baringo and Mt. Elgon which had high levels of genetic diversity should be delineated and managed as *in-situ* conservation areas. There is need to introduce genotypes from distinct population of Gwasii, Mt. Elgon Mau and Baringo to help improve the male/female ratio of the species in the natural and hence allow for increase in their regeneration capacity and increase genetic diversity of the populations.

2. Since most of the genetic diversity resides within populations, it means that all the populations are important. This calls for protection and preservation of the other five populations from destruction and degradation to increase the populations sizes.

3. An *ex-situ* conservation program based on proper representative populations sampling methods should be initiated with selection targeting mainly Gwasii and Mt. Elgon populations as well as including some good trees from the rest of the populations. This will ensure conservation of full array of genetic diversity within the species and restoration of its full genetic diversity necessary for its survival, adaptation and evolution.

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APPENDICES

APPENDIX i: ANOVA for reproductive success among treatments

			Sum of Squares	df	Mean Square	F	Sig.
Fruiting * Treatment	Between Groups	(Combined)	57.867	2	28.933	40.062	.000
	Within Groups		19.500	27	.722		
	Total		77.367	29			
Ripening * Treatment	Between Groups	(Combined)	64.867	2	32.433	47.335	.000
	Within Groups		18.500	27	.685		
	Total		83.367	29			
Full * Treatment	Between Groups	(Combined)	49.267	2	24.633	47.170	.000
	Within Groups		14.100	27	.522		
	Total		63.367	29			

APPENDIX ii: PCR thermocycler programme for ISSR primers

Cycle	$^{\circ}$C	Minutes	Stage
1	94	5	Hot start
40	94	30	Denaturisation
3	Range 45-47	45 sec	Annealining
4	72	2	Polymerization
5	72	10	Final extension

APPENDIX iii: DNA quanntification by Bio-photometer.

DNA Quantification

Bio-photometer(cuvets) Dilution factor dsDNA 2:100 TE

Sample No.	Gwasii Blank 0.00 µl	260/280	Baringo 0.00 µl	260/280	Meru 0.00 µl	260/280	Kitui 0.00 µl	260/280	Wundanyi 0.00 µl	260/280	Mt.Elgon 0.00 ng/µl	260/280	Mau 0.00 µl	260/280
1	68	1.02	238	0.85	716	1.16	400	1.02	144	1.44	399	1.07	42	1.52
2	17	0.81	546	1.12	822	1.34	50	1.29	165	1.39	457	1.16	64	1.42
3	29	0.97	248	1.42	705	1.32	99	1.08	351	1.45	678	0.93	43	1.46
4	100	1.11	205	1.84	1613	1.34	34	1.56	123	1.44	341	0.99	50	1.41
5	58	1.03	227	1.56	1492	1.29	34	1.77	87	1.58	326	1.04	38	1.4
6	46	1.01	141	1.84	920	1.22	18	1.64	161	1.39	201	1.04	39	1.64
7	154	1.11	227	1.67	676	1.25	16	1.56	218	1.32	747	1.16	52	1.42
8	104	1.05	485	1.61	552	1.49	32	1.47	496	1.43	426	1.13	44	1.48
9	95	1.04	482	1.58	410	1.43	25	1.54	711	1.23	308	1.07	6	1.05
10	346	1.09	424	1.39	611	1.44	37	1.42	176	1.4	249	1.46	21	1.6
11	46	1.03	369	1.32	320	1.52	22	1.68	208	1.44	55	1.07	13	1.9
12	259	1.06	229	1.1	887	1.34	31	1.54	218	1.47	45	0.81	115	1.28
13	175	1.10	219	1.36	1264	1.38	64	1.43	328	1.5	369	1.41	59	1.5
14	180	1.15	198	1.66	945	1.34	80	1.44	249	1.41	34	1.15	43	1.5
15	113	1.05	239	1.73	340	1.45	55	1.26	141	1.63	127	1.01	32	1.6
16	97	1.06	571	1.68	449	1.46	73	1.53	349	1.33	48	1.12	40	1.59
17	162	1.07	7249	1.07	1732	1.18	56	1.48	535	1.4	66	1.07	56	1.73
18	142	1.04	271	1.81	967	1.37	108	1.35	239	1.52	86	1.07	39	1.4
19	70	1.02	301	1.6	432	1.46	44	1.48			172	1.08	40	1.47
20	141	1.08	254	1.45	1281	1.19	50	1.38			68	0.92	58	1.42
21	24	0.89	299	1.25							72	1.04	48	1.4
22	43	1.01	212	1.2							209	1.08	50	1.49
23	25	0.96	244	1.37							153	1.27	50	1.31
24	21	0.97	663	1.14							153	1.27	39	1.5
25	19	0.67	557	1.14							368	1.03	51	1.41
26	61	1.16	208	1.4							216	1.24	60	1.24
27	49	1.01	231	1.46							316	1.23	58	1.25
28	28	0.62	284	1.46							280	1.21	65	1.74
29	29	1.00	207	1.2							470	1.08	41	1.64
30	30	0.61	397	1.49							328	1.02	34	1.52