ANALYSIS OF THE GENETIC STRUCTURE OF FIVE *Eucalyptus grandis* POPULATIONS AND GENERATION OF F1 PROGENIES FROM *E. grandis* × *E. urophylla*

BY

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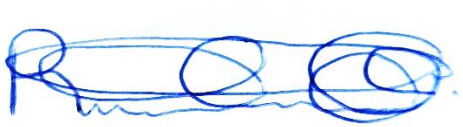
Signature  Date: 6th August 2022

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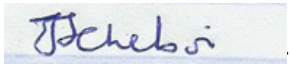
Signature…  ……... Date….10th August,2022……

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DEDICATION

I dedicate this thesis to my father, Eng. Fredrick Ouma Nyawalo and mother, Pamela Musundi Omondi for their great inspiration to take up this task and their continuous support throughout the study period.

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ABBREVIATIONS AND ACRONYMS

AEZ : Agro Ecological Zones

AFLP : Amplified Fragment Length Polymorphism

ANOVA : Analysis of Variance

AMOVA : Analysis of Molecular Variance

CPT : Candidate Plus Tree

CTAB : Cetyl Trimethyl Ammonium Bromide

CV : Coefficient of Variation

DBH : Diametre Breast Height

DNA : Deoxyribonucleic Acid

GC : Guanine-Cytosine

GCA : General Combining Ability

GMT : Greenwich Mean Time

ISSRs : Inter Simple Sequence Repeats

JOG : Joint Operation Graphics

KEFRI : Kenya Forestry Research Institute

LSD : Least Significant Differences

NCD : North Carolina Design

OD : Optical Density

PCoA : Principal Coordinate Analysis

PIC : Polymorphic Information Content

PCR : Polymerase Chain Reaction

PPG : Percentage of Pollen Germinated

RAPD : Randomly Amplified Polymorphic DNA

RFLP : Restriction Fragment Length Polymorphism

SCA : Specific Combining Ability

SSRs : Simple Sequence Repeats

UPMDC : Un-weighted Pair Group Method using Dissimilarity Coefficient

UTC : Coordinated Universal Time

UTM : Universal Transverse Mercator

GENERAL ABSTRACT

Eucalypts account for about 39600 ha of land in Kenya while other exotic species, *Cupressus* and *Pinus*, at 28,900 and 6,800 ha of land, respectively in the year 1999 though in 2009, the total area under eucalypts cultivation stood at 100, 000 ha. Nonetheless, in 2012, wood supply was estimated to be 31 million cubic meters while the demand was about 42 million cubic meters but, future forecasting shows that wood supply is set to increase to about 36 million cubic meters and a demand of about 51 million cubic meters by the year 2032. This demand could be addressed using planation forestry with trees such as eucalypts, but there is no active breeding program in Kenya due to insufficient information on genetic diversity and pollination techniques. The objectives of this study were to: i) determine the *E. grandis* genetic structure from selected candidate plus trees and their progenies using microsatellite markers from five main seed collection centers; and ii) generating F1 progenies from *E. grandis* and *E. urophylla* genotypes in Muguga, Kenya as a strategy of increasing genetic diversity. The genetic fingerprinting work, entailed genomic DNA extraction using CTAB method, DNA quality check and quantification using Nano drop nucleic acid analyzer, model Shimadzu Bio Spec-nano 206-26300-48, SSR screening and reconstitution using fluorescently labeled EMBRA primers, PCR amplification using Veriti™ 96-well thermal cycler and capillary electrophoresis by Applied Biosystems 3500 Genetic Analyzer. Data on allele frequency, allelic richness, gene diversity, heterozygosity and Polymorphic Information Content (PIC) then analyzed by Power Marker v3.2.5 Genetic distance matrices were generated by GenAlEx v6.5 and used for computing Principal Coordinates Analysis (PCoA) to visualize variations and similarities. Neighbor-joining trees were constructed using DARwin v6.0.21 where the resulting trees were visualized. Variation partitioning within and among group components was computed using Analysis of Molecular Variance (AMOVA) in GenAlEx v6.5 which facilitated estimation of broad sense heritability (H^2), standardized allelic patterns across different

families and populations and components of variance significance levels using 999 permutations. From analysis, results generally indicated high genetic diversity of 0.844 suggesting high genetic variability for possible exploitation in future breeding programs. The work on pollination involved collecting ripe flowers at anthesis stage of development, pollen extraction and examination of its characteristics, in-vitro pollen germination on liquid media to determine viability, pollen short-term storage at 4ºC, controlled pollination by conventional and one-stop techniques and lastly seed extraction at maturity. Data was also collected on various parameters including morphological traits of pollen and mother parents, pollen germination rate on nutrient agar medium, flower diameter and length of artificially and naturally pollinated flowers. From this analysis, a success rate of around 28.6% was realized indicating that more Eucalyptus breeding programs can be established using Kenyan germplasm. Conclusively, the study confirmed that there is need for maximum utilization of genotypes from the main seed collection zones for heterosis or targeting of specific traits, and also ratified that different breeding objectives such as fast growth and disease resistance can be achieved through controlled pollination.

Key words: Eucalypts, genetic diversity, controlled pollination

CHAPTER ONE: INTRODUCTION

1.1 Background Information

The Genus *Eucalyptus* is a very diverse and broad genus of flowering shrubs and trees in Myrtaceae family (Coppen, 2005). There are approximately 700 species of Eucalyptus majority of them being native to Australia with a small percentage in adjacent areas of Indonesia and New Guinea (Paine et al., 2011). There are fifteen species found outside Australia and nine of them are pre dominantly non-Australian (Blake, 2019; Davis et al., 1997).

Eucalypts are considered successful due to rapid burgeon rate, massive bioenergy production, ability to thrive in non-identical types of environments, creating bioenergy plantations and spiffing timber quality (Warren et al., 2009; Xu and Dell, 2002). They are also important in making repellents and bio-pesticides especially in United States of America (Batish et al., 2008), natural anti-microbial in Australia (Ali et al., 2010; Sadlon et al., 2010), as antiseptics across Australia (Barbosa et al., 2016), dental hygiene products in Japan (Nagata et al., 2008), solvents for removing sticky residue and grease in China (Coppen and John, 2002) and pulpwood (Nanko, 2005). Eucalypts are important ornamentals, timber, firewood, fence posts and in charcoal making (WWI, 2007).

Eucalypts mainly grow and generally spread in tropical and temperate agro-ecological zones including: Europe, America, Middle-East, Mediterranean regions, Africa, India and China subcontinent. The leading Eucalyptus growing countries across the world are China, India and Brazil (ICFRE, 2010; Liu and Li, 2010; Stape, 2002). South Africa has the biggest area dominated by Eucalyptus estates in Africa (Teketay, 2003).

In Kenya, about 100 Eucalyptus species were introduced in 1902 with the aim to identify fast growing species that would be used in construction of Kenya-Uganda railway line. In 2009, the total area under eucalyptus cultivation was estimated at approximately 100, 000 ha with 35,000 ha was under private ownership by large companies, 15,000 ha was under gazette forests while 50,000 ha of land was possessed by local authorities and different agrarians (KFS, 2009). The current area is not documented but it is presumably higher due to demand for commercial reasons, especially in private farms. In general, its cultivation is projected to rise owing to the mounting necessity for firewood, timber, carbon sequestration, renewable energy and mitigation of climate change (FAO, 2009; Binkley and Stape, 2004) owing to the reducing areas under natural forests and the need to produce wood using fast growing species.

Currently, there is a huge demand for wood and its products in Kenya but there is insufficient supply. In 2012, wood supply was estimated to be 31 million cubic meters while the demand was about 42 million cubic meters, (Cheboiwo and Githiomi, 2012). Forecasting future wood supply is set to increase to about 36 million cubic meters and a demand of about 51 million cubic meters by the year 2032 (Cheboiwo and Githiomi, 2012). This demand could be addressed using natural or plantation forests but former is overexploited due to high deforestation. Plantation forestry is the other option, which includes use of indigenous and exotic species but the former takes longer to mature (Oballa et al., 2010). For fast establishment to deal with the rising demand for wood and its merchandises, exotic plantation species are widely grown.

There are three main exotic species used for plantation forestry in Kenya including *Eucalyptus*, *Cupressus* and *Pinus* species produced on 39600, 28,900 and 6,800 ha of land, respectively (KFDP, 1999). Eucalyptus improvement was selected based on its high production in Kenya and relatively easy manipulation of flowers for controlled pollination compared to the other two.

1.2 Statement of the problem

Many introductions of Eucalypts were made to Kenya in 1902 but over time, more introductions have also been made aimed at increasing the genetic base of the germplasm where *E. grandis* and *E. urophylla* were among them. There are few known pure Eucalyptus stands that serve as seed collection centres for commercial purposes. This is due to high logging of public plantations partly from rotation period ranging 18-25 years (Oballa et al., 2010), to deforestation for commercial gain or urbanization. Eucalypts are outcrossing species (Potts, 2004) and seed collected could be affected by contamination from neighboring eucalyptus stands but there is no documentation on the probable level of contamination that would affect seed quality of *E. grandis*. This is because, the seed collected after each flowering season could be a mixture of species. If progeny seeds from these pure stands are mixed with other Eucalyptus species, they affect quality and yield of seeds sold to farmers and other stakeholders hence creating a need to determine the genetic structure of *E. grandis* from the pure stands which serve as the main seed collection centres.

On the other hand, there is no *Eucalyptus spp.* hybridization program in Kenya although it is important. This is so because Kenya has previously introduced hybrids from South Africa but they did not perform better than pure species from the GEI studies conducted (Retief and Stanger, 2009; Wamalwa et al., 2007). Artificial or controlled pollination is one method to increase diversity but eucalyptus trees take at least 24 months to start flowering followed by 6-12 months for seed development (Potts, 2004) and since they are cross pollinated, targeted controlled is required to incorporate superior qualities, which is a deliberate effort needed but lacking in natural pollination. Therefore, this confirms the need to carry out controlled pollination on *Eucalyptus spp*. in Kenya as a way of increasing genetic diversity.

1.3 Justification of the study

The differences in genetic structure is the key driver of genetic variations within and between species in an ecosystem (Vellen and Geber, 2005). In plantation forestry, *E. grandis* is one of the main species introduced to Kenya but its outcrossing nature may compromise seed quality and yield sold to farmers. Eucalypts are reported to have a limited genetic structure (Grattapaglia and Kirst, 2008) hence restraining genetic improvement aimed at enhancing breeding programs. Examination of genetic structure in *E. grandis* was previously done on Kenyan genotypes using five ISSRs (Okun et al., 2008) but the numbers were too few to draw a conclusive report. This study aimed at genotyping superior performing trees from five main *E. grandis* seed collection stands using 22 SSR markers (Butler, 2017) in order to obtain comprehensive genetic structure and differentiation. Since Eucalypts have a low population structure (Grattapaglia and Kirst, 2008), other methods like controlled pollination have been reported to increase genetic diversity (Potts and Dungey, 2004) but this has not been done in Kenya before but in other countries with different species as corresponding controlled pollination partners (Retief and Stanger, 2009; Bison et al., 2006; Potts and Dungey, 2004). The F1 progenies developed in those countries have resulted to better performing trees with different traits. For example, *E. grandis* is vulnerable to fungal infections like cankers in tropics but their F1s with compatible tree partners such as *E. urophylla* have facilitated development of superior progenies which are fast-growing and disease resistant (White et al., 2007). For breeding efforts to develop superior germplasm in Kenya, similar protocols were used to perform controlled pollination to determine the level of success on Kenyan germplasm for the sake of future Eucalyptus species improvement programs.

1.4 Objectives of the study

1.4.1 Overall Objective

- To contribute on broadening genetic composition and controlled pollination knowledge of Kenyan *E. grandis* and *E. urophylla* genotypes to enhance future breeding programs.

1.4.2 Specific Objectives

1. To determine *E. grandis* genetic structure from selected candidate plus trees and their progenies using microsatellite markers from five main seed collection centres.

2. To generate F1 progenies from *E. grandis* and *E. urophylla* genotypes in Muguga, Kenya as a strategy of increasing genetic diversity.

CHAPTER TWO: LITERATURE REVIEW

2.1 Evolution, Origin and Distribution of Eucalyptus species

Eucalypts are presumed to have evolved from rainforest precursors in response to various changes in soils, climate and landscapes. Most Eucalyptus species originated from Australia but nine are predominantly non Australian which include: *E. urophylla* native to the Lesser Sunda Islands of Indonesia, *E. confertiflora* native to East and West Papua New Guinea, *E. alba* native to Flores Island in Indonesia, *E. deglupta* native to the Philippines, Indonesia and Papua New Guinea, *E. pellita* native to West Papua, *E. brassiana* native to Woroi and Wipim in Papua New Guinea, *E. tereticornis* and *E. leptopheleb* innate to Papua New Guinea and *E. polycarpa* innate to the northern parts of Mexico and southwestern regions of United States (Blake, 2019; Davis et al., 1997; Srivastava, 1996).

Outside its original habitat, Eucalyptus was first cultivated in Portugal about 400 years ago. Over time, it became widely distributed across Latin America, Europe, Asia and Africa. The distribution resulted to over 10 million ha within the tropics (FAO, 2009).

Eucalypts grow naturally at latitude of 7ºN and 43ºS and this essentially explains why it is well adapted to a broad range of climatic conditions, product uses, management systems and different type of sites (Eldridge et al., 1993). In Kenya, the main cultivation areas for Eucalypts are Rift Valley, Western, Coast, Eastern and Central regions (Oballa et al., 2010).

2.2 Botany and genetics of Eucalyptus

Eucalypts originate from a mutual antecedent known as 52 MYA (Thornhill et al., 2015) with similar chromosome denoted as $n = 11$ (Crisp et al., 2011), and this is highly maintained across many Myrtaceae species (Grattapaglia et al., 2012). Eucalypts generally have sleek, rugged, tough or lanky bark leaves with oil glands (Boland et al., 2006). Their petals and sepals are fused to form a cap like structure known as operculum over the stamens that is merged with the receptacle

(Boland et al., 2006). The resulting fruit formed after fertilization is a woody capsule ordinarily known as a gumnut (Boland et al., 2006).

Eucalypts have multiple unique traits that all play a role in its dominance. These include high levels of heat and drought tolerance, ability to quickly coppice after widespread fires and ability to deploy effective mechanism to defend themselves against attack by herbivores (Boland et al., 2006).

2.3 Current uses of Eucalyptus species

Forest plantations have become very critical source in terms of supplying vital renewable energy resources due to escalating and immense pressure on indigenous forests from an expanding population globally (Oliveira, 2018). Eucalypts are mainly cultivated for commercial purposes escalating demand for timber and its products (Turnball, 1999). Eucalypts are economically significant and a key cash crop in Peru where locals are low income earners (Luzar, 2007). The trees are also fast growing species to meet the short-term wood supply in many countries (Luzar, 2007). Other uses include ornamentals and pulpwood production, charcoal, fencing posts and cellulose extraction for making biofuels (Maundu and Tengnans, 2005). Eucalypts tremendous fast growth trait makes them suitable windbreakers and agents of minimizing soil erosion and degradation (Luzar, 2007). Eucalypts oil is used as industrial solvent, antiseptics, deodorants and mosquito repellents (Fradin et al., 2009).

In Kenya, eucalypts are mostly used for construction and fuel purposes (Maundu and Tengnans, 2005). The tree is also used for pulpwood, plywood, harvesting of essential Eucalyptus oil and fencing (Kituyi et al., 2001). Bigger and mature trees are harvested for the purposes of utility while smaller immature trees are harvested for use in the construction industry and of lately for furniture making purposes (Maundu and Tengnans, 2005).

2.4 Eucalyptus Species grown in Kenya

The major Eucalyptus spp. cultivated in Kenya include; *E. grandis, E. globulus, E. saligna,* E. *camaldulensis* and *E. urophylla*. Other species cultivated on a smaller scale include: *E. paniculata*, *E. regnans*, *E. maculata* and *E. citriodora*. Eucalyptus hybrids and clones are now cultivated extensively in Kenya (RELMA, 2006; Muchiri et al., 2005) but the performance is wanting.

2.4.1 Eucalyptus camaldulensis

Eucalyptus camaldulensis has Australian mainland as its origin, capable of attaining heights of about 40.5m and a diametre of 2.5m (Oballa et al., 2010). Its branches spread widely with flaky bark. The best growth performance is obtained at lower elevations of below 1400m above sea level with 600-1000 mm of rainfall per annum. This species can thrive in areas with prolonged dry seasons and poor saline soils (Oballa et al., 2010).

2.4.2 *Eucalyptus saligna*

It is a tall tree capable of achieving heights of 40-50m and a diametre of about 1.5m (Oballa et al., 2010). This Eucalyptus spp. grows at an altitude ranging from 1600-2500m above sea level (Maundu and Tengnas, 2005). The growth of this species is faster than that of *E. grandis* under these climatic conditions.

2.4.3 *Eucalyptus globulus*

The species has large blue-gray juvenile leaves. When leaves mature they become narrow, dark, glossy-green and sickle-shaped. In Kenya, it performs best in high altitude regions above 2000m above sea level (Maundu and Tengnas, 2005). *E. globulus* species are however, susceptible to gonoptera beetles that usually leads to reduced production (Mwangi, 2014).

2.4.4 *Eucalyptus citriodora*

This species grows innately along Northern and Central coastlines of Queensland, Australia. It can achieve heights of up to 45m and a diameter of 1.3m when fully matured. It has a pleasant white-red or bluish bark that appears to be faint. *E. citriodora* has several desirable traits such as good timber quality, faster rate of growth and excellent bole form, therefore this species is rapidly being cultivated in different agro ecological zones (NAS, 1980).

2.4.5 *Eucalyptus maculata*

Eucalyptus maculata has Eastern Australia as its centre of origin. It can attain heights of up to 40m and a diametre of over 1.2m. It has analogous features like *E. citriodora* but the difference is that its leaves do not have the lemon-like aroma. It normally thrives in dry high altitude regions of above 2000m (Oballa et al., 2010).

2.4.6 *Eucalyptus paniculata*

Eucalyptus paniculata has New South Wales in Australia as its centre of origin. This species is straight and can achieve heights of above 30m but cannot exceed 35m therefore efficient for utility poles production. The growth rate of this species is comparatively lower than that of *E. saligna* and *E. grandis* and performs better in dry highlands. Its fully developed wood is very robust and highly resistant to degeneration by microorganisms (Oballa et al., 2010).

2.4.7 *Eucalyptus regnans*

Eucalyptus regnans has Victoria and Tasmania in Australia as its centre of origin. This species is the tallest known flowering plant species and can attain heights of about 100m. It is appropriate in zones with well-drained soils at an elevation of about 2500-3000m above sea level (KFD, 1996) and is very suitable for sawn lumber and pulpwood (Oballa et al., 2010) but is not very commonly cultivated in Kenya.

2.4.8 *Eucalyptus grandis*

This species majorly grows in Queensland, Australia along the coastal areas and also in New South Wales, England, at altitudes of about 600 m above sea level with sparsely distributed populations also present within the tropics of northern Queensland where it performs well at altitudes of 1100m above sea level (Boland et al., 2006; Slee et al., 2006). It is naturally favoured by temperatures ranging from 2 to 29ºC on average per month and annual rainfall ranging from 725 to 3750 mm per annum (Oballa et al., 2010). It prefers moist well drained soils, but is still capable of growing in many other types of soils (FAO, 1979).

In Kenya, *E. grandis* is the most common in production, it has high rate of growth especially in the highlands, on an altitude between 1400 and 2200 m above sea level with mean annual rainfall of 900 mm per annum. Kenya Forestry Research Institute (KEFRI) was able to develop fast-growing trees with straight boles capable of attaining height growth of 5 m within 3 to 5 years and volume above 45 m^3ha-1yr-1 through re-introduction, selection and breeding (Oballa and Giathi, 1996) but there are no hybrids so far produced to further improve on the germplasm. Genetic information especially on *E. grandis* is vital in coming up with an efficient hybridization strategy.

2.4.9 *Eucalyptus urophylla*

This is one of the few species which is not native to Australia (Blake, 2019), and naturally grows on 7 Indonesian islands; Alor Adonara, Timor, Lembata, Flores, Pantar, and Wetar. It thrives well at altitudes of between 300 and 1100 m above sea level apart from the species in Timor which prefer altitudes of 2960m above sea level. Their favorable temperatures ranges from 27 to 30°C on average per month, but the temperatures are capable of dropping to around 17-21°C at 1900m above sea level (Oballa et al., 2010).

In Kenya, evaluation performance trials have proved *E. urophylla* to be suitable for Nyanza, Eastern and Coastal areas with 1000 mm rainfall per annum. *E. urophylla* set seeds much well in areas with great elevation of 2000m above sea level but the tree form and growth is poor on such altitudes (Oballa et al., 2010).

This species has been cultivated as a pure species or as a hybrid partner by some organizations since the beginning of 1970's (Wright and Osorio, 1996). It is a very popular species especially in wet tropical climates (Eldridge et al., 1993). It is mainly used as a hybrid partner with other eucalypt species such as *E. grandis* and is known to produce progenies with very good hybrid vigor especially for growth (Hodge and Dvorak, 2015).

2.4.10 Eucalyptus hybrids

Tree breeding programs for Eucalyptus have mainly focused on the enhancement of less unadulterated species (Eldridge et al., 1993). The largest genetic improvements in forestry plantations was attributed to clonal utilization of eucalyptus hybrid genetic plant materials (Griffin et al., 2000) and several reports have shown superiority of interspecific hybrids (Vigneron and Bouvet, 2009; Bison et al., 2006). Hybrids developed using eucalypts have the capability of producing genotypes which have special attributes such as high growth vigor and yield (Hettasch et al., 2005)

Hybrids between *E. grandis* and *E. urophylla* have been utilized in some planted forests for quite some time predominantly in Congo (Vigneron and Bouvet, 2000), Brazil (Bison et al., 2006) and moderately in other parts of the world including Indonesia, South America and also China (Dungey and Nikles, 2000). Development of hybrids using *E. urophylla* is becoming important especially for offering disease resistance and improving yields (White et al., 2007).

In Kenya, introductions of about eighteen *E. grandis* and *E. camaldulensis* (GCs) and *E. grandis* and *E. urophylla* (GUs) hybrids were made in 2002 by Tree Biotechnology Project where KEFRI was tasked to evaluate their performance. The hybrids combined tremendous growth rate of *E. grandis,* drought tolerance of *E. camaldulensis* and fungal resistance of *E. urophylla.* From the 18 hybrids introduced only 3 performed well and stable across low altitude areas (Oeba et al., 2009; Wamalwa et al., 2007). Due to these results, there was a need to develop hybrids using local germplasm for better performance but the genetic composition of the genotypes in Kenya is unknown.

2.5 Eucalyptus improvement program

2.5.1 Controlled Pollination

Controlled pollination in forestry plantations especially for commercial gains have been reported worldwide (Bison et al., 2006; Kerr et al., 2004; Potts and Dungey, 2004; Vigneron and Bouvet, 2000) for example between *E. grandis* or *E. urophylla* and *E. tereticornis* (He et al., 2012; Vigneron and Bouvet, 2000), *E. globulus* (Griffin et al., 2000), *E. pellita* (Vigneron and Bouvet, 2000) and *E. dunnii* (Griffin et al., 2000). The following are the main techniques used in controlled pollination:

2.5.1.1 Conventional Technique (CT)

This technique involves the following stages:

2.5.1.1.1 Emasculation

It involves cutting stamens of flowers at anthesis (when operculum changes colour to yellow and starts separating from the receptacle) while being careful to avoid injuring the style or stigma (Potts and Gore, 1995). This helps to avoid flower abortion or infection by pathogens (Potts and Gore, 1995).

2.5.1.1.2 Isolation

Isolation entails using a special celluloid or terylene bag with a transparent window which aids in monitoring the progress of flowers and allows easy application of pollen on receptive stigmas (Potts and Gore, 1995). During bagging, one half of the leaf lamina should be removed to ensure proper fitting, while leaves at the end of the branches should be left intact in order to allow supply of water and other essential nutrients (Potts and Gore, 1995). Apart from bagging, isolation can also be done using a plastic drinking straw or a rubber tubing of between 3-6mm in diameter. This allows saving of time and cost.

2.5.1.1.3 Pollination

This involves application of viable pollen by use of tools such as pollen gun, fine paint brush or sterilized toothpick. Pollen application time varies from species to species depending on whether the stigmas have attained receptivity or not. For example, *E. grandis* and *E. urophylla* take 5-7 days, *E. globulus*, 4-8 days, *E. nitens*, 5-8 days, *E. gunnii*, 5-8 days and *E. regnans*, 10-14 days (Potts and Gore, 1995).

2.5.1.1.4 Debagging

This stage involves removal of the isolation bags usually 4-8 weeks after pollination depending on the involved species (Potts and Gore, 1995). At this point, there is an abscission layer which has developed at the bottom of the style. (Potts and Gore, 1995).

2.5.1.1.5 Harvesting

This stage entails collecting mature fruits with a brown star shaped pattern (Potts and Gore, 1995). For example, in Colombia, *E. urograndis* (*E. grandis* x *E. urophylla*) maturity takes about 16-24 weeks (Potts and Gore, 1995).

2.5.1.2 One Stop Pollination technique (OSP)

The OSP technique involves carrying out emasculation, pollination and isolation the same day (Randall et al., 2014; Potts and Gore, 1995). Pollination is achieved by making a small cut (1 mm below stigma) in order to release a sticky exudate which traps the pollen grains during its application (Randall et al., 2014; Potts and Gore, 1995).

2.5.1.3 Artificially Induced Protogyny (AIP)

The AIP technique does not require emasculation (Assis and Harwood, 2005). It involves making a cut at the tip of the operculum on mature flower bud just before anthesis where the cut is meant to get rid of the stigma (Randall et al., 2015; Assis and Harwood, 2005). It is then seconded by application of target pollen to the exposed cut-surface on the upper style (Randall et al., 2015; Assis and Harwood, 2005).

2.5.2 Natural Pollination

Natural pollination mainly occurs by wind and to a smaller extent by insects due to self-incompatibility and protandrous nature of eucalypts (Bhattachar, 2005) but Hingston and Potts, (1998) reported birds to be more effective than insects. Natural pollination in *E. grandis* and *E. urophylla* has been reported as of major importance for commercial seed production (Horsley and Johnson, 2007).

2.6 Mating designs for eucalypts improvement

Although, these mating designs have not been applied before to produce hybrids especially using the Kenyan germplasm, they can still be used by developing a hybridization protocol for it.

2.6.1 Bi parental

Bi parental mating design involves selecting many plants at random then crossing them in duos so as to have full-sibs (Acquaah, 2012). The resulting progenies are then tested, and the detected

variation apportioned by ANOVA (Hill et al., 1998). This mating design is simple to execute but it is marred with limitations. Its biggest limitation is the incapability to provide relevant information required to evaluate all parameters (Acquaah, 2012). For example, Muneera et al. (2021) reported application of this design on *Eucalyptus camaldulensis* x *E. Tereticornis*, Jones et al. (2005) on *E. morrisbyi* and Mimura et al. (2009) on *E. globulus*.

2.6.2 Polycross

Polycross involves intermating an assemblage of genotypes by making natural crosses in a segregated block. It is very suitable for cross-pollinating species such as sweet potato and sugarcane but can still be used for trees such as eucalypts (Acquaah, 2012). This mating design provides an equal opportunity for each of the involved clones or parents to cross naturally amongst themselves in a given block, hence barring self-pollination (Nduwumuremyi, 2013). However, polycross mating design results to non-randomness due to lack of flowering synchronization and statistical data generated is usually not sufficient enough to estimate all parameters (Nduwumuremyi, 2013). Shelbourne et al. (2007) reported that complementary mating designs of polycrossing is essential for pair crossing to generate full-sib families for forward selections and estimation of breeding values in trees such as *E. nitens*. Lambeth et al. (2001) used complementary polycross mating design while Grattapaglia et al. (2004) used it in an exceptional case of inter-specific hybridization in eucalypts. Bouffier et al. (2019) also evaluated an *E. nitens* forest which used polycross mating design as one of the breeding strategies.

2.6.3 Top cross design

It involves having selected plant materials crossed with a mutual pollen parent whose genetic background and performance is well known especially in an open pollination set up (Aly et al., 2011). The major advantage is that it can greatly facilitate early evaluation of genetic stocks since

it requires very low crossing load, and can facilitate assessment of GCA and SCA of different inbred lines (Mosa, 2010). Its major limitation is that F1's experience segregation hence difficult to identify plants with superior traits (Mosa, 2010). Hill et al. (1998) reported that top cross design has been used in trees like eucalypts after successful modification from polycross mating design.

2.6.4 Line x tester design

This design is essentially a modification of the top cross, in the sense that it involves usage of more than one tester. Line x tester basically entails hybridization between selected lines and comprehensive based testers so as to generate hybrids (Sharma, 2006). This mating design is very simple and provides both half-sibs and full-sibs concurrently. This design is used in assessing numerous types of genetic activities essential in manifestation of quantitative aspects, and also provides SCA of each cross, and GCA of the involved testers (Rashid et al., 2007; Sharma, 2006). However, its limitation is that it requires proper understanding of statistical analysis (Sharma, 2006). Phillips and Aradhya, (1995) reported that this design is suitable in tree improvement especially in the estimation of combining ability.

2.6.5 Diallel design

A comprehensive diallel design permits parents to undergo crossing in all likely combinations (Schlegel, 2010), inclusive of reciprocals together with selfs. This mating design is unique in that it facilitates achievement of equilibrium in Hardy-Weinberg fashion within a given population (Acquaah, 2012). The full diallel design comprises of parents, one assemblage of F_1 generations and reciprocal F_1 generations. Full diallel results to giving n^2 genotypes (Griffing, 1956b). This design provides equal chance of mating with every other parent and measures maternal effects (Nduwumuremyi, 2013). Limitation is that it is laborious and requires more experimental area for evaluation (Nduwumuremyi, 2013). Half diallel design includes progenitors and one assemblage

of F_1 generation with no reciprocals. This system of mating design results to *p(p+1)/2* genotypes (Griffing, 1956b). This system allows equal opportunity of mating particularly half mating and requires less experimental area for evaluation (Nduwumuremyi, 2013). However, its limitation is that it cannot measure maternal effects (Nduwumuremyi, 2013). Wu et al. (2019) used full diallel-mating designs among six *E. urophylla* parents while Bison et al. (2007) used six *E. grandis* x *E. urophylla* elite clones which were crossed with ten *E. globulus* clones in a half-diallel mating design and the resultant hybrids were evaluated in a randomized complete block designs.

2.6.6 North Carolina

North Carolina design (NCD) was established subsequently after using diallel for a long time in order to minimize the labor required to obtain information on combining ability.

2.6.6.1 North Carolina Design I

The NCDI entails a unit of parents serving as males being crossed with a different unit of parents serving as females (Nduwumuremyi, 2013). It is a stratified design with unrelated parents being integrated into related parents (Acquaah, 2012). However, this design requires large experimental area and it is influenced by maternal effects (Nduwumuremyi, 2013). Hill et al. (1998) noted that NCDI has been successfully used in tree breeding like eucalypts where collection of massive amounts of pollen, possess no practical problems.

2.6.6.2 North Carolina Design II

NCDII involves different male parents being mated to a group of similar female parents in a factorial mating scheme. This design is very suitable to multi-flowered plants where each can either be a male or female recurrently (Nduwumuremyi, 2013). It requires much less experimental area compared to NCDI and is capable of estimating GCA and SCA. However, this design cannot determine non allelic interactions and it is influenced by maternal effects (Nduwumuremyi, 2013).

Zhu et al. (2017) performed inter-specific controlled pollinated crosses between 6 *E. urophylla* clones and 6 *E. camaldulensis* CPTs where the design resulted in each *E. camaldulensis* parent being crossed with all *E. urophylla* group hence creating reciprocals.

2.6.6.3 North Carolina Design III

NCDIII involves a sample from F_2 generation being backcrossed to the two original inbred lines from which this very F_2 was generated from. NCDIII is the most robust of all the three NC mating systems. This design is capable of testing GCA, SCA and epistatic interactions (Acquaah, 2012), requires much less area compared to NCDI and NCDII and it is not affected by maternal effects (Nduwumuremyi, 2013). The major limitation with this design is that it consumes a lot of time and resources to set up (Nduwumuremyi, 2013). However, NCDIII has not been reported in eucalypts and also Fasahat, (2016) stated that this design has been used very limitedly in plant breeding despite it, being the most powerful.

2.7 Marker Assisted Selection

2.7.1 Restriction Fragment Length Polymorphism (RFLP)

They are made by isolation of pure DNA with bacteria-retrieved restriction enzymes (Nadeem, 2017). The enzymes essentially slice DNA at explicit loci resulting in a massive number of fragments that fluctuate in length (Nadeem, 2017). RFLPs are polymorphic and co dominant but however, they demand large amounts of pure DNA and are very difficult to automate. For example, genetic diversity studies have been conducted on *E. nitens* using 40 RFLP markers (Bryne et al., 1998) and also on *E. camaldulensis* using 33 markers (Butcher et al., 2002). Elliott and Byrne, (2003) also used 30 markers on *E. occidentalis* to study genetic diversity in natural populations.

2.7.2 Randomly Amplified Polymorphic DNA (RAPD)

RAPDs were advanced by amplification of gDNA by using a single, short random primer (Jiang, 2013). RAPDs exhibit high levels of polymorphism require small amounts of DNA, easy to automate and they require no blotting hybridization (Bruno et al., 2006). However, these markers have limitations in the sense that they are dominant and can never be reproduced. For example, Tiwari et al. (2013) used 10 RAPD markers on *E. tereticornis* while Li, (2004) used 18 on *E. microtheca* and Osman et al. (2012) used 5 on *E. camaldulensis, E. gomphocephala, E. citridora* and *E. resinfera* to carry out genetic diversity studies.

2.7.3 Amplified Fragment Length Polymorphism (AFLP)

These markers utilize RFLPs and PCR technology (Lynch and Walsh, 1998). It is developed by use of two restriction enzymes to cut the DNA resulting to fragments at each end which is then ligated using oligonucleotides (Madhumati, 2014). AFLPs are inexpensive because they don't need prior sequencing information and they allow good quality and moderately degraded DNA to be utilized (Blears, 1998). Moreover, they are dominant markers, densely clustered around the genome and they produce too much data that is not easy to interpret (Madhumati, 2014). Poltri et al. (2003) used 4 markers on *E. dunii* for selecting appropriate seed orchards based on genetic diversity while Mo et al. (2009) used 18 markers on *E. globulus* to analyse somaclonal variations diversity.

2.7.4 Inter Simple Sequence Repeats (ISSRs)

They are made by amplifying DNA segments amid two contrasting but identical SSR recurrent regions within fairly reasonable expanse which permits amplification. The primers involved in their synthesis may be loose (Gupta, 1994) or more inclined towards the 3′ or 5′ end. ISSRs are easy to cognize and they need no preceding acquaintance of DNA sequence (Kar et al., 2005).

However, they cannot discriminate homozygotes from heterozygotes because they are dominant markers with minimal reproducibility (Semagn et al., 2006). Teixeira et al. (2020), Ballesta et al. (2015) and Okun et al. (2008) used 9, 8 and 5 markers on *E. urophylla* × *E. microcorys;* E. *cladocalyx* × *E. grandis*, respectively, for genetic diversity studies in different stands.

2.7.5 Simple Sequence Repeats (SSRs)

SSRs are repeat tandems of about 1–6 nucleotide bases that are abundantly present and widely distributed in the genome (Rajendrakumar et al., 2007). Microsatellites are a vivid manifestation of lower repetition with great levels of polymorphism (Zane et al., 2002). This great level of polymorphism is due to abundant repeat numbers in the SSR regions which can be easily detected by PCR (Kalia et al., 2011). Data analysis and interpretation of bands are scored by assessing the PCR products in order to investigate polymorphism (Röder, 1998). SSR markers are simple, co-dominant, highly polymorphic, automatable, locus specific, reproducible and widely distributed in the genome (Kalia et al., 2011) but they require sequencing and primer information. Many SSRs have been developed and used for genotyping eucalypts in many countries including Brazil, Australia and South Africa (Grattapaglia et al., 2012) but this has not been done in Kenya. Torres-Dini et al. (2011), ChangRong et al. (2016) and Lv et al. (2020) used 8, 21 and 12 markers on *E.* globulus, *E. pellita*, and *E. cloeziana*, respectively, for genetic diversity studies.

2.8 Clustering Methods

2.8.1 Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

This is a simple, hierarchical clustering method used for building an ultra-metric phylogenetic tree (Pavlopoulos, 2010). Its major merit is that it constructs rooted phylogenetic trees (Pavlopoulos, 2010). However, its main disadvantage is that it assumes the distances from the root to every

branch are similar hence indicating that the degree of mutations in these ancestries are constant over time (Pavlopoulos, 2010).

2.8.2 Neighbor Joining (NJ)

This is the latest agglomerative clustering method used for constructing phylogenetic trees. However, it's fast and efficient in analyzing large sets of data (Attenson, 1997), constructs the correct tree topology (Mihaescu, 2009) and does not assume all ancestries evolve at the same rate (Kuhner and Felsenstein, 1994). Its major demerit is that it works by constructing unrooted phylogenetic trees (Pavlopoulos, 2010).

2.8.3 Unweighted Neighbor Joining (UNJ)

This technique constructs a dendogram (rooted tree) using simple coefficient of dissimilarity matrix in order to cluster genotypes into major and minor sets (Ondabu et al., 2017). The clustering is achieved by having the nearest minor clusters combined into one major cluster and the distance between any two clusters is taken to be the average of all distances (Olmstead, 1996). It's a simple, reliable method but its main drawback is that it does not consider mutations (Nasrollah and Milad, 2016).

2.9 Hardy-Weinberg Theory and other genetic diversity parameters

Hardy-Weinberg equilibrium states that the genetic variation in a population will remain constant from one generation to the next provided there is no random mating, mutation, gene flow, selection or an infinite population size (Edwards, 2008). Disruption of the equilibrium will indicate that the populations have experienced one or more disruptive forces which normally results to change of genetic variation (Edwards, 2008).

On the different parameters, genetic differentiation indicates the status of population structure or degree of genotype similarity where F_{ST}<0.05 means low, F_{ST} 0.05-0.15, moderate, F_{ST} 0.15-0.25,

moderate while F_{ST}>0.25 as high (Lu *et al*., 2018). Eucalypts, in general, have a limited population structure owing to low F_{ST} values, mostly less than 0.06 as discussed by Grattapaglia and Kirst (2008). Intra-inbreeding (F_{is}) and inter-inbreeding (F_{it}) values indicate the extent of sharing genetic material within and between populations, respectively. Furthermore, it is expected that if populations obey the Hardy-Weinberg equilibrium then Fis=0 and Fit=Fst (Guries and Ledig, 1979). Number of migrations (Nm) indicates how much of external alleles infiltrates a population where Nm>1 indicate high genetic diversity as a result of gene flow that helps prevent genetic drift (Wright, 1965). Broad sense heritability (H^2) indicates the degree of phenotypic variations due to genetic influences which may include dominance and epistasis effects where values close to 0 signify high environmental influence while those close to 1 are entirely due to genetic influence (Wray and Visscher, 2008). The Principal Coordinate Analysis (PCoA) and Principal Component Analysis (PCA) signify genetic variations which could be explained by PC1 and PC2 scores suggestive of other factors affecting genetic diversity (Shi et al., 2020; Lever and Altman, 2017). Allele frequency is the occurrence of gene variants at a given locus in a particular population where values close to 0 signify low occurrence of gene variants while those close to 1 indicate high occurrence of gene variants at a given locus (Gillespie et al., 2004). Gene diversity Index and heterozygosity determines the long-term persistence in a population and its fitness potential where values close to 0 indicate a weak population while those close to 1 signify a robust population (Cavalcante et *al.*, 2019; Frankham, 2005). Polymorphic Information Content (PIC) indicates the ability of given markers to discriminate genotypes where values >0.5 are noted to be highly informative while those between 0.25 and 0.5 are moderately informative (Botstein et al., 1980). Nei's genetic distance and identity are used to analyse the degree of genetic differentiation in

populations where longest genetic distance are those values close to 1 while shortest distances are those values close to 0 (Tomiuk & Graur, 1988)

CHAPTER THREE: GENETIC STRUCTURE OF FIVE *E. grandis* POPULATIONS IN KENYA USING MICROSATELLITE MARKERS.

3.1 Abstract

Eucalypts are mainly outcrossing species and if in production over long periods of time, seeds collected after each harvest may be a mixture of species especially in areas where other eucalypts are also grown. This affects quality and yield of seeds sold to farmers and other stakeholders hence the need to determine genetic structure from pure seed collection stands within the country. Screening of genetic structure in Kenyan eucalypts has previously been done using 5 ISSRs but the numbers were too few to draw a conclusive report on the germplasm. The objective of this study was to determine the *E. grandis* genetic structure from selected candidate plus trees and their progenies using microsatellite markers from five main seed collection centers. The aim was to genotype the superior performing trees from five main *E. grandis* seed collection stands, James Finlay, Londiani-Kamara, Nyeri-Kabarage, Nyeri-Kiandanguro and Turbo, using 192 *E. grandis* genotypes and 17 SSR markers. Data was collected on Polymorphic Information Content (PIC), genetic diversity, allelic frequency and heterozygosity, which differed between sites. The SSR markers had an average PIC of 0.826 suggesting that they were highly informative and had the capability of discriminating the 192 *E. grandis* genotypes from the 5 seed collection zones, and genetic diversity of 0.844 indicating high levels of genetic variability for possible exploitation in future breeding programmes. The overall contribution within individual difference was 86%, the fixation index (F_{ST}=0.036), intra inbreeding (Fis=0.106), inter inbreeding (Fit=0.138) and migration number per generation (*N*m=6.761) suggesting that the genetic diversity among the genotypes is satisfactorily large enough for exploitation in a given breeding program in Kenya. The unweighted neighbor-joining tree categorized test genotypes into two main clusters: all the genotypes were present in the two clusters hence confirming high genetic diversity among the

Kenyan genotypes. The study ratifies there is need for maximum utilization of genotypes from the main seed collection zones for heterosis or targeting of specific traits such as disease resistance through development of hybrids.

Keywords: allelic frequency, fixation index, genetic diversity, heterozygosity,

3.2 Introduction

The Genus *Eucalyptus* consists of widely cultivated plantation species in temperate and tropical regions due to good quality timber, wide adaptability and relatively low susceptibility to biotic factors (Warren et al., 2009; Danusevicius and Lindgren, 2003; Xu and Dell, 2002). The genus *Eucalyptus* is made up of more than 700 species occurring worldwide. In Kenya it is grown as a plantation species in both the public and private sector. Nine species are widely grown in Kenya including *E. camaldulensis, E. saligna, E. globulus, E. urophylla, E. paniculata, E. regnans, E. maculata, E. citriodora* and *E. grandis* (APC, 2019; Oballa et al., 2010). It is categorized among the three main plantation species alongside pines and cypress (PSRA; KFDP, 1999) with seed stands used for commercial purposes. Eucalyptus species are mainly outcrossing (Potts, 2004), but since introduction into Kenya in 1902, their genetic composition has not been undertaken to establish purity of each species. This has hindered efforts made towards genetic improvement of eucalyptus germplasm in Kenya, as sufficient germplasm information is crucial for improvement programs (Ondabu et al., 2017). Tree improvement programmes in Kenya have adopted breeding through recurrent mass selection of Candidate Plus Trees (CPTs) and *E. grandis* is the only species from the Genus *Eucalyptus* with an intensive tree improvement programme in Kenya. Although many CPTs have been selected by breeders at Kenya Forestry Research Institute (KEFRI), since the introduction of *E. grandis* in Kenya, the need to genetically fingerprint the CPTs has not been much emphasized. Genetic fingerprinting of *E. grandis* has previously been done in Kenya using

five inter simple sequence repeats (ISSRs) (Okun et al., 2008) but due to its attribute as non-homologous and non-repeatability (Godwin et al., 1997), SSRs primers were used. Our study aimed at genotyping 10 CPTs together with their respective progenies from five main *E. grandis* seed collection stands namely Nyeri-Kiandanguro, Nyeri-Kabarage, Londiani-Kamara, James Finlay Limited and Turbo using 22 SSR markers. The number of SSR markers were selected based on availability and optimal performance. The objective of the study was to determine the genetic composition and structure of selected candidate plus trees and progenies of *E. grandis* in Kenya using microsatellite markers to support advanced breeding programmes.

3.3 Materials and methods

3.3.1 Plant materials and germplasm collection

Forty-five CPTs were selected from five seed stands namely Nyeri-Kiandanguro, Nyeri-Kabarage, Londiani-Kamara, James Finlay and Turbo (Table 3.1) based on morphological characteristics for tree height and diametre breast height (DBH). Leaf tissues and seeds were collected from each of the CPTs for advanced improvement program of the species. For the present study, ten CPTs from the forty-five were selected and genotyped together with at least 18 of their respective progenies.

Image 3.1. *E. grandis* (A) seedling, 3 weeks after sowing with young leaves ready for genomic DNA extraction.

Table 3.1 Candidate plus tree selections of *E. grandis* from five seed collection sites in Kenya

CPT No.	Location	Origin	Height (m)	DBH (cm)	Age of plantation (years)
EG7	Nyeri-Kiandanguro	Kenya	54	57	16
EG9	Nyeri-Kiandanguro	Kenya	52	56	16
EG12	Nyeri-Kabarage	Kenya	58	50	16
EG14	Nyeri-Kabarage	Kenya	63	32	16
EG19	Londiani Kamara	Kenya	37	41	14
EG20	Londiani Kamara	Kenya	33	40	14
EG27	James Finlay	Kenya	42	44	9
EG29	James Finlay	Kenya	39	33.6	9
EG43	Turbo	Kenya	43	34	12
EG47	Turbo	Kenya	42	41	12

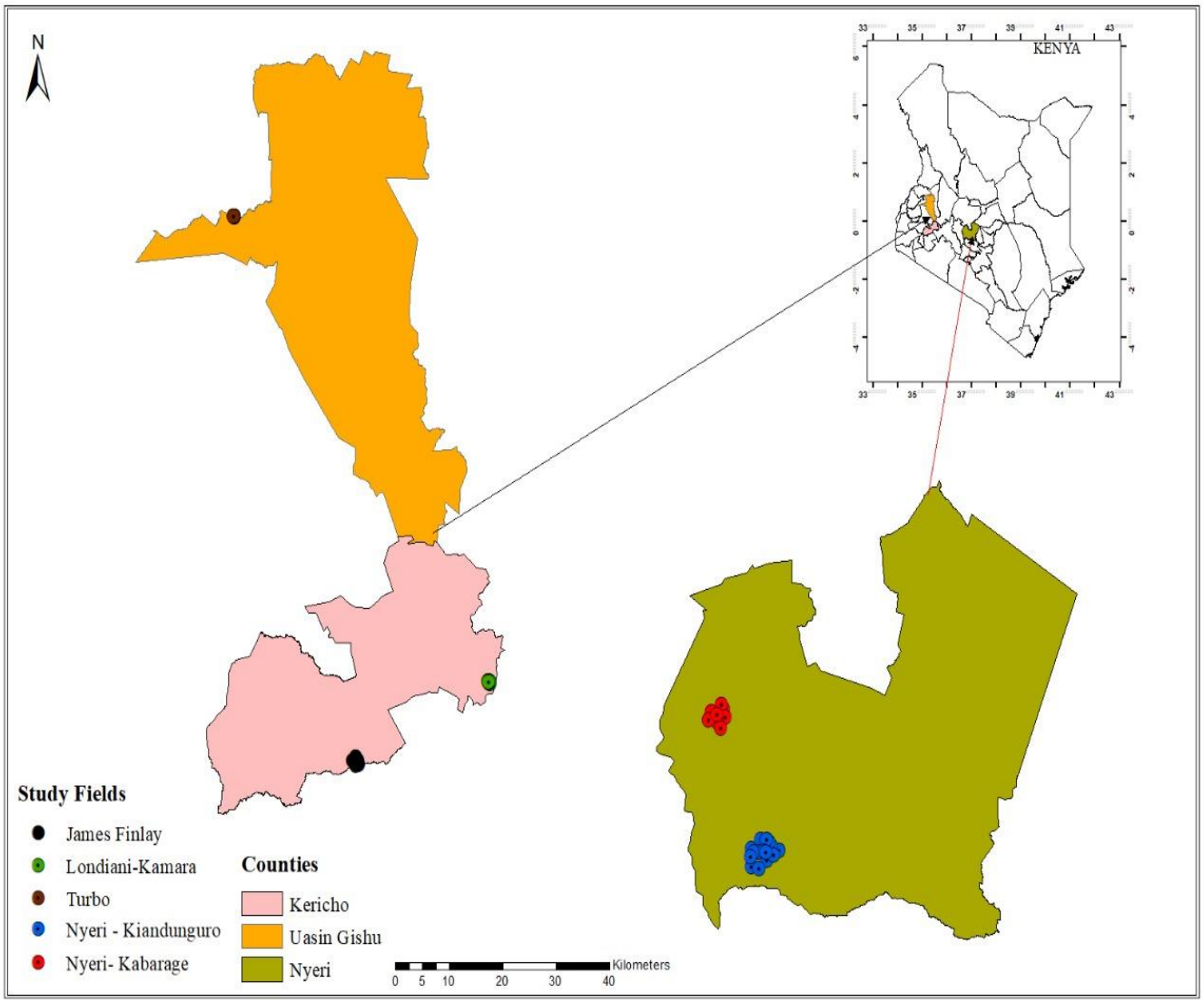

Figure 3.1. Map of Kenya showing the tree seed sources of the CPTs for the study

3.3.2 Genomic DNA Extraction

Young leaf samples were collected from each of the 10 CPTs (Table 3.1) preserved on silica gel and then taken to the laboratory in KEFRI. From each CPT, at least 18 seedlings were sampled for leaf tissue collection after being germinated and raised in the tree nursery until sufficient leaves were obtained for DNA isolation using CTAB method according to the protocol described by Hanaoka et al. (2012). One gram of young leaf samples was used for the process which involved lysis by retsch grinder, DNA separation from cellular debris by centrifugation, DNA precipitation by cold isopropanol, DNA cleaning using 70% ethanol and DNA pellet dilution using 200 µL nuclease-free water.

3.3.3 DNA quality check and quantification

DNA quantification was done using Nano drop nucleic acid analyzer, model Shimadzu Bio Spec-nano 206-26300-48 according to the protocol described by Hanaoka et al., (2012). 1 µL of DNA was used for the process which involved placing DNA samples on the measurement window, pressing the start button on the machine, determination of optical density/nucleic acid concentration (ng/µL) and discarding of samples automatically.

3.3.4 SSR screening and reconstitution using fluorescently labeled primers

Twenty-two primers were screened for this study, however, 5 (EMBRA19, EMBRA75, EMBRA8, EMBRA34 and EMBRA46) did not work and were excluded from the downstream analysis (Appendix 11). The 17 remaining functional SSR primers were then reconstituted in 6 multiplexes for genotyping.

Table 3.2 Microsatellite markers, primer sequences, annealing temperature (Ta), allele sizes, and number of repeat motifs

Microsatellite	Forward Primer (5' to 3')	Reverse Primer (5' to3')	Ta (°C)	Expected Product Size (bp)	Repeat motif	Summary Statistics	
						No. of Allele	PIC
EMBRA1	gATAgAACTTTCCTATTTgATCg	gTAggATTTgATgTCTgCAA	56	127	$(AG)^{33}$	6	0.50
EMBRA2	CgTgACACCAggACATTAC	ACAAATgCAAATTCAAATgA	56	121	$(AG)^{15}$	13	0.85
EMBRA3	gATCggATTggAggAgAC	AATTCAATTCATCCAAAgC	56	123	$(AG)^{19}$	12	0.82
EMBRA6	AgAgAATTgCTCTTCATggA	gAAAAgTCTgCAAAgTCTgC	56	98	$(AG)^{19}$	16	0.81
EMBRA7	CACACCgTgTCAgTTAgC	AATAAggAggATTCCATgg	56	115	$(AG)^{15}$	19	0.84
EMBRA12	AggATTTgTggggCAAgT	gTTCCCCATTTTCATgTCC	56	98	$(AG)^{22}$	18	0.88
EMBRA23	ggTTgTTTCATCTTTTCCATg	AgCgAAggCAATgTgTTT	56	118	$(AG)^{16}$	15	0.85
EMBRA26	CCCACAACAAAAggAAAg	AgAggTgTTCgATTCAATTC	56	120	$(AG)^{19}$	14	0.84
EMBRA28	CAAgACATgCATTTCgTAgT	ACTCTTgATgTgACgAgACA	56	178	$(AG)^{25}$	17	0.89
EMBRA36	TTACgTCAATTCTTgCTTg	AATTCAgCTCAAgATTTTggT	56	155	$(AG)^{29}$	15	0.84
EMBRA41	ATgATTTTgTgCgTggAC	TCAggTgAAAggATggAg	56	198	$(AG)^{13}$	14	0.85
EMBRA43	TCCAggTTCATATTCACATC	CATCTCAAgTTCCTCCCT	56	145	$(AG)^{14}$	13	0.86
EMBRA45	gTCATTTgCACACAgTTTTC	AgTTCATAgAATgCAgAAAATg	56	102	$(AG)^{18}$	17	0.88
EMBRA114	AggCgATgACTgTTATCAA	ACTTCCAAAATTCCCCAC	56	130	$(GA)^{26}$	17	0.76
EMBRA158	gTgCAgATATCACCACCT	CATTCAgTTCCCAgTACC	56	125	$(CT)^{26}$	17	0.87
EMBRA194	AAgATAggTggCgCTTgAg	gggCATgTAgAAACTCTTCg	56	146	$(CT)^{17}$	18	0.84
EMBRA204	CTCgTgTggTTATgTgAACT	gCTTgTCTACTATgCACATgA	56	147	$(TC)^{25}$	12	0.88
	Mean					14.9	0.83

3.3.5 PCR amplification and genotyping by capillary electrophoresis

The master mix components were prepared and aliquoted to a final volume of 6.9 μL and then placed on Veriti™ 96-well thermal cycler, the final concentrations of the primer mixes averaged to 0.8μM. Temperature regime used was initial denaturing the template DNA was 95ºC for 15 minutes, denaturation at 94ºC for 30 seconds, annealing at 57ºC for 90 seconds, extension at 72ºC for 60 seconds and final extension at 60ºC for 30 minutes. This was done for 35 cycles. The multiplexed PCR products were precisely mixed with 8.83 μL Hi-Di-formamide together with 0.134 μL fluorescent-labeled GeneScan™ LIZ size standard in a 96-well microtiter plate (Hanaoka et al., 2012). The mixed products were denatured at 95ºC for 5 min and chilled on ice for 5 min to avoid the formation of double-strand DNA (Hanaoka et al., 2012). The products were loaded to Applied Biosystems 3500 Genetic Analyzer for genotyping.

3.3.6 Data collection and analysis

Power Marker v3.2.5 used for haploids and diploids (Liu and Muse, 2005) was used to collect data including allele frequency, allelic richness, gene diversity, and heterozygosity. Polymorphic Information Content value for individual loci were calculated between each pair of lines. Genetic distance matrices of the 192 *E. grandis* genotypes (10 parents and 182 progenies) across the 5 sites (Table 4.1) generated by GenAlEx v6.5 (Peakall and Smouse 2006, 2012) were used for computing Principal Coordinates Analysis (PCoA) to visualize variations and similarities. Neighbor-joining trees were constructed using DARwin v6.0.21 (Perrier and Jacquemoud, 2006) where the resulting trees were visualized. Variation partitioning within and among group components was computed using Analysis of Molecular Variance (AMOVA) in GenAlEx v6.5 (Peakall and Smouse 2006, 2012) which facilitated estimation of broad sense heritability (H^2), standardized allelic patterns

across different families and populations (Meirmans, 2007) and components of variance significance levels using 999 permutations.

3.4 Results

3.4.1 DNA Quantity and Quality of different families and populations

DNA from different sites had variations in quantity and quality which was estimated in terms of nucleic concentrations and Optical Density ratios respectively. The highest quantity of DNA was from James Finlay and lowest from Nyeri-Kiandanguro while the highest quality of DNA was from Londiani Kamara and lowest from James Finlay (Table 3.3).

Table 3.3 Means of Nucleic Acid Concentration and OD ratios of *E. grandis* genotypes

Parent No.	Site	Nucleic Acid Conc.(ng/µL)	OD260/280	OD260/230	OD260	OD280	OD230
27	James Finlay	8.5	1.21	1.55	0.19	0.17	0.15
29	James Finlay	106.6	1.28	0.61	3.52	3.22	5.19
19	Londiani Kamara	82.7	1.80	1.00	2.32	1.59	2.53
20	Londiani Kamara	10.4	1.03	1.07	0.24	0.20	0.26
12	Nyeri Kabarage	54.1	1.68	0.75	2.00	1.59	2.41
14	Nyeri Kabarage	38.3	1.56	0.71	1.12	0.84	1.33
7	Nyeri Kiandanguro	7.6	1.49	-0.13	0.27	0.23	0.34
9	Nyeri Kiandanguro	5.4	1.57	0.80	0.22	0.18	0.26
43	Turbo	31.7	1.78	0.83	0.92	0.65	0.58
47	Turbo	48.6	1.80	0.94	1.38	0.97	1.70
	Overall mean	39.4	1.52	0.81	1.22	0.97	1.47

3.4.2 Genetic fingerprinting

3.4.2.1 Allele Frequency of different families and populations

Allele Frequency for 192 *E. grandis* genotypes from 5 sites with 2 different families (Table 3.1; Figure 3.1) per site were summarized so as to estimate how much of the gene represented by the SSR was present. James Finlay parents had major allele frequencies between 0.25 and 0.75 (Table 3.4) while the progenies ranged from 0.19 to 0.63 (Table 3.5). Londiani Kamara parents were between 0.25 and 1.00 (Table 3.6) and the progenies ranged from 0.17 to 0.58 (Table 3.7). Nyeri-Kabarage parents were between 0.25 and 0.5 (Table 3.8) and the progenies ranging from 0.21 to 0.51 (Table 3.9). Nyeri-Kiandanguro parents were between 0.25 and 0.75 (Table 3.10) and the progenies ranged from 0.23 to 0.58 (Table 3.11). Turbo parents were between 0.25 and 1.00 (Table 3.12) and the progenies ranged from 0.18 to 0.61 (Table 3.13). The allele frequencies among all populations ranged from 0.17 to 0.58 (Table 3.14). Turbo had the highest allele frequency (Table 3.12) while Nyeri-Kabarage had the lowest (Table 3.8) among parents. Secondly, Nyeri-Kiandanguro had the highest allele frequency (Table 3.11) while James Finlay had the lowest (Table 3.5) for the progenies.

3.4.2.2 Allelic richness of different families and populations

Allelic richness for 192 *E. grandis* genotypes from 5 sites with 2 different families (Table 3.1; Figure 3.1) per site were summarized in order to determine current status of the biodiversity. James Finlay parents had alleles of between 2 and 4 (Table 3.4) and the progenies ranged from 4 to 14 (Table 3.5). Londiani Kamara parents were between 1 and 4 (Table 3.6) and progenies ranged from 4 to 13 (Table 3.7). Nyeri-Kabarage parents were between 2 and 4 with a mean of 3.2941 (Table 3.8) and progenies ranged from 5 to 13 (Table 3.9). Nyeri-Kiandanguro parents were between 2 and 4 (Table 3.10) and progenies ranged from 6 to 11 (Table 3.11). Turbo parents were between 2

and 4 (Table 3.12) and progenies ranging from 4 to 12 (Table 3.13). The total number of alleles among all populations ranged from 6 to 19 (Table 3.14). Nyeri-Kabarage had the highest number of alleles (Table 3.8) while Turbo had the lowest (Table 3.12) among parents. Nyeri-Kabarage had the highest number of alleles (Table 3.9) while Nyeri-Kiandanguro had the lowest (Table 3.11) for the progenies.

3.4.2.3 Gene Diversity Index of different families and populations

Gene Diversity Index for 192 *E. grandis* genotypes from 5 sites with 2 different families (Table 3.1; Figure 3.1) per site were summarized in order to determine the overall fitness of different populations. James Finlay parents' gene diversity was between 0.375 and 0.750 (Table 3.4) and the progenies ranged from 0.51 to 0.87 (Table 3.5). Londiani Kamara parents were between 0.00 and 0.75 (Table 3.6) and the progenies ranged from 0.56 to 0.88 (Table 3.7). Nyeri-Kabarage parents were between 0.50 and 0.75 (Table 3.8) and the progenies ranged from 0.57 to 0.87 (Table 3.9). Nyeri-Kiandanguro parents were between 0.38 and 0.75 (Table 3.10) and the progenies ranged from 0.59 to 0.84 (Table 3.11). Turbo parents were between 0.00 and 0.75 (Table 3.12) and the progenies ranged from 0.56 to 0.87 (Table 3.13). Gene diversity index among all populations ranged from 0.56 to 0.90 (Table 3.14). Turbo had the gene diversity index (Table 3.12) while Nyeri- Kiandanguro had the lowest (Table 3.10) among parents. James Finlay (Table 3.5) had the highest gene diversity index while Nyeri-Kiandanguro had the lowest (Table 3.11) for the progenies.

3.4.2.4 Heterozygosity of different families and populations

Heterozygosity for 192 *E. grandis* genotypes from 5 sites with 2 different families (Table 3.1; Figure 3.1) per site were summarized so as to determine the levels of heterozygosity within populations. James Finlay parents' heterozygosity were between 0.0 and 1.0 (Table 3.4) and progenies ranged from 0.38 to 1.00 (Table 3.5). Londiani Kamara parents were between 0.0 and 1.0 (Table 3.6) and progenies ranged from 0.18 to 0.94 (Table 3.7). Nyeri-Kabarage parents were between 0.0 and 1.0 (Table 3.8) and progenies ranged from 0.22 to 0.97 (Table 3.9). Nyeri-Kiandanguro parents were between 0.0 and 1.0 (Table 3.10) and progenies ranged from 0.08 to 1.00 (Table 3.11). Turbo parents were between 0.0 and 1.0 (Table 3.12) and progenies ranged from 0.21 to 1.00 (Table 3.13). The heterozygosity among all populations ranged from 0.25 to 0.97 (Table 3.14). Nyeri-Kabarage had the highest heterozygosity (Table 3.8) while Turbo had the lowest (Table 3.12) among parents. Among the progenies, James Finlay had the highest heterozygosity (Table 3.5) while Londiani-Kamara had the lowest (Table 3.7).

3.4.2.5 Polymorphic Information Content (PIC) of different families and populations

The PIC for 192 *E. grandis* genotypes from 5 sites with 2 different families (Table 3.1; Figure 3.1) per site were summarized in order to estimate the ability of the SSR markers in differentiating genotypes. James Finlay parents' PIC were between 0.31 and 0.71 (Table 3.4) and the progenies ranged from 0.41 to 0.86 (Table 3.5). Londiani Kamara parents were between 0.0 and 0.71 (Table 3.6) and the progenies ranged from 0.47 to 0.84 (Table 3.7). Nyeri-Kabarage parents PIC were between 0.38 and 0.70 (Table 3.8) and the progenies ranged from 0.48 to 0.84 (Table 3.9). Nyeri-Kiandanguro parents were between 0.30 and 0.70 (Table 3.10) and the progenies ranged from 0.53 to 0.82 (Table 3.11). Turbo parents were between 0.0 and 0.70 (Table 3.12) and progenies ranged from 0.50 to 0.86 (Table 3.13). The PIC among all populations ranged from 0.49 to 0.89 (Table

3.14). Nyeri-Kabarage had the highest PIC (Table 3.8) while Turbo had the lowest (Table 4.12) among parents. On the other hand, Londiani Kamara (Table 3.7) had the highest PIC while Nyeri-Kiandanguro had the lowest (Table 3.11) for the progenies.

Table 3.4. Summary statistics of James Finlay parents

Marker	MAF	Genotype No.	Sample Size	No.of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.50	1	2	2	2	1.0	0.50	1.0	0.38
EMBRA2	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA3	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA6	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA7	0.75	2	2	2	2	1.0	0.38	0.5	0.30
EMBRA12	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA23	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA26	0.50	2	2	2	2	1.0	0.50	0.0	0.38
EMBRA28	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA36	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA41	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA43	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA45	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA114	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA158	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA194	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA204	0.25	2	2	2	4	1.0	0.75	1.0	0.70
Mean	0.43	1.82	2.0	1.88	3.06	0.94	0.63	0.85	0.55

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.5. Summary statistics of James Finlay progenies

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.63	4	36	36	4	1.0	0.50	0.75	0.41
EMBRA2	0.28	14	36	36	8	1.0	0.81	0.92	0.79
EMBRA3	0.27	18	36	35	11	0.9	0.84	0.80	0.82
EMBRA6	0.37	11	36	19	8	0.5	0.77	0.89	0.74
EMBRA7	0.33	13	36	36	10	1.0	0.82	0.94	0.80
EMBRA12	0.29	16	36	36	11	1.0	0.82	1.00	0.80
EMBRA23	0.19	20	36	36	10	1.0	0.85	0.97	0.84
EMBRA26	0.25	15	36	32	9	0.9	0.82	0.38	0.80
EMBRA28	0.37	11	36	19	10	0.5	0.81	1.00	0.78
EMBRA36	0.29	15	36	36	11	1.0	0.82	1.00	0.80
EMBRA41	0.21	20	36	36	11	1.0	0.85	0.92	0.84
EMBRA43	0.36	12	36	36	8	1.0	0.76	0.92	0.73
EMBRA45	0.28	8	36	25	8	0.7	0.81	0.76	0.78
EMBRA114	0.31	8	36	35	9	0.9	0.77	0.80	0.74
EMBRA158	0.21	22	36	36	14	1.0	0.87	0.81	0.86
EMBRA194	0.28	17	36	36	14	1.0	0.81	0.64	0.78
EMBRA204	0.25	19	36	36	10	1.0	0.86	0.97	0.85
Mean	0.30	14.29	36	33	9.76	0.92	0.80	0.85	0.77

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.6. Summary statistics of Londiani Kamara parents

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.50	1	2	2	2	1.0	0.50	1.0	0.38
EMBRA2	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA3	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA6	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA7	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA12	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA23	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA26	0.50	2	2	2	2	1.0	0.50	0.0	0.38
EMBRA28	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA36	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA41	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA43	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA45	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA114	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA158	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA194	1.00	1	2	1	1	0.5	0.00	0.0	0.00
EMBRA204	0.25	2	2	2	4	1.0	0.75	1.0	0.70
Mean	0.38	1.82	2.0	1.88	3.24	0.94	0.63	0.85	0.57

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.7. Summary statistics of Londiani Kamara progenies

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.58	4.	36	36	4	1.0	0.55	0.83	0.47
EMBRA2	0.31	12	36	35	7	0.9	0.78	0.71	0.75
EMBRA3	0.37	12	36	35	8	0.9	0.77	0.71	0.73
EMBRA6	0.26	15	36	31	10	0.9	0.82	0.84	0.80
EMBRA7	0.34	12	36	28	11	0.8	0.81	0.75	0.79
EMBRA12	0.35	13	36	26	12	0.7	0.82	0.73	0.80
EMBRA23	0.24	16	36	36	10	1.0	0.83	0.86	0.81
EMBRA26	0.44	11	36	33	11	0.9	0.75	0.18	0.73
EMBRA28	0.17	13	36	30	12	0.8	0.88	0.73	0.87
EMBRA36	0.28	11	36	25	10	0.7	0.81	0.92	0.78
EMBRA41	0.28	15	36	36	10	1.0	0.81	0.81	0.78
EMBRA43	0.29	16	36	35	9	0.9	0.80	0.83	0.77
EMBRA45	0.23	12	36	33	13	0.9	0.85	0.82	0.84
EMBRA114	0.36	11	36	35	9	0.9	0.78	0.89	0.75
EMBRA158	0.30	14	36	35	10	0.9	0.82	0.94	0.80
EMBRA194	0.36	15	36	36	10	1.0	0.79	0.56	0.77
EMBRA204	0.19	14	36	26	9	0.7	0.86	0.92	0.84
Mean	0.31	12.7	36	32.41	9.71	0.9	0.80	0.77	0.78

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.8. Summary statistics of Nyeri-Kabarage parents

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA2	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA3	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA6	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA7	0.25	2	2	2	4	1.0	0.75	1.0	0.71
EMBRA12	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA23	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA26	0.50	2	2	2	2	1.0	0.50	0.0	0.38
EMBRA28	0.50	2	2	2	2	1.0	0.50	0.0	0.38
EMBRA36	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA41	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA43	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA45	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA114	0.50	1	2	2	2	1.0	0.50	1.0	0.38
EMBRA158	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA194	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA204	0.50	2	2	2	3	1.0	0.63	1.0	0.55
Mean	0.37	1.88	2.0	1.94	3.29	0.97	0.66	0.88	0.59

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.9. Summary statistics of Nyeri-Kabarage progenies

Markers	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.51	5	36	36	5	1.0	0.57	0.97	0.48
EMBRA2	0.30	13	36	35	8	0.9	0.77	0.94	0.74
EMBRA3	0.27	14	36	35	8	0.9	0.81	0.74	0.79
EMBRA6	0.33	13	36	36	9	1.0	0.76	0.92	0.72
EMBRA7	0.30	15	36	33	10	0.9	0.82	0.85	0.79
EMBRA12	0.27	21	36	33	14	0.9	0.85	0.94	0.84
EMBRA23	0.25	19	36	36	12	1.0	0.86	0.78	0.84
EMBRA26	0.26	11	36	36	8	1.0	0.79	0.22	0.76
EMBRA28	0.21	16	36	36	12	1.0	0.87	0.78	0.86
EMBRA36	0.39	15	36	33	10	0.9	0.77	0.85	0.75
EMBRA41	0.40	16	36	36	10	1.0	0.77	0.83	0.74
EMBRA43	0.42	15	36	36	9	1.0	0.75	0.83	0.72
EMBRA45	0.24	19	36	35	13	0.9	0.85	0.63	0.84
EMBRA114	0.31	12	36	35	11	0.9	0.80	0.80	0.77
EMBRA158	0.26	17	36	35	10	0.9	0.85	0.91	0.83
EMBRA194	0.34	14	36	35	8	0.9	0.77	0.66	0.73
EMBRA204	0.28	18	36	34	10	0.9	0.83	0.97	0.81
Mean	0.32	14.88	36	35	9.82	0.9	0.79	0.80	0.77

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.10. Summary statistics of Nyeri-Kiandanguro parents

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA2	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA3	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA6	0.75	2	2	2	2	1.0	0.38	0.5	0.30
EMBRA7	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA12	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA23	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA26	0.75	2	2	2	2	1.0	0.38	0.5	0.30
EMBRA28	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA36	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA41	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA43	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA45	0.50	2	2	2	2	1.0	0.50	0.0	0.38
EMBRA114	0.50	1	2	2	2	1.0	0.50	1.0	0.38
EMBRA158	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA194	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA204	0.25	2	2	2	4	1.0	0.75	1.0	0.70
Mean	0.46	1.89	2.0	1.9	3.0	0.97	0.61	0.76	0.54

MAF=Major Allele Frequency; No.of obs=Number of observations; PIC=Polymorphic Information Content

Table 3.11. Summary statistics of Nyeri-Kiandanguro progenies

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.58	6	37	37	6	1.0	0.59	0.84	0.53
EMBRA2	0.23	16	37	37	9	1.0	0.84	0.95	0.82
EMBRA3	0.36	12	37	36	8	0.9	0.75	0.75	0.72
EMBRA6	0.50	11	37	28	7	0.8	0.66	0.68	0.61
EMBRA7	0.31	7	37	37	7	1.0	0.81	1.00	0.78
EMBRA12	0.41	13	37	37	9	1.0	0.76	0.92	0.73
EMBRA23	0.30	16	37	37	8	1.0	0.80	0.89	0.77
EMBRA26	0.58	13	37	37	10	1.0	0.63	0.27	0.61
EMBRA28	0.31	13	37	29	9	0.8	0.81	0.93	0.79
EMBRA36	0.36	13	37	36	7	0.9	0.79	0.92	0.76
EMBRA41	0.36	16	37	37	10	1.0	0.79	0.84	0.77
EMBRA43	0.35	19	37	36	10	0.9	0.80	0.75	0.78
EMBRA45	0.46	7	37	24	6	0.6	0.67	0.08	0.61
EMBRA114	0.43	5	37	34	7	0.9	0.67	0.91	0.61
EMBRA158	0.32	13	37	36	7	0.9	0.76	0.89	0.73
EMBRA194	0.26	17	37	37	11	1.0	0.84	0.92	0.82
EMBRA204	0.27	16	37	37	8	1.0	0.83	1.00	0.81
Mean	0.38	12.53	37	34.82	8.18	0.9	0.75	0.81	0.72

MAF=Major Allele Frequency;No. of obs.=Number of observations; PIC=Polymorphic Information Content

Table 3.12. Summary statistics of Turbo parents

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.50	1	2	2	2	1.0	0.50	1.0	0.38
EMBRA2	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA3	0.75	2	2	2	2	1.0	0.38	0.5	0.30
EMBRA6	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA7	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA12	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA23	0.50	2	2	2	3	1.0	0.63	1.0	0.55
EMBRA26	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA28	0.75	2	2	2	2	1.0	0.38	0.5	0.30
EMBRA36	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA41	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA43	0.25	2	2	2	4	1.0	0.75	1.0	0.70
EMBRA45	1.00	1	2	1	1	0.5	0.00	0.0	0.00
EMBRA114	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA158	0.50	1	2	1	2	0.5	0.50	1.0	0.38
EMBRA194	0.50	2	2	2	3	1.0	0.63	0.5	0.55
EMBRA204	0.25	2	2	2	4	1.0	0.75	1.0	0.70
Mean	0.51	1.71	2.0	1.76	2.71	0.88	0.55	0.74	0.48

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.13. Summary statistics of Turbo progenies

Marker	MAF	Genotype No	Sample Size	No. of obs.	Allele No	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.61	4	37	37	4	1.0	0.56	0.78	0.50
EMBRA2	0.34	14	37	37	9	1.0	0.76	0.86	0.73
EMBRA3	0.31	11	37	29	8	0.8	0.78	0.86	0.75
EMBRA6	0.43	11	37	34	7	0.9	0.69	0.68	0.64
EMBRA7	0.44	13	37	36	9	0.9	0.72	0.86	0.68
EMBRA12	0.35	14	37	24	10	0.6	0.79	0.92	0.76
EMBRA23	0.49	12	37	37	8	1.0	0.70	0.81	0.67
EMBRA26	0.43	7	37	28	4	0.8	0.62	0.21	0.54
EMBRA28	0.19	19	37	36	15	0.9	0.88	0.81	0.87
EMBRA36	0.26	18	37	33	10	0.9	0.82	0.91	0.80
EMBRA41	0.45	13	37	37	10	1.0	0.75	0.89	0.73
EMBRA43	0.24	20	37	37	12	1.0	0.85	1.00	0.84
EMBRA45	0.38	12	37	33	8	0.9	0.77	0.70	0.75
EMBRA114	0.29	9	37	28	9	0.8	0.83	0.96	0.82
EMBRA158	0.18	19	37	37	11	1.0	0.87	0.81	0.86
EMBRA194	0.29	14	37	33	8	0.9	0.80	0.82	0.77
EMBRA204	0.25	16	37	34	9	0.9	0.82	0.94	0.80
Mean	0.35	13.29	37	33.53	8.88	0.9	0.77	0.81	0.73

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

Table 3.14. Summary statistics of all parents and progenies among all the populations

Marker	MAF	Genotype No.	Sample Size	No. of obs.	Allele No.	Availability	Gene Diversity	Heterozygosity	PIC
EMBRA1	0.58	6	192	191	6	0.99	0.56	0.84	0.49
EMBRA2	0.22	45	192	190	13	0.99	0.86	0.88	0.85
EMBRA3	0.26	35	192	180	12	0.94	0.84	0.77	0.82
EMBRA6	0.32	42	192	156	16	0.81	0.82	0.80	0.80
EMBRA7	0.22	37	192	180	19	0.94	0.86	0.89	0.84
EMBRA12	0.19	53	192	165	18	0.86	0.89	0.91	0.88
EMBRA23	0.25	46	192	192	15	1.00	0.86	0.87	0.85
EMBRA26	0.25	34	192	176	14	0.92	0.85	0.25	0.84
EMBRA28	0.19	48	192	158	17	0.82	0.90	0.82	0.89
EMBRA36	0.27	41	192	172	15	0.90	0.85	0.92	0.84
EMBRA41	0.22	49	192	192	14	1.00	0.86	0.85	0.85
EMBRA43	0.18	50	192	190	13	0.99	0.87	0.87	0.86
EMBRA45	0.18	35	192	159	17	0.83	0.89	0.61	0.88
EMBRA114	0.34	29	192	177	17	0.92	0.79	0.87	0.76
EMBRA158	0.19	48	192	188	17	0.98	0.88	0.87	0.87
EMBRA194	0.21	43	192	186	18	0.97	0.86	0.71	0.84
EMBRA204	0.17	50	192	177	12	0.92	0.89	0.97	0.88
Mean	0.25	40.65	192	178.18	14.88	0.93	0.84	0.81	0.83

MAF=Major Allele Frequency; No.of obs. =Number of observations; PIC= Polymorphic Information Content

3.4.2.6 Genetic differentiation and relationships of parents and progenies in James Finlay

Principal Coordinate Analysis (PCoA) established genetic differentiation by using genetic distance matrix among the *E. grandis* genotypes. The first and second coordinates explained 22.41% and 6.31% (Fig 3.2) of the overall variations within the genotypes respectively, but unweighted neighbor joining tree algorithm of the 38 genotypes established distinct genetic relationship by grouping the genotypes into two distinct clusters (Fig 3.3). Cluster I was inclusive of all genotypes from parent 27 while cluster II included all genotypes from parent 29 but only one progeny from parent 27 appeared in cluster II (Fig 3.3) indicating a distant relationship.

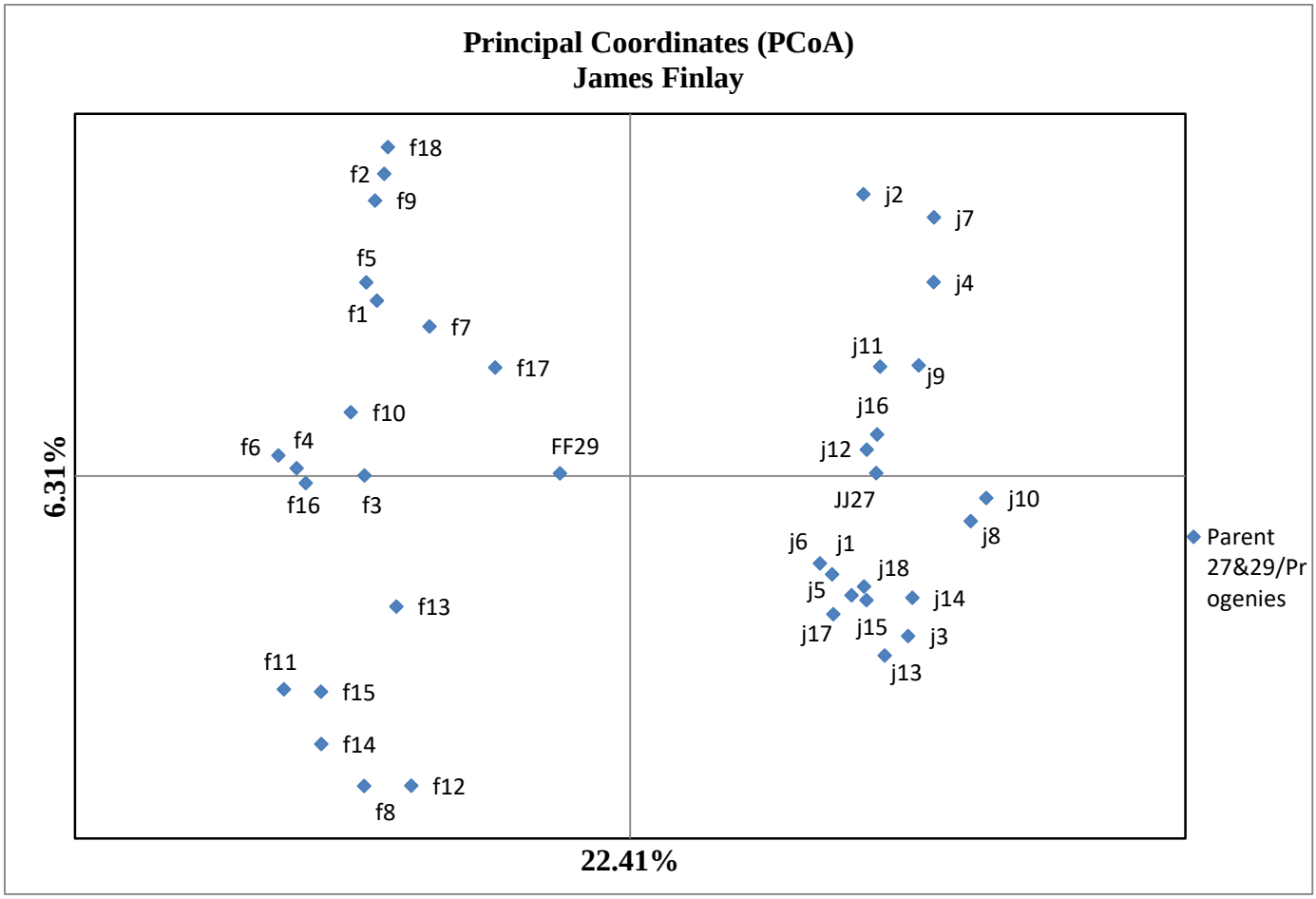

Figure 3.2. Principal coordinates analysis (PCoA) biplot showing the clustering of 38 genotypes (including 2 parents; P19 and P20) from James Finlay. Variation percentages explained by PC1 and PC2 were 22.41% and 6.31%, respectively.

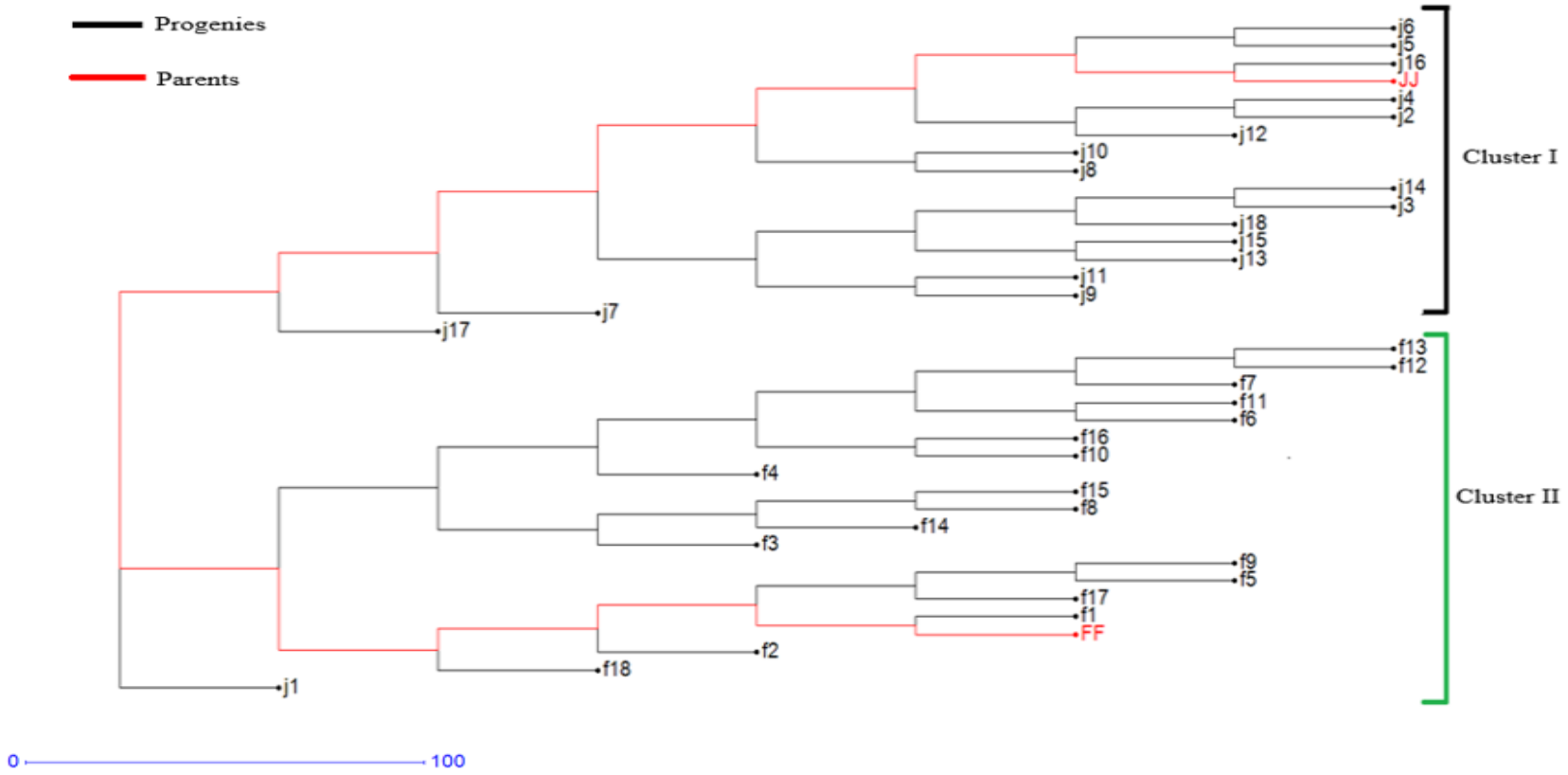

Figure 3.3. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 38 *E. grandis* genotypes from James Finlay.

3.4.2.7 Genetic differentiation and relationships of parents and progenies in Londiani Kamara

The first and second coordinates in the PCoA explained 20.55% and 8.29% (Fig 3.4) of the overall variations genetically within the genotypes respectively but unweighted neighbor joining tree of the 38 genotypes grouped them into two distinct clusters (Fig 3.5). Cluster I included 12 progenies and parent 20 while cluster II included 24 progenies and parent 19 but the progenies from both parents appeared in all the clusters (Fig 3.5) indicating a close relationship among the genotypes.

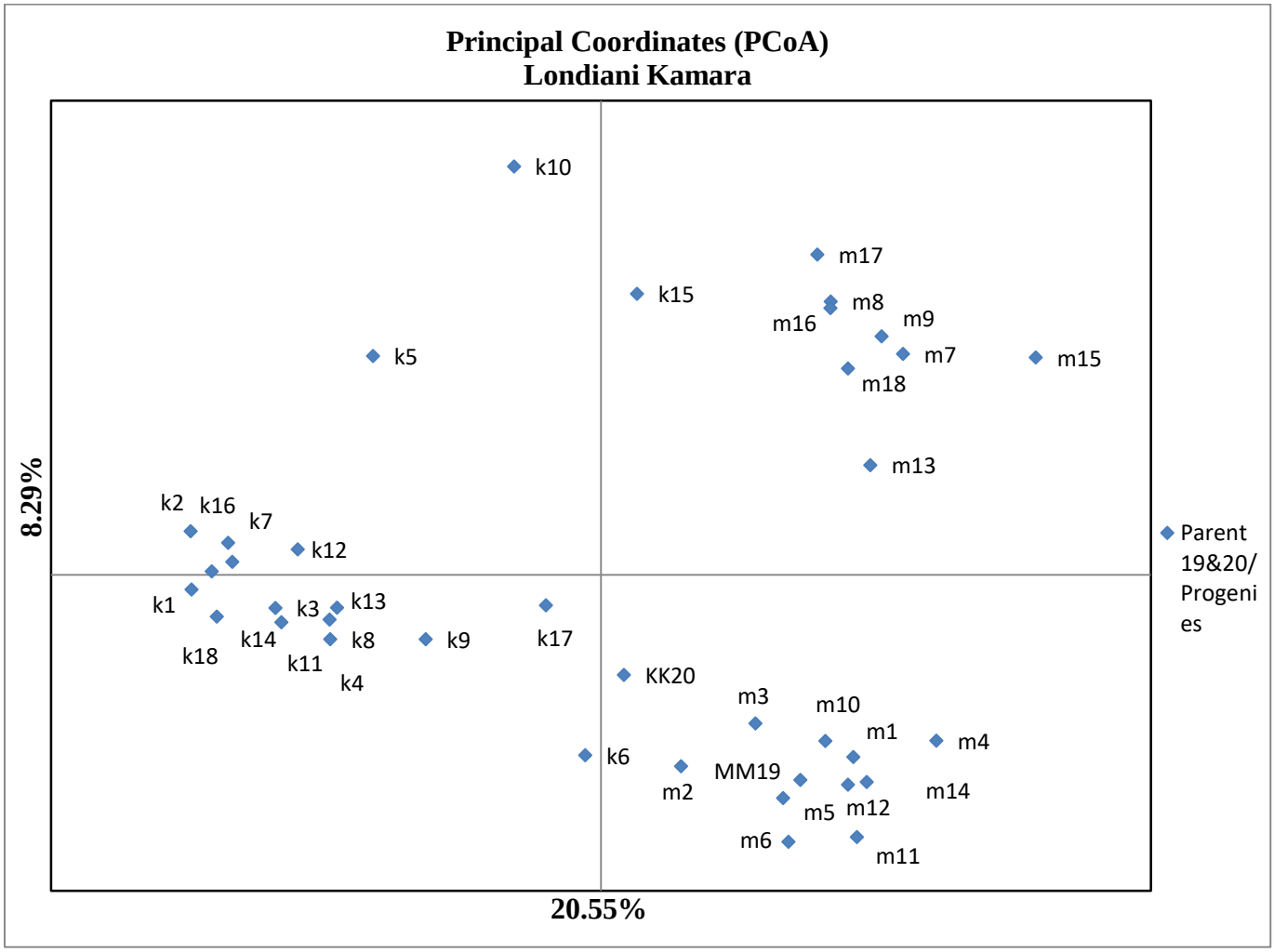

Figure 3.4. Principal coordinates analysis (PCoA) biplot showing the clustering of 38 genotypes (including 2 parents, P19 and P20) from Londiani Kamara. Variation percentages explained by PC1 and PC2 were 20.55% and 8.29% respectively.

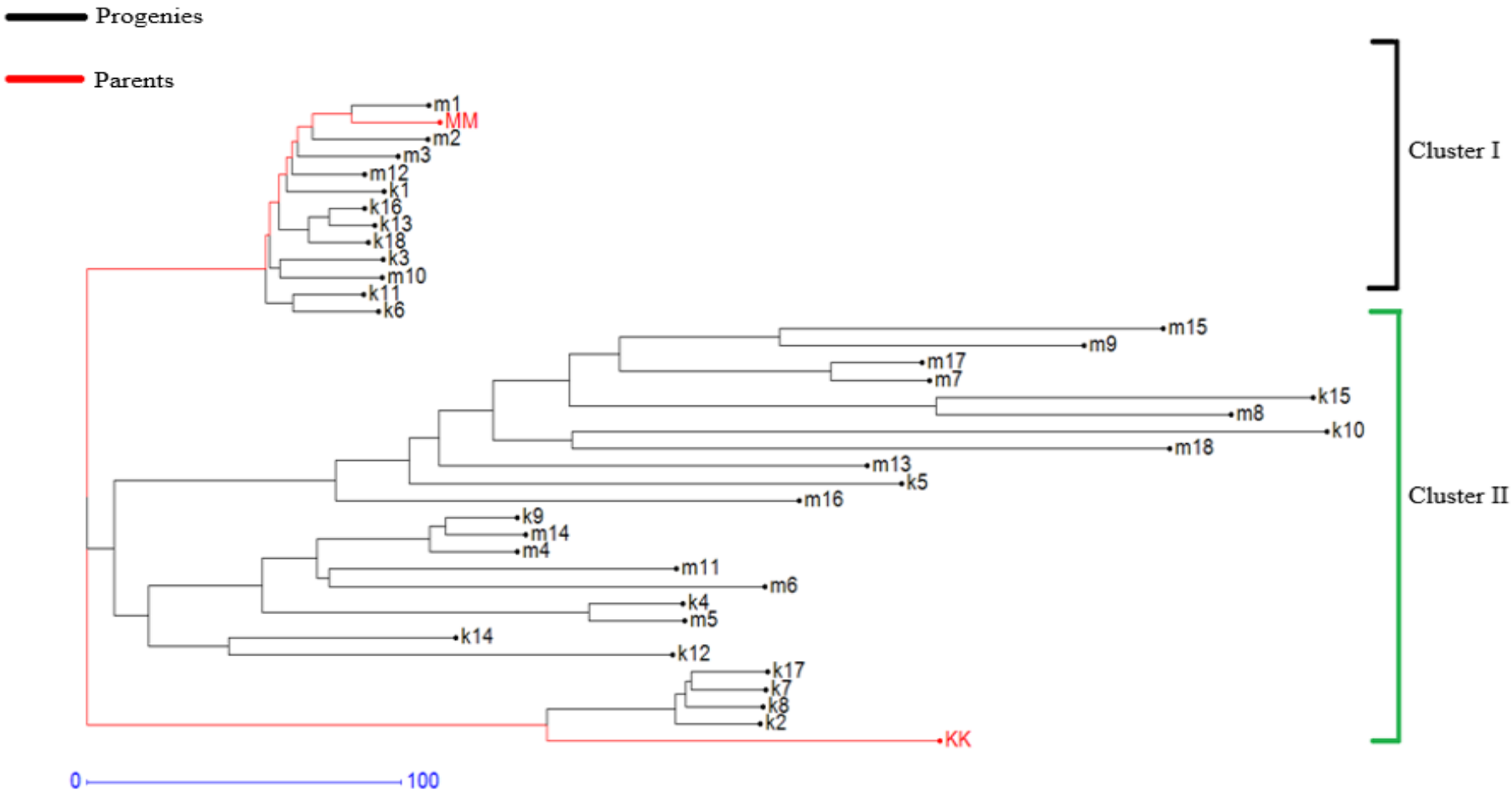

Figure 3.5. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 38 *E. grandis* genotypes from Londiani Kamara.

3.4.2.8 Genetic differentiation and relationships of parents and progenies in Nyeri Kabarage

The first and second coordinates in the PCoA explained 15.51% and 6.53% (Fig 3.6) of the overall variations genetically within the genotypes respectively but unweighted neighbor joining tree of the 38 genotypes grouped them into two distinct clusters (Fig 3.7). Cluster I included 21 progenies and parent 12 while cluster II included 16 progenies and parent 14 but the progenies from both parents appeared in all the clusters (Fig 3.7) indicating a close relationship among the genotypes.

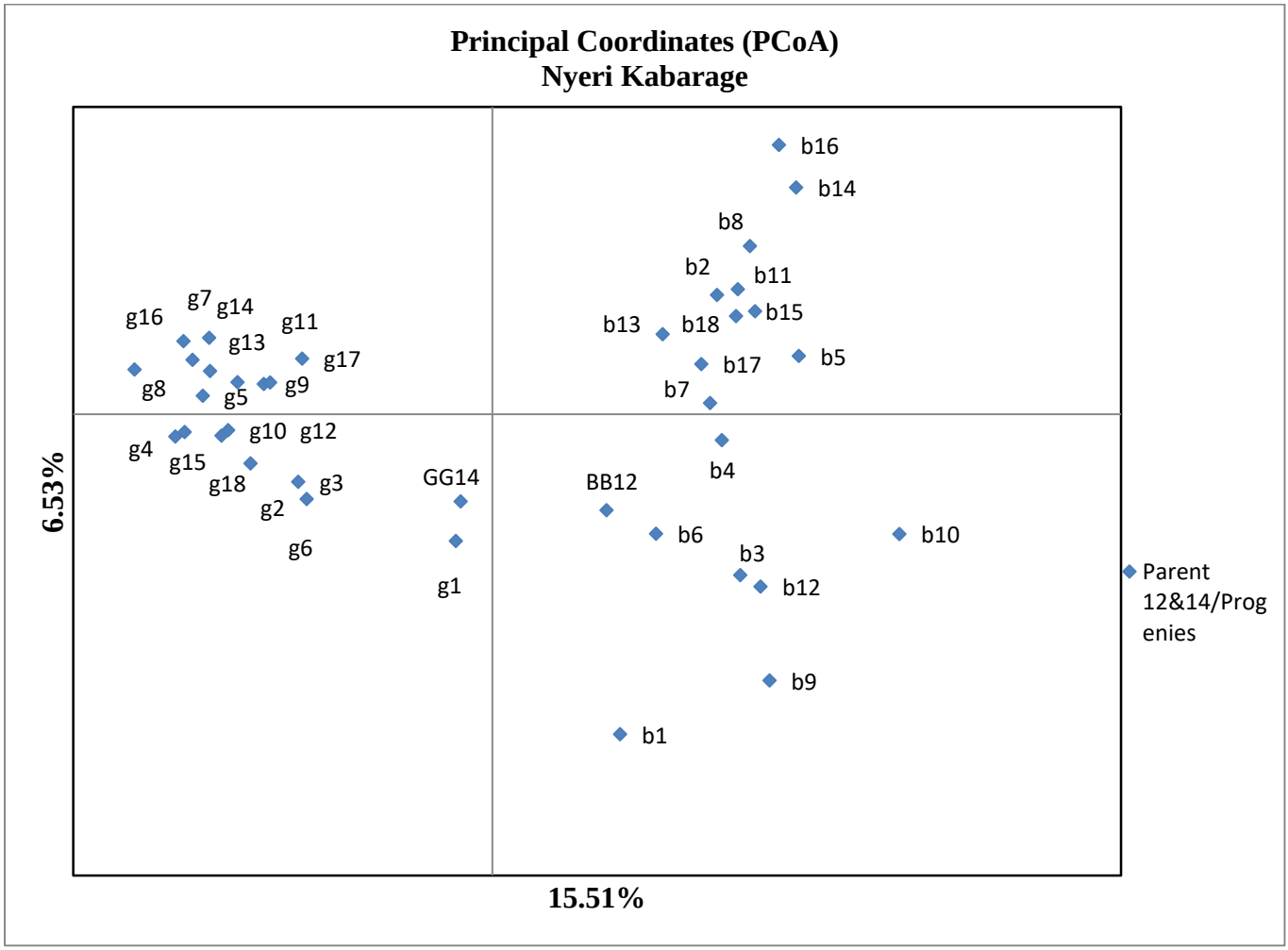

Figure 3.6. Principal coordinates analysis (PCoA) biplot showing the clustering of 38 genotypes (including 2 parents; P12 and P14) from Nyeri Kabarage. Variation percentages explained by PC1 and PC2 were 15.51% and 6.53% respectively

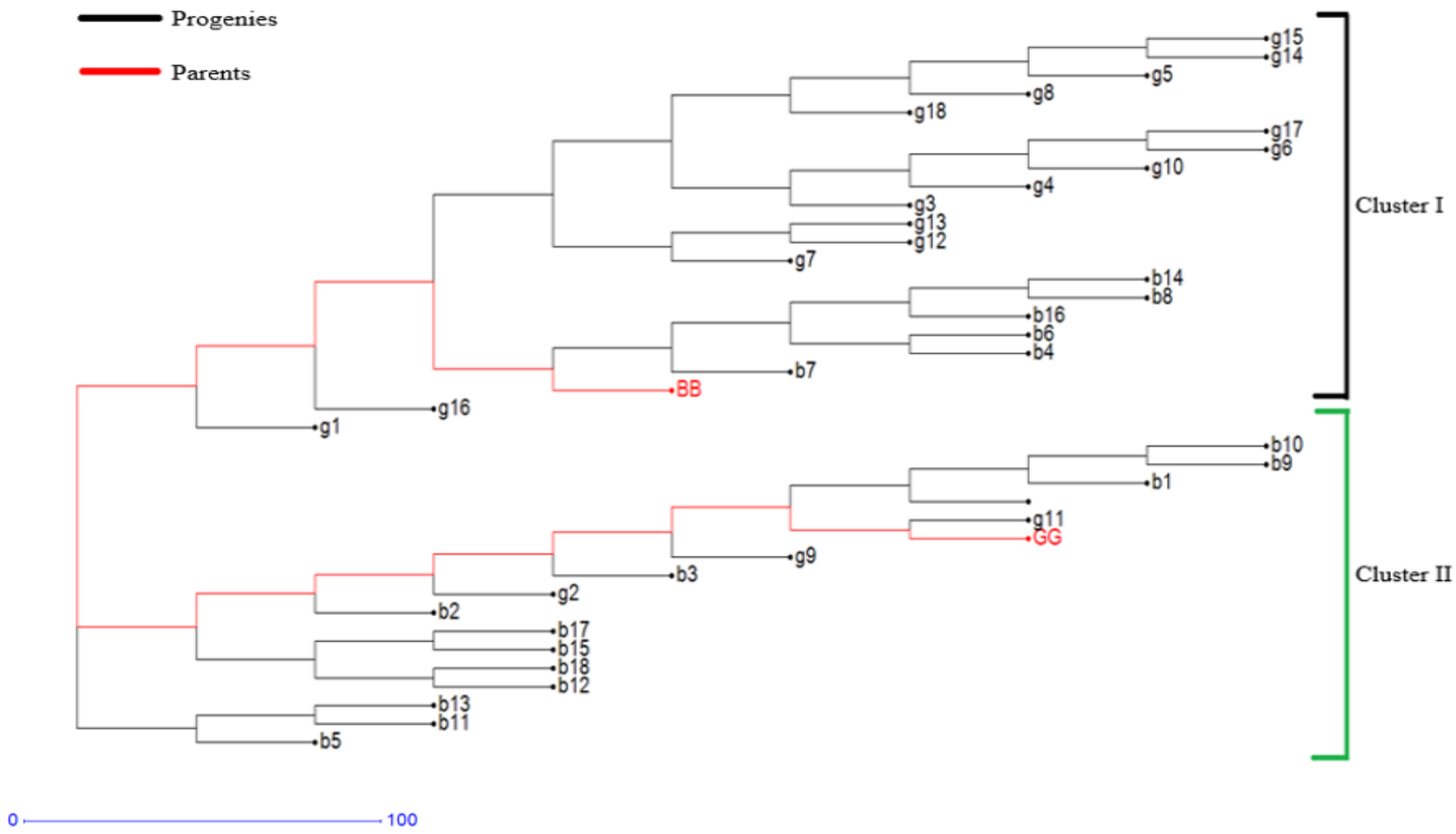

Figure 3.7. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 38 *E. grandis* genotypes from Nyeri Kabarage

3.4.2.9 Genetic differentiation and relationships in Nyeri Kiandanguro

The first and second coordinates in the PCoA explained 18.41% and 8.91% (Fig 3.8) of the overall variations genetically within the genotypes respectively but unweighted neighbor joining tree of the 38 genotypes grouped them into two distinct clusters (Fig 3.9). Cluster I included 13 progenies and parents 7 and 9 while cluster II included 26 progenies but they appeared in all the clusters (Fig 3.9) indicating a close relationship among the genotypes.

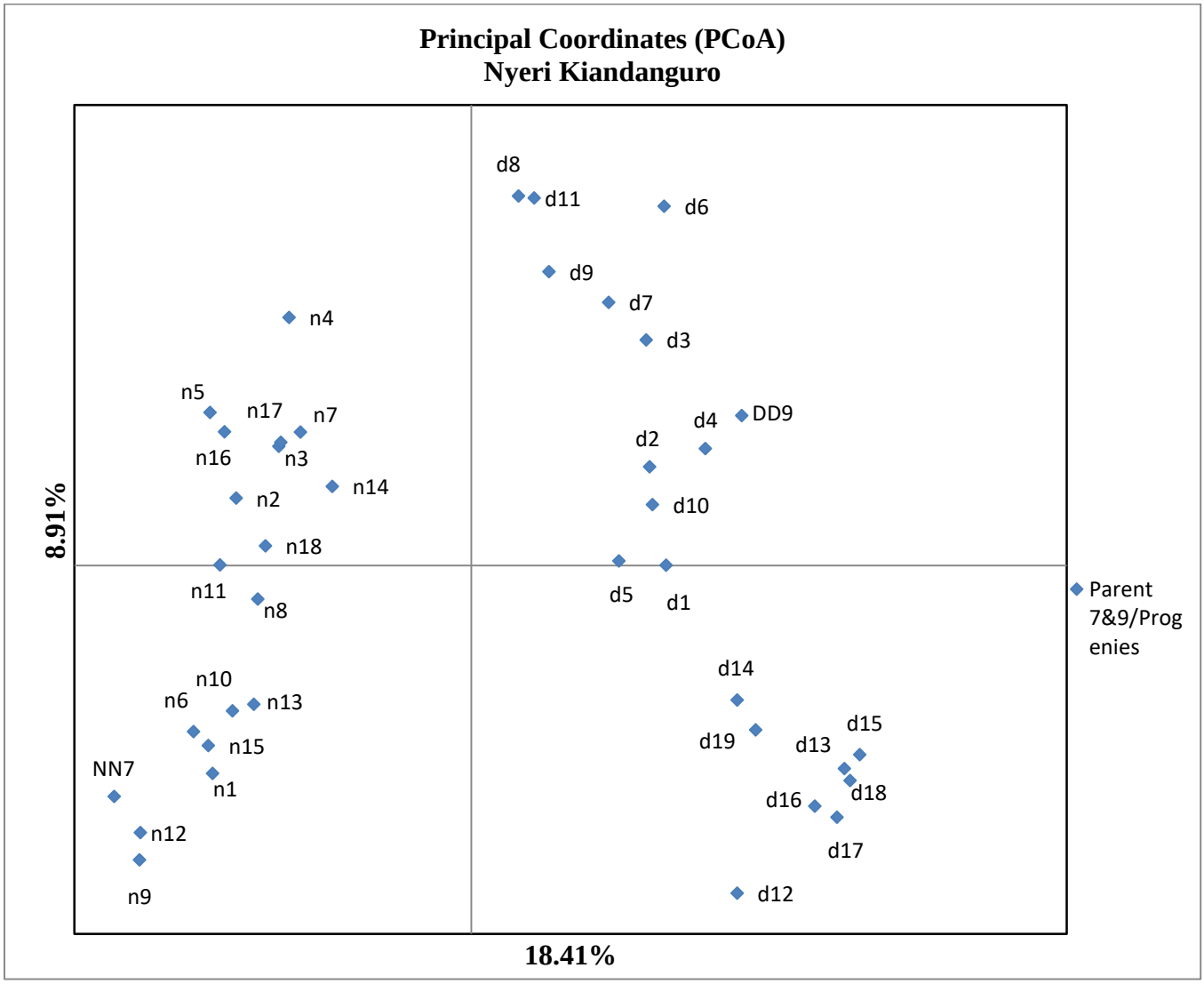

Figure 3.8. Principal coordinates analysis (PCoA) biplot showing the clustering of 39 genotypes (including 2 parents; P7 and P9) from Nyeri Kiandanguro. Variation percentages explained by PC1 and PC2 were 18.41% and 8.91% respectively

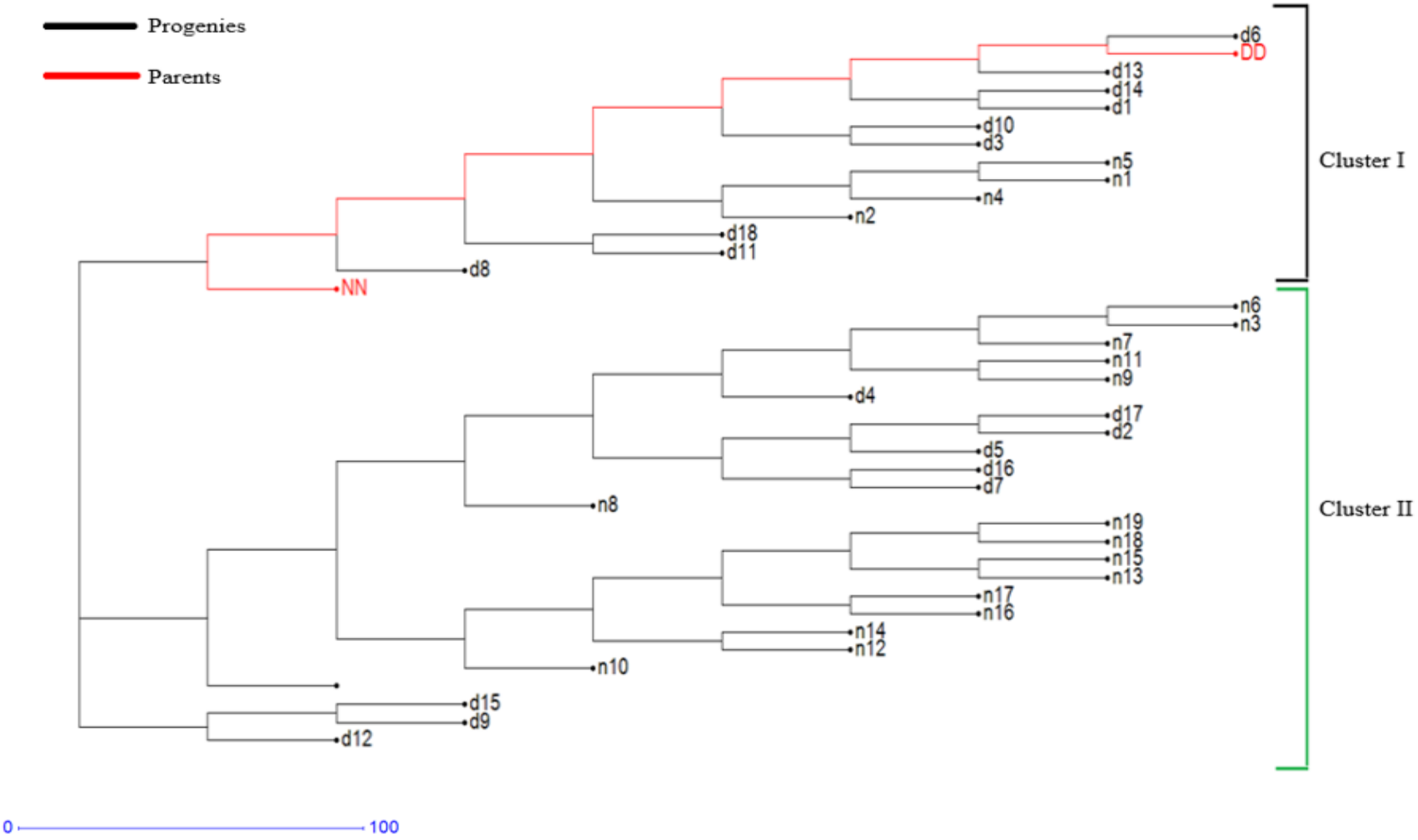

Figure 3.9. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 39 *E. grandis* genotypes from Nyeri Kiandanguro.

3.4.2.10 Genetic differentiation and relationships in Turbo

The first and second coordinates in the PCoA explained 16.53% and 7.57% (Fig 3.10) of the overall variations genetically within the genotypes respectively but unweighted neighbor joining tree of the 38 genotypes grouped them into two distinct clusters (Fig 3.11) Cluster I included 13 progenies and parent 47, cluster II included 15 progenies and parent 43 while cluster III had 9 progenies but they appeared in all the clusters (Fig 3.11) indicating a close relationship among the genotypes.

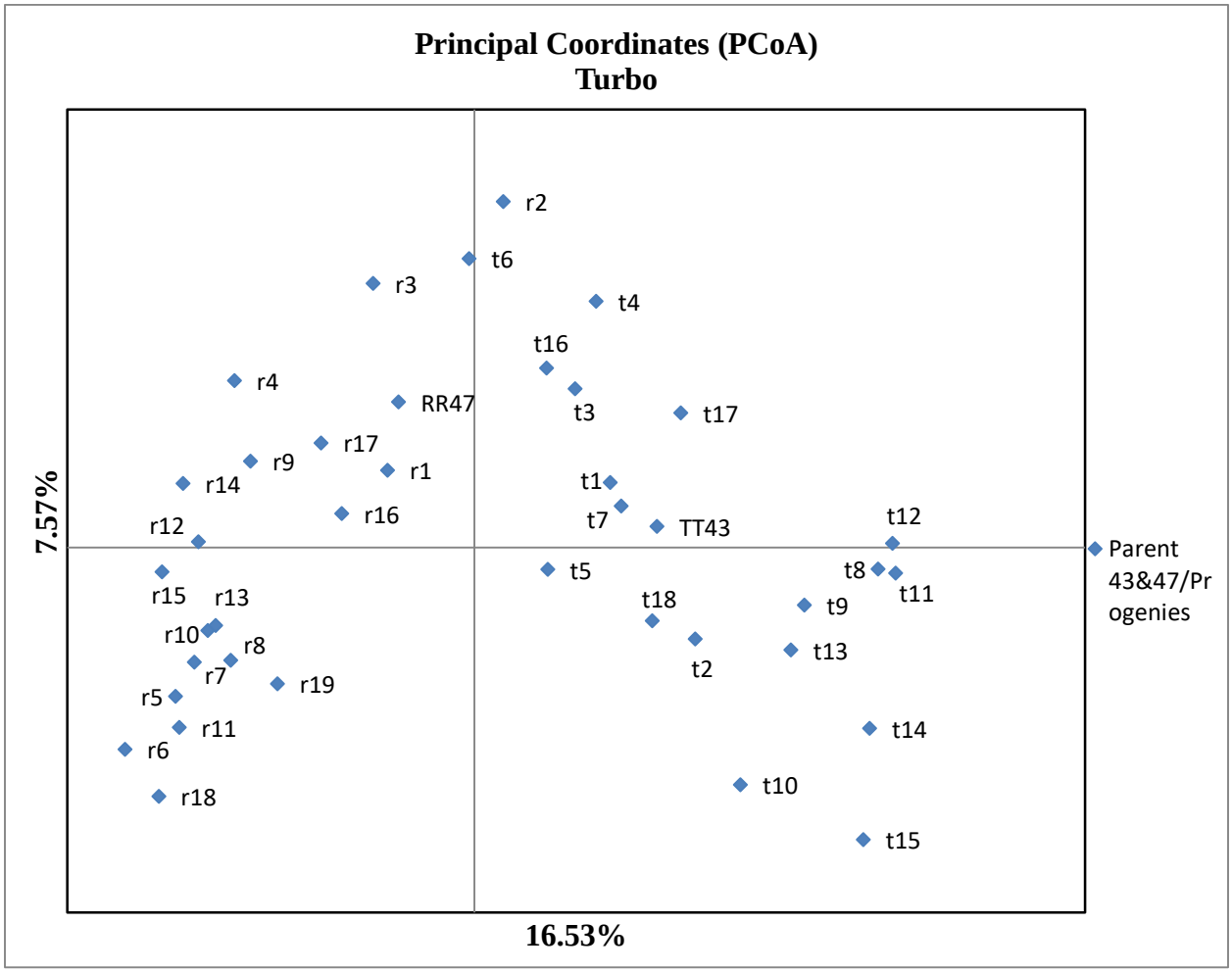

Figure 3.10. Principal coordinates analysis (PCoA) biplot showing the clustering of 39 genotypes (including 2 parents; P43 and P47) from Turbo. Variation percentages explained by PC1 and PC2 were 16.53% and 7.57% respectively

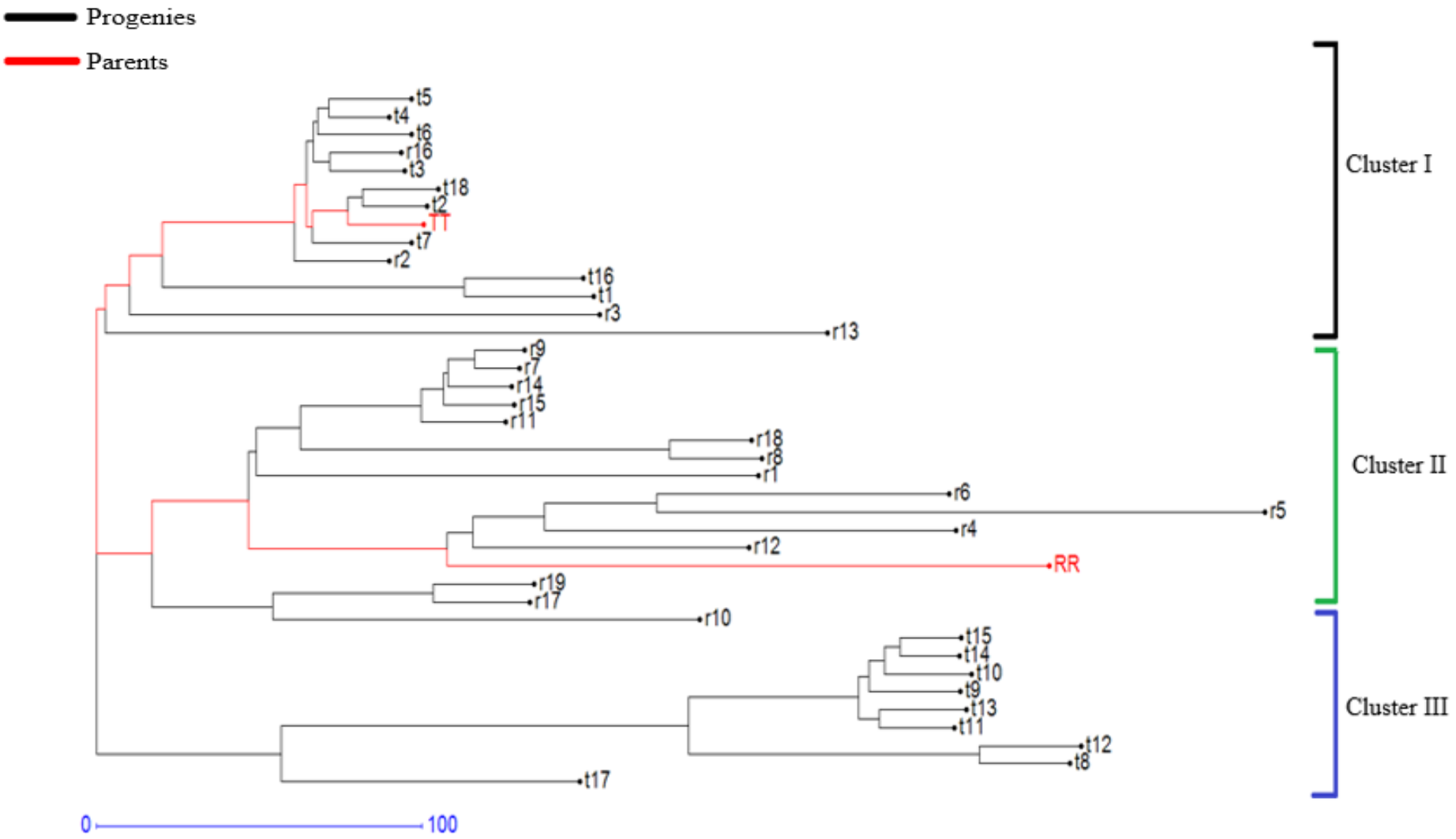

Figure 3.11. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 39 *E. grandis* genotypes from Turbo.

3.4.2.11 Genetic differentiation and relationships among all populations

The first and second coordinates in the PCoA explained 5.99% and 5.75% (Fig 3.12) of the overall variations genetically within the genotypes respectively but unweighted neighbor joining tree of the 192 genotypes grouped them into two distinct clusters (Fig 3.13). Cluster I included 68 progenies and parents 7,9,12,20,27 and 47 while cluster II included 114 progenies and parents 14,19,29 and 43 but the progenies appeared in all the clusters (Fig 3.13) indicating a generally close relationship among genotypes from all the sites.

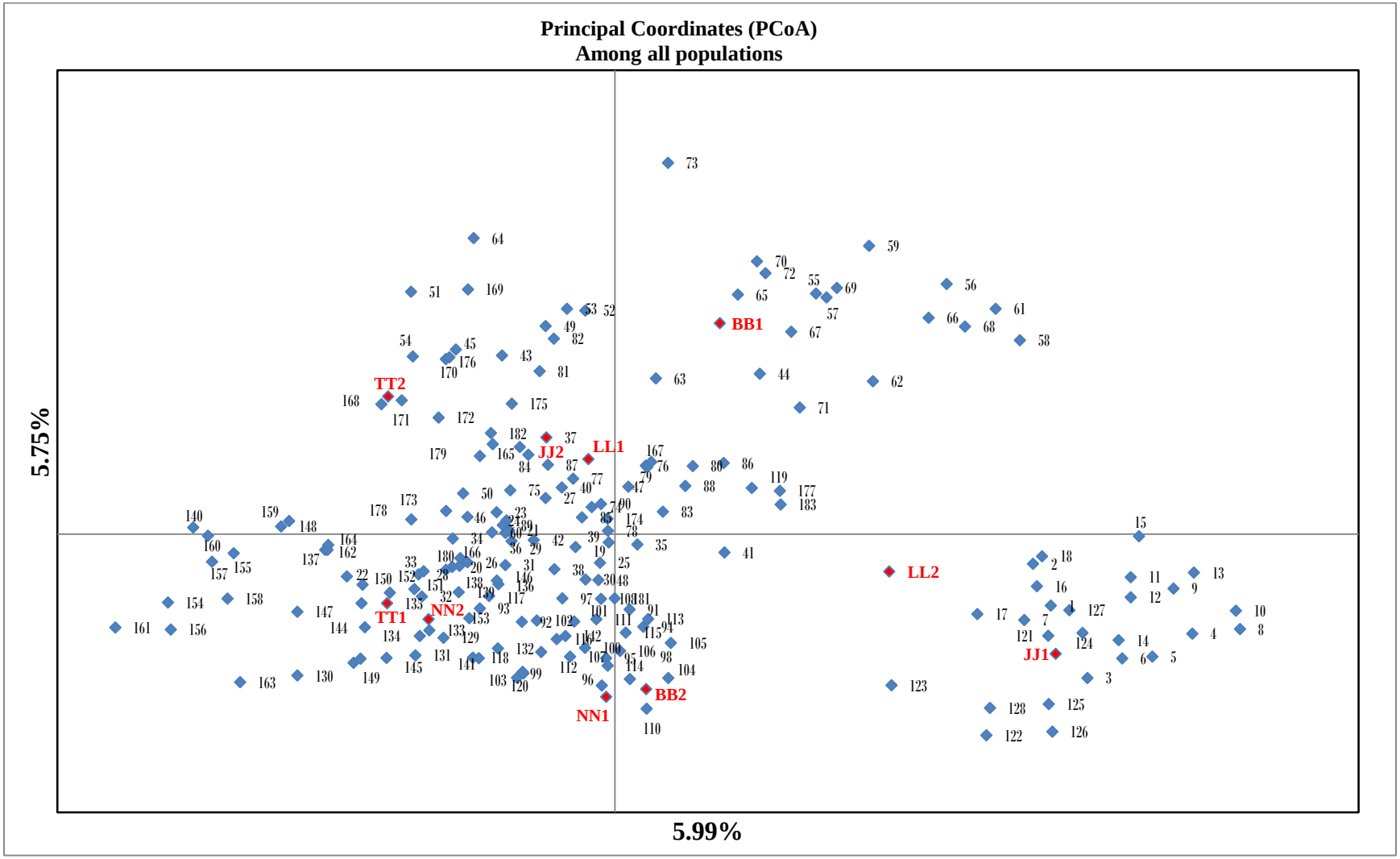

Figure 3.12. Principal coordinates analysis (PCoA) biplot showing the clustering of 192 *E. grandis* genotypes (including 10 parents; JJ, TT, BB, LL and NN) from the 5 sites. Variation percentages explained by PC1 and PC2 were 5.99% and 5.75% respectively. Numbers 1-36 indicate James Finlay progenies from parents JJ1 and JJ2; 37-72 Londiani Kamara progenies from LL1 and LL2; 73-108 Nyeri Kabarage progenies from BB1 and BB2; 110-146 Nyeri Kiandanguro progenies from NN1 and NN2;147-183 Turbo progenies from TT1 and TT2.

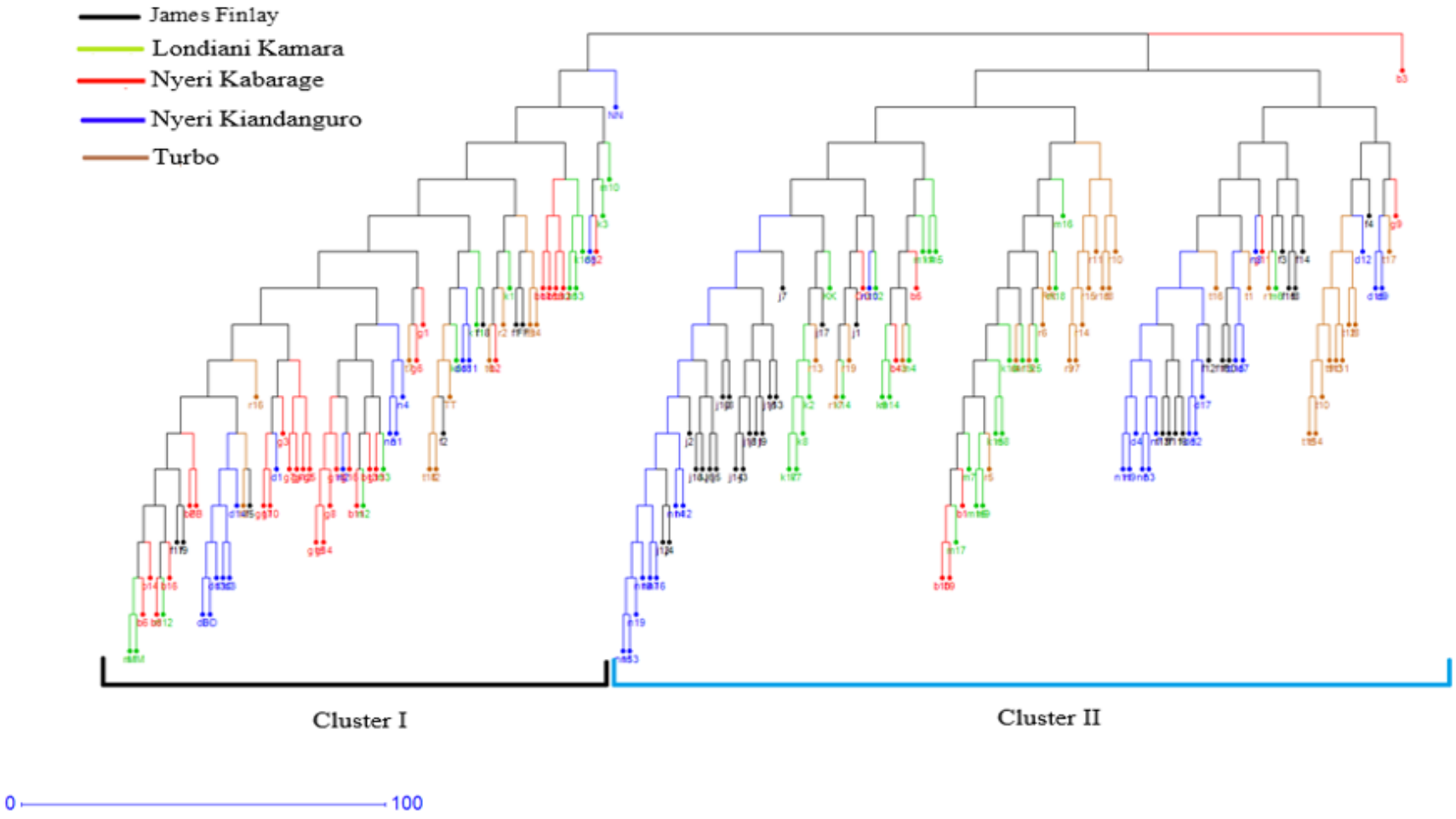

Figure 3.13. Unweighted neighbor joining tree using simple coefficient of dissimilarity based on 17 microsatellite loci for 192 *E. grandis* genotypes comprising parents and progenies from 5 main seed collection zones (black=James Finlay, green=Londiani Kamara, red=Nyeri Kabarage, blue=Nyeri Kiandanguro and brown=Turbo)

3.4.3.1 Analysis of Molecular Variance of James Finlay *E. grandis* genotypes

James Finlay AMOVA for 2 families along with their distribution (Fig 3.14) were summarized in (Table 3.15). The genetic variability of 17% was attributed to differences among populations, 0.0% among individuals and 83% within individuals (Table 3.15). The Fixation index (F_{ST}) was 0.178 indicating moderate genetic differentiation, intra inbreeding value (F_{is}) -0.072 signifying no in breeding among individuals, inter inbreeding value (F_{it}) 0.119 suggesting high genetic variations among populations, number of migration per generation (*N*m) 1.156 indicating very low gene flow and broad sense heritability (H^2) 0.1181 showing low genetic influence on the genotypes.

Table 3.15 AMOVA table indicating variations in James Finlay of *E. grandis* genotypes

Source	df	SS	MS	Est. Var.	%
Among Pops	1	56.618	56.618	1.339	17%
Among Indiv	36	206.842	5.746	0.000	0%
Within Indiv	38	252.000	6.632	6.632	83%
Total	75	515.461		7.970	100%
F_{ST}=0.178 Fis= -0.072	Fit=0.119	*N*m=1.156	H^2=0.1181		

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation; H^2=Broad Sense Heritability

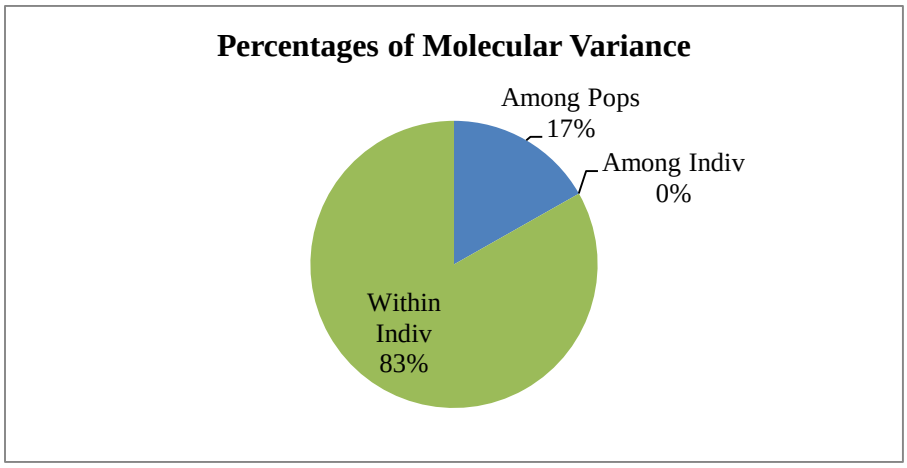

Figure 3.14. AMOVA pie chart indicating genotype variations in James Finlay

3.4.3.2 Analysis of Molecular Variance of Nyeri Kabarage *E. grandis* genotypes

Nyeri Kabarage AMOVA for 2 families along with their distribution (Fig 3.15) were summarized in (Table 3.16). The genetic variability of 11% was attributed to differences among populations, 0.0% among individuals and 89% within individuals (Table 3.16). The Fixation index (F_{ST}) was 0.116 indicating moderate genetic differentiation, intra inbreeding value (F_{is}) -0.019 signifying no in breeding among individuals, inter in breeding value (F_{it}) 0.099 suggesting high genetic variations, number of migration per generation (*N*m) 1.912 indicating very low gene flow and broad sense heritability (H^2) 0.094 showing low genetic influence on the genotypes.

Table 3.16. AMOVA table indicating variations in Nyeri Kabarage of *E. grandis* genotypes

Source	df	SS	MS	Est. Var.	%
Among Pops	1	38.803	38.803	0.853	11%
Among Indiv	36	230.316	6.398	0.000	0%
Within Indiv	38	252.500	6.645	6.645	89%
Total	75	521.618		7.497	100%
F_{ST}=0.116 Fis= -0.019	Fit=0.099	*N*m=1.912	H^2=0.0904		

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation; H^2=Broad Sense Heritability

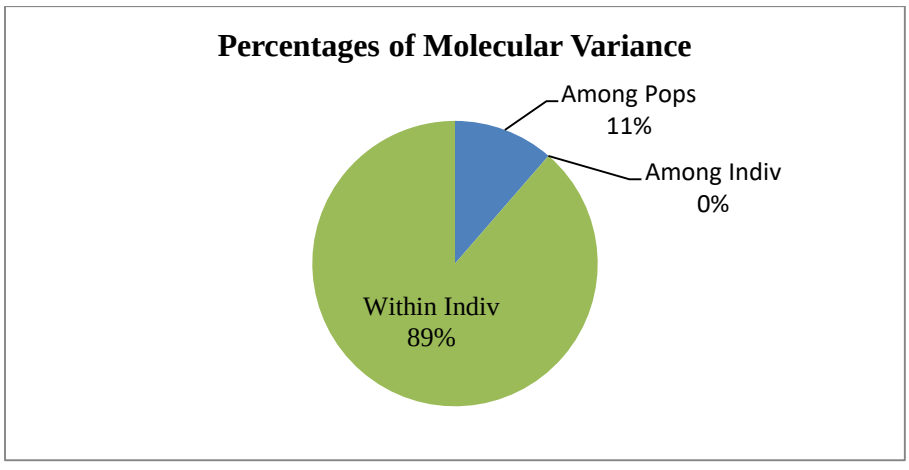

Figure 3.15. AMOVA pie chart indicating genotype variations in Nyeri Kabarage

3.4.3.3 Analysis of Molecular Variance of Nyeri Kiandanguro *E. grandis* genotypes

Nyeri Kiandanguro AMOVA for 2 families along with their distribution (Fig 3.16) were summarized (Table 3.17) The total genotype genetic variability of 3.0% was attributed to differences among populations, 3.0% among individuals and 94% within individuals. (Table 3.17). The Fixation index (F_{ST}) was 0.034 indicating very low genetic differentiation, intra inbreeding value (F_{is}) 0.026 and inter in breeding value (F_{it}) 0.06 suggesting high genetic variations, number of migration per generation (*N*m) 7.081 indicating moderate gene flow and broad sense heritability (H^2) 0.0532 showing low genetic influence on the genotypes.

Table 3.17. AMOVA table indicating variations in Nyeri Kiandanguro of *E. grandis* genotypes

Source	df	SS	MS	Est. Var.	%
Among Pops	1	19.111	19.111	0.281	3%
Among Indiv	37	302.082	8.164	0.210	3%
Within Indiv	39	302.000	7.744	7.744	94%
Total	77	623.192		8.235	100%
F_{ST}=0.034 Fis= 0.026	Fit=0.06	*N*m=7.081	H^2=0.0532		

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation, H^2=Broad Sense Heritability

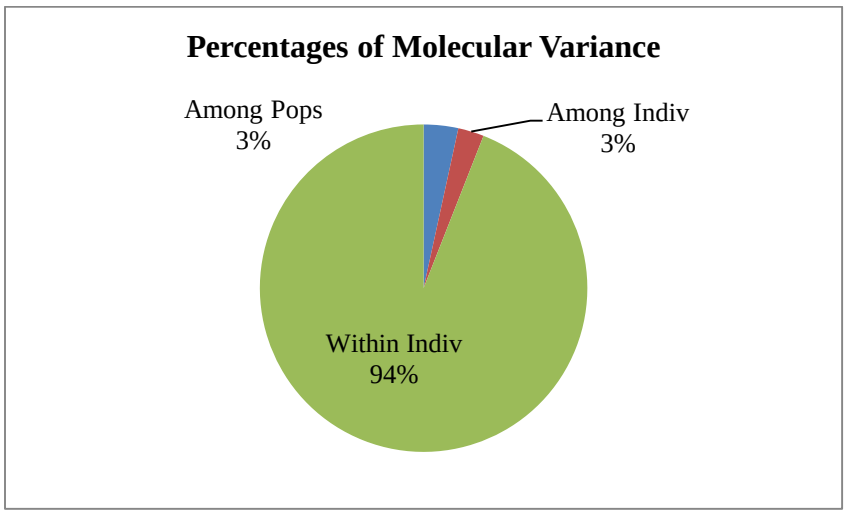

Figure 3.16. AMOVA pie chart indicating genotype variations in Nyeri Kiandanguro

3.4.3.4 Analysis of Molecular Variance of Londiani Kamara *E. grandis* genotypes

Londiani Kamara AMOVA for 2 families along with their distribution (Fig 3.17) were summarized (Table 3.18) The genetic variability of 3.0% was attributed to differences among populations, 0.0% among individuals and 97% within individuals (Table 3.18). The Fixation index (F_{ST}) was 0.034 indicating moderate genetic differentiation, intra inbreeding value (F_{is}) -0.011 signifying no in breeding among individuals, inter in breeding value (F_{it}) 0.024 suggesting high genetic variations, number of migration per generation (*N*m) 8.358 indicating moderate gene flow and broad sense heritability (H^2) 0.538 showing low genetic influence on the genotypes.

Table 3.18. AMOVA table indicating variations in Londiani-Kamara of *E. grandis* genotypes

Source	df	SS	MS	Est. Var.	%
Among Pops	1	18.697	18.697	0.284	3%
Among Indiv	36	284.737	7.909	0.000	0%
Within Indiv	38	307.000	8.079	8.079	97%
Total	75	610.434		8.363	100%
F_{ST}=0.034 Fis= -0.011	Fit=0.024	*N*m=8.358	H^2=0.0538		

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation; H^2=Broad Sense Heritability

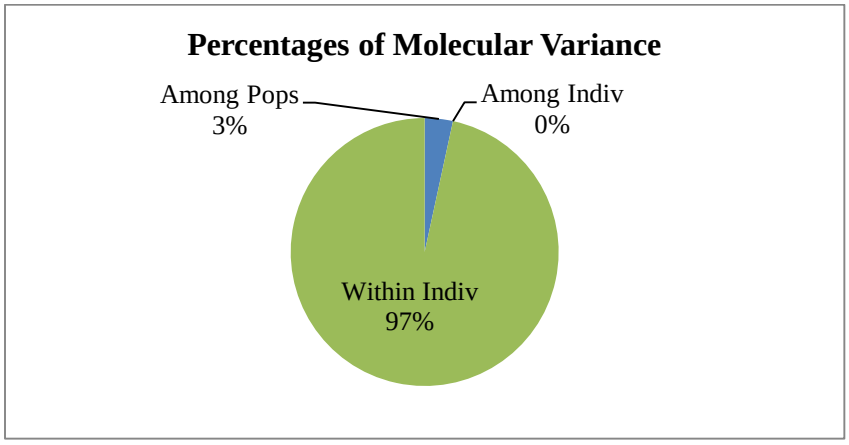

Figure 3.17. AMOVA pie chart indicating genotype variations in Londiani-Kamara

3.4.3.5 Analysis of Molecular Variance of Turbo *E. grandis* genotypes

Turbo AMOVA for 2 families along with their distribution (Fig 3.18) were summarized (Table 3.19). The genetic variability of 3% was attributed to differences among populations, 2.0% among individuals and 95% within individuals (Table 3.19). The Fixation index (F_{ST}) was 0.029 indicating very low genetic differentiation, intra inbreeding value (F_{is}) 0.019 and inter in breeding value (F_{it}) 0.047 suggesting high genetic variations, number of migration per generation (*N*m) 8.358 indicating moderate gene flow and broad sense heritability (H^2) 0.0508 showing low genetic influence on the genotypes.

Table 3.19. AMOVA table indicating variations in Turbo of *E. grandis* genotypes

Source	df	SS	MS	Est. Var.	%
Among Pops	1	17.411	17.411	0.238	3%
Among Indiv	37	300.422	8.120	0.150	2%
Within Indiv	39	305.000	7.821	7.821	95%
Total	77	622.833		8.208	100%
F_{ST}=0.029 Fis=0.019	Fit=0.047	*N*m=8.358	H^2=0.0508		

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation; H^2=Broad Sense Heritability

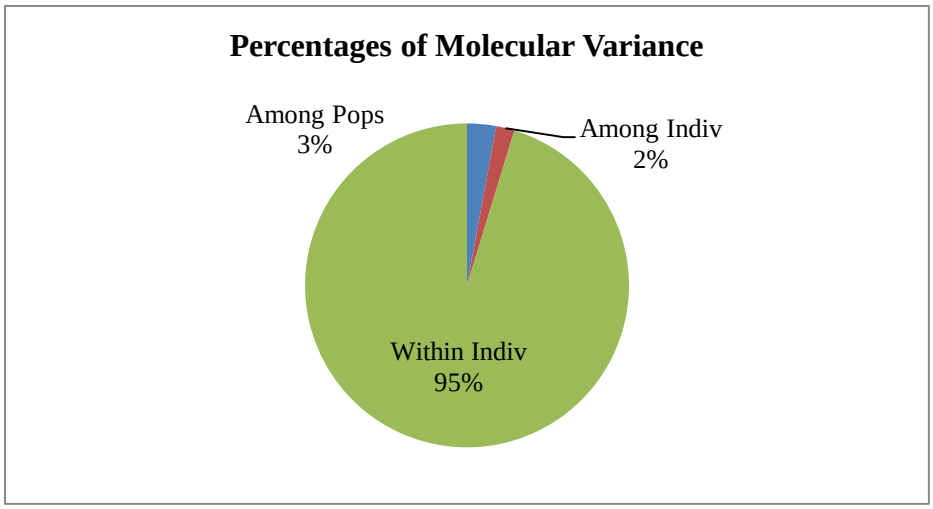

Figure 3.18. AMOVA pie chart indicating genotype variations in Turbo

3.4.3.6 Analysis of Molecular Variance among all populations

The AMOVA for 10 families along with their distribution (Fig 3.19) were summarized for all populations (Table 3.20). The total genotype genetic variability of 4.0% was attributed to differences among populations across the 5 sites, 10% among individuals and 86% within individuals (Table 3.20). The Fixation index (F_{ST}) was 0.036 indicating very low genetic differentiation, intra inbreeding value (F_{is}) 0.106 and inter in breeding value (F_{it}) 0.138 suggesting high genetic variations, number of migration per generation (*N*m) 6.761 indicating moderate gene flow and broad sense heritability (H^2) 0.0255 showing low genetic influence on the genotypes.

Table 3.20. AMOVA table indicating *E. grandis* genotypes variations among the 5 sites

Source	df	SS	MS	Est. Var.	%	*p* values
Among Pops	4	58.594	58.594	0.264	4%	0.001
Among Indiv	190	1500.833	7.899	0.758	10%	0.001
Within Indiv	192	1225.500	6.383	6.383	86%	0.001
Total	383	2784.927		7.405	100%	
F_{ST} = 0.036 Fis=0.106 Fit=0.138 *N*m = 6.761 H^2=0.0255						

df = degree of freedom; SS = sum of squares; MS = squares; Est. var. = estimate of variance, (%) = percentage of total variation; F_{ST} = Fixation index; Fis= Intra inbreeding value; Fit= Inter inbreeding value; *N*m = Number of migration per generation, H^2=Broad Sense Heritability

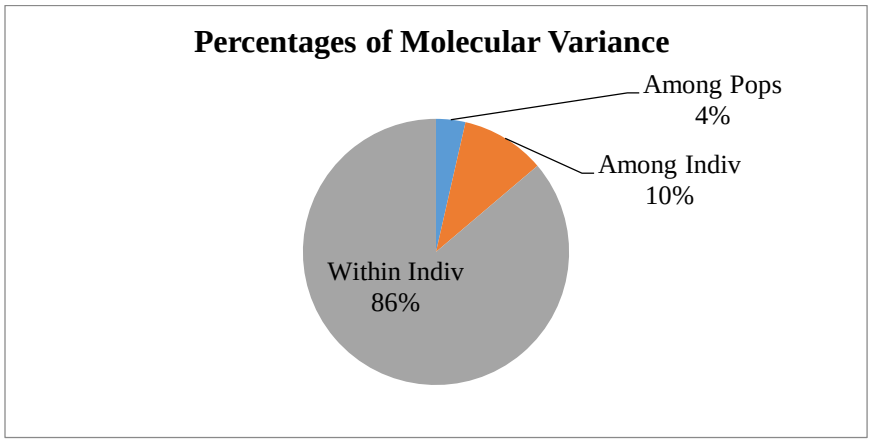

Figure 3.19. AMOVA pie chart indicating genotype variations among the 5 sites

3.5 Discussion

DNA of *E. grandis* parent and progeny genotypes was of fairly good quality and quantity

The DNA from the 5 sites had a mean concentration of 39.408 ng/μL, absorbance ratios OD 260/280 was as 1.54 while OD 230/260nm was 0.81, which varied with the expected ranges of OD 260/280 from 1.6 to 2.0 while OD230/260 ratios < 0.6 (O'Neill et al., 2011). The OD values indicated presence of very little DNA contamination which can be attributed to minor mishandling during isolation, but microsatellites are capable of using little quantity and low quality DNA for genetic fingerprinting because it is a PCR-based marker (Selkoe and Toonen 2006; Brondani et al., 1998; McDonald and Potts, 1997).

Low allele frequency indicated possible future exposure of Kenyan *E. grandis* genotypes to increased homozygosity

The allele frequency defined as the rate of gene variant incidence at a given locus in a particular population (Gillespie et al., 2004) was low for the Kenyan genotypes indicating possible future exposure to increased homozygosity. Major allele frequency values for the 17 SSR markers across the 5 sites for 192 *E. grandis* genotypes averaged to 0.43 for parents and 0.33 for progenies indicating low occurrence of gene variants (Gillespie, 2004). Similar low variability of 0.3 was previously reported by Brondani et al. (1998) for *E. grandis* and *E. urophylla* concluding that low allelic frequency with moderate homogenous distribution has a significant effect on expected heterozygosity. Song et al. (2016) reported an overall mean of 0.075 concluding that low allelic frequency contributes to a weak population structure in *E. grandis*. Comparatively, major allelic variations within populations (0.45) was higher compared to between populations (0.25), signifying that genotypes within populations have got a higher genetic potential for hybridization programs compared to among populations (Pojskic, 2018; Meyers et al., 2005). Generally, low

allele frequency indicated that the probability of particular alleles to occur severally in populations across the main seed collection zones was minimal.

High allelic richness indicated Kenyan germplasm is suitable enough for future hybridization programmes

High allelic richness which is used to measure loss of diversity as a result of genetic bottleneck within populations (Luikart et al., 1998; El Mousadik and Petit, 1996) indicated Kenyan germplasm is suitable enough for future hybridization programmes. The allelic richness for both parents and progenies averaged to (Rt = 14.9) indicating high allelic diversity among genotypes, Kim et al. (2010) suggested that genotypes with average allelic numbers ranging from (Rt=11 to 26) can be considered to have high allelic variability. Additionally, relatively similar value of Rt = 14.3 has previously been reported by Jones et al., (2006) concluding that high allelic richness provides evidence of non-exposure to bottle neck effects in different populations and vice versa. Brondani et al. (1998) and Kirst et al. (2005) reported higher values of Rt = 16.3 and Rt =19.8, respectively, concluding that some allelic variability were attributed to the fact that SSRs may be favorably positioned in low-copy transcribed regions along plant genomes (Morgante et al., 2002). Conclusively, high allelic richness indicated that the *E. grandis* genotypes across the main seed collection zones in Kenya is sufficient to initiate germplasm enhancement for future breeding programs.

The CPTs have high genetic diversity suited for enhancing Eucalyptus breeding programs

High gene diversity and heterozygosity of the *E. grandis* genotypes, which determines the fitness potential and eventual long-term persistence in a population with possible creation new genotypes with desirable traits (Cavalcante et al., 2019; Frankham, 2005) indicated high levels of genetic variability for possible exploitation in future breeding programmes. The gene diversity value for

parents was 0.45 while progenies was 0.72, which indicated low fitness potential among parents, and high fitness potential among progenies. Slightly higher value of 0.71 was reported by Brondani et al. (1998) and Chaix et al. (2003) who concluded that *E. grandis* generally has high genetic variability. Similar results were reported for *E. marcucci* and *E. dunii*, with high gene diversity of 0.78 and concluded that the involved breeding populations had low levels of inbreeding (Poltri et al., 2003). These results indicate that progenies are more diverse than parents owing to hybridization with other *E. grandis* genotypes within sites and the progeny seemed fitter and more vigorous (Szczecinska, 2016; IIves et al., 2013; Leimu et al., 2006) suggestive of enhanced adaptability (De Villemereuil, 2019; Frankham, 2005). On the other hand, heterozygosity showed how much of external alleles have been introduced to the populations (Frankham, 2005). Heterozygosity values for the parents averaged to (H= 0.81) and progenies (H= 0.80) indicating very high genetic variability. Astorga et al. (2004) who reported (H=0.80) concluding that *E. grandis* generally has low chances of incurring inbreeding depression while Chaix et al. (2003) reported slightly lower value (H=0.71) concluding that *E. grandis* is prone to contamination due to high outcrossing levels. The high heterozygosity values can be attributed to vigorous healthy trees which can attain maximum heights hence attracting efficient pollinators, ability to produce many flowers and discriminate selection of planting materials from base populations before planting (Willmer, 2011; Jones et al., 2006). Decisively, the high genetic variability indicated that the vital traits are still well preserved among the Kenyan *E. grandis* genotypes which can be exploited in future breeding programmes to develop hybrids with good performance.

Select highly informative SSR markers important for genotype discrimination

High PIC levels indicated adequate differentiation power among SSRs to discriminate *E. grandis* genotypes. The PIC values for the 17 SSR markers were 0.55 for parents and 0.75 for progenies with a total of 253 alleles, indicative of the abundance of the markers in the *E. grandis* genome. Previous studies have documented nearly similar results (Chen et al., 2020; El-Awady, 2012; Kirst et al., 2004; Brondani et al., 1998) concluding the SSR markers being highly informative and had the capability of discriminating 192 *E. grandis* genotypes from seed collection zones. Tillault and Yevtushenko, (2019) reported slightly lower value of 0.675 concluding that the markers were also informative. Despite the markers being very informative, there were challenges in distinguishing between true and repetitive peaks, which are in agreement with reports by Bousalem et al. (2006) and Dewoody et al. (2006).

Low genetic differentiation of *E. grandis* genotypes between sites indicates high similarity between genotypes

The significant genetic differentiation within individuals of 86% in contrast to low, 4%, genetic differentiation indicated the high similarity between genotypes across sites showing low genetic differentiation. The low genetic differentiation could be attributed to similarity of germplasm planted across the sites while the high variation within sites was attributed to natural hybridization between genotypes. Similar reports have been made by Leitch, (2008), Valbuena-Carbaña, (2008) and Cheplick and Kane, (2004) who attributed low genetic variability to reduced pollen viability and mobility, poor coppicing ability or stiff competition among genotypes. Earlier studies (da Costa et al., 2013; Jones et al., 2006; Steane et al., 2006) have also reported similar results for *E. grandis* populations and concluded that possible polycross occurrences in the field may have resulted to creation of new genotypes hence affecting genetic diversity within and among populations. Since *E. grandis* is an outcrossing species, it is likely that higher genetic exchange

occurred among individuals within experimental sites (Utomo et al., 2009). Evidently, the genetic diversity among the genotypes is satisfactorily large enough to facilitate exploitation in future Kenyan breeding programs meant in developing superior *E. grandis* hybrids.

Low broad sense heritability indicates high environmental influence

The low broad sense heritability which is the relationship between phenotypic and genetic composition in a given population (Fonseca et al., 2010), could have been as a result of epistasis or dominance (Wray and Visscher, 2008). The broad sense heritability across the sites was very low (H^2=0.026) and was attributed to minimal genetic variation at the appropriate loci, or similarity of phenotypes from the environments where the genotypes were developed (Andrews, 2010). This shows that Kenyan genotypes have low repeatability in different environments, since genotype trait performance is robustly linked to environmental influence (Frank et al., 2016).

3.6 Conclusion

The study documented high level of genetic and allelic diversity using microsatellites standing at 0.84 and 14.9 respectively. Genotypes from James Finlay were the most diverse hence they would be useful sources for heterosis in terms of greatly enhancing of height and DBH. Genotypes from Nyeri-Kiandanguro, Nyeri-Kabarage, Londiani-Kamara and Turbo were closely related signifying they are good in targeting specific desirable traits in a particular breeding program. Therefore, the information generated from this study will build a platform for eucalypts improvement in Kenya for commercial and environmental conservation purposes.

CHAPTER FOUR: GENERATION OF F1 PROGENIES FROM *E. grandis* x *E. urophylla*

4.1 Abstract

Tree breeding programs for Eucalyptus have mainly focused on the improvement of pure species but the largest genetic gains in forestry plantations have been attributed to hybrid plant materials. Currently, there is no *Eucalyptus* spp. hybridization program in Kenya although it is important. This is so because Kenya has previously introduced hybrids from South Africa but their performance did not fulfill the genotype by interaction effect leading to their overall poor performance for some clones. Hybridization of *E. grandis* with other species has previously been done in other countries. The hybrids developed in those countries have resulted to better performing trees with different traits. The objective of the study was to determine the success level of interspecific hybridization between *E. grandis* and *E. urophylla* from Muguga, Kenya. A total of 2 mature *E. grandis* trees served as male along with 21 different mature *E. urophylla* trees as the female parents. Data was collected on various parameters including morphological characteristics of pollen and mother parents, pollen parents' germination rate on nutrient agar medium, flower diameter and length of artificially and naturally pollinated flowers. From the total of 21 hybridized mother parents, 6 produced hybrids by conventional technique and they comprise trees EU2, EU3, EU4, EU7, EU13 and EU16 with a success rate of around 28.6% indicating that more Eucalyptus breeding programs can be established using Kenyan germplasm. This study confirms that different needs such as fast growth and disease resistance can be addressed through hybridization of different eucalyptus species which have got desirable traits.

Key words: conventional technique, hybrids, interspecific hybridization

4.2 Introduction

Eucalyptus is a broad genus of flowering shrubs and trees in Myrtaceae family (Coppen, 2005) with over 700 species (Brooker, 2000) and many hybrid combinations (Ravi 2015), most of them are native to Australia. Eucalypts are considered successful due to their rapid growth rate, massive bioenergy production and ability to thrive in non-identical types of environments (Oliveira, 2018; Luzar, 2007; Maundu and Tengnans, 2005). Introduction of Eucalyptus species to Kenya was made in 1902 and among the species were *Eucalyptus grandis* known for fast growth at altitudes of between 1400 and 2200 m above sea level (Oballa et al., 2010) and *E. urophylla*, with fungal disease resistance such as Chrysoporthe canker (Soares et al., 2018; Gryzenhout et al., 2006), prevalent in Kenya (Nakabonge, 2006). Due to climate change effects (Romm, 2018), increased demand of wood and its products (Nyangena, 2018) and few number of plantation species (pines, eucalypts and cypress) used in Kenya (Oeba et al., 2017), there was need to enhance Eucalyptus germplasm diversity through selection, introduction and hybridization (Drake, 2014). This notwithstanding, to date, there have been no public sector hybridization programs for Eucalyptus species and yet it is ranked among the three most important plantation species in Kenya (KFDP, 1999; PSRA 1999).

Selection of candidate plus trees and introductions have previously been made by Kenya Forestry Research Institute (KEFRI) and private companies such as Komaza Forestry Limited (Cheboiwo, 2011). Introduction of select hybrid clones, 16 *E. grandis* by *E. camaldulensis* (GCs) and 3 *E. grandis* by *E. urophylla* (GUs), was made in 2002 by Tree Biotechnology and KEFRI was tasked to evaluate their performance over several agro-ecological zones (AEZ). Results showed good performance and suitability for three clones across AEZ and this was attributed to few select clones used and variability in environmental conditions from the source. In the case of hybridization, it is important because it results to improved heterosis, adaptability and complementarity compared to

pure species (Kain, 2003). Many Eucalyptus hybrids have been developed worldwide, aimed at addressing different needs. *Eucalyptus grandis* x *E. camaldulensis* combines fast growth and drought tolerance (Shen, 2000), *E. grandis* x *E. globulus* combines fast growth and good pulp quality (Griffin et al., 2000), *E. urophylla* x *E. globulus* combines fungal disease resistance and good pulp quality (Shen, 2000) while *E. grandis* x *E. urophylla* are widely produced for good quality wood, high pulp yield and wide adaptability across AEZ (Bison et al., 2006; Vigneron and Bouvet, 2000). Nonetheless, when producing *Eucalyptus* hybrids there is a limitation of how to produce them for maximum returns (Dungey and Nikles, 2000).

There are several methods used for control pollination including conventional technique, one stop pollination and artificial induced protogyny (Randall et al., 2015; Collins and Callister, 2010). Conventional technique uses natural flower receptivity which varies among species, one-stop induces receptivity by making a small cut on the stigma and artificial induced protogyny is a new technique that does not require emasculation (Randall et al., 2012; Horsley et al., 2010). These techniques have been used in Brazil for *E grandis* × *E. urophylla, E. grandis* × *E. globulus, E. urophylla* × *E. globulus* under nursery conditions with success rates from 39.8 to 79.2% while in Australia under greenhouse conditions for *E. grandis* × *E. camaldulensis* showed 21.2 to 44.3% success (Assis, 2005). Other studies have also reported success on these techniques ranging from 4 to 90% (Dickinson et al., 2010; Patterson et al., 2004; Barbour and Spencer 2000; Harbard et al., 2000 a, b; Williams et al., 1999). This study focused on *E. urophylla* x *E. grandis* combination using conventional and one-stop pollination techniques in Muguga, Kenya to determine the effect of controlled pollination between *E. grandis* and *E. urophylla* genotypes on seed production to enhance future breeding programmes.

4.3 Materials and methods

4.3.1 Plant materials

A pure *E. urophylla* stand located at KEFRI Muguga was used owing to closeness to the research station and ease of access to the flowers since the stand was established using grafted material hence shorter trees. The pollen parent used was *E. grandis* also from a presumably pure tree stand of the species in KEFRI Muguga. The pollen parent was selected based on its closeness to KEFRI research station but the trees were tall. Two *E. grandis* trees as pollen parents and 21 *E. urophylla* female parents were used in the experiments using North Carolina Design I (NCDI) to improve success chances of the controlled pollination. Each parameter was assessed by either 2 or 3 replicates with a sample size (n=853) and the hybridization period lasted between August and January 2019 but the first and second harvest was done in January and February 2020 respectively to allow the hybridized seeds to mature.

Two *E. grandis* genotypes were selected as pollen parents of 5.5 years in age and 21 *E. urophylla* (maternal parents) of 4 years in age from Muguga, KEFRI orchard. The trees were selected by keen consideration on floral copiousness and accessibility of mature flowers at anthesis for hand-pollinations (Horsley et al., 2009). The progeny of the crosses made were then planted in a screen house to obtain leaves for assessment of their genetic composition.

4.3.2 Trial establishment site

The experiments were carried out on mature trees situated in Muguga, Kenya plantation stand orchard (Image 4.1). Muguga is typically made up of V-Vegetation, situated in Kiambu County. Its coordinates are 1°13'0" S and 36°37'60" or -1.21667 and 36.6333. Its UTM location is BU36 and JOG reference is SA37-05 with ordinary zonal time as UTC/GMT+3. It is located at an elevation of 2,070 m asl with average temperature of 15.8ºC, rainfall 991mm per annum

(climatedata.eu) and dominant soil type as nitisols which have good aeration and moisture storage capacity (Gachene and Kimaru, 2003).

Image 4.1 Experimental site for the controlled pollination studies of *E. grandis* and *E. urophylla*

4.3.3 Pollen collection, extraction and storage

Pollen was sourced from two *E. grandis* genotypes where 428 flowers from 26 branches were selected on the first pollen source (EG1) while 239 flowers from 17 branches on second pollen source (EG2) since they were flowering over the collection period. To prevent contamination, open flowers were detached from the branches and the ripe closed ones left in a desiccator with silica gel to dry for about 48 h at room temperature. The dried opened flowers were gently agitated on a 150-micron sieve with aluminum foil beneath to collect fine pollen which was kept in falcon tubes and stored at 4°C for short-term preservation and excess stored at -16°C for long-term preservation as described by Horsley et al., (2007).

4.3.4 *In-vitro* germination

Liquid medium consisting of 50g sucrose, 1.25g agar complemented by 0.0375 mg L^{-1} boric acid for 250 mL were used for *in-vitro* germination as described by Shivanna, (2019). Pollen germination rate was scored using optimus light microscope (model D21-CB) after 24 hours at 400x (10x eye piece lens and 40x objective lens) magnification as similarly used by Barth et al., (2010). Pollen germination rate was estimated according to the protocol described by Shivanna, (2019). The following formula was used to calculate the germination rate:

Pollen germination rate (PGR) = Number of germinated pollen/Total number of pollen *100

4.3.5 Seed set and seed extraction

All mature fertilized fruits after pollination were harvested after 26-30 weeks especially when the brown star-shaped pattern has formed on top of the fruit after the styles dried out. The harvested fruits were kept in envelopes and petri-dishes so that drying takes place in order to release the hybrid seeds. Viable seeds in each mature fertilized fruit were totaled up. Irregular, solid and dark seeds were considered viable as opposed to flat, light-brown chaff (Image 4.2). The seeds produced were planted for the purposes of molecular fingerprinting.

Image 4.2. Photo A showing dark irregular shaped GU seeds under 40X magnification by optimus light microscope (model D21-CB) and B showing flat light-brown chaff.

4.3.6 Raising GU hybrids and naturally pollinated seeds as checks

The hybrids generated as a result of crossing *E. grandis* with *E. urophylla* were raised by sterilizing sand at 121ºC under 15 psi for 60 minutes to eliminate any harmful microorganisms such as fungi and bacteria and then allowed to cool for 48 hours. The sterilized sand was kept in labelled pots according to their respective families and saturated with sterilized water which was prepared through reverse osmosis. The seeds were spread on sand surfaces on all pots and regular watering was done daily using a hand spray in order to facilitate germination. The germinated seedlings were then transferred to seedling pots 4 weeks later in order to allow the leaves to attain the required sizes for molecular fingerprinting work.

4.3.7 Data collection

Data collected was broadly grouped into morphological characteristics of pollen and mother parents, pollen collection germination and hybridization, seed setting, harvesting and seed emergence rate.

4.3.7.1 Morphological characteristics of the parents

I. Height (m), DBH (cm) and total number of flowers per individual mother and pollen trees were estimated.

4.3.7.2 Pollen collection germination and hybridization

I. Total number of flowers from the pollen parents were counted and their weight measured using a sensitive electric balance.
II. Physical appearance of the pollen from the parents were compared and contrasted visually.
III. Pollen germination rate was estimated by recording the germinated against non-germinated pollen grains on the liquid media.

Pollen Germination Rate= Number of germinated pollen/Total number of pollen *100

IV. Pollination methods success rate was estimated by assessing the number of total branches and flowers that survived up to harvesting compared to the ones present during emasculation.

4.3.7.3 Seed setting

I. Diametre and length changes were estimated by using digital caliper in controlled pollinated flowers against the naturally pollinated flowers.

II. Style health performance was scored visually at the seed set period phase on the scale (1-5) where 1=green and healthy styles due to complete withering and 5 =total aborted styles.

Table 4.21 Scoring scale for style health performance during seed setting

Score	Visual rating on style health	Response to hybridization
1	Green colour	Healthy
2	Green-yellow	Relatively healthy
3	Yellow and shriveled	Moderately unhealthy
4	Brown and shriveled	Severely unhealthy
5	Aborted	Total withering

Source: KEFRI-Kenya Forestry Research Institute

III. Stigma performance was estimated by visual counting of the remaining ones against the withered.

4.3.7.4 Harvesting

I. Mature fruits and hybrid seeds were visually counted after seed set and compared to the healthy flowers at the onset of hybridization in order to estimate success rate of the entire intraspecific hybridization.

4.3.7.5 Seed emergence rate

I. Seed germination rate comparison was obtained by counting number of seeds sowed against the number of seeds germinated.

   Seed Germination Rate= Number of germinated seeds /Total number of sowed seeds*100

4.3.8 Data analysis

Coding was done in Microsoft excel and analysis carried out using GenStat (15^{th} Edition) where analyzed data was presented in form of tables. The level of significance was conducted at $p<0.01$, $p<0.05$ and $p>0.05$ using Analysis of Variance (ANOVA) and t-test**,** the means were compared using Fisher's protected Least Significant Difference (LSD) at 5% level.

4.4 Results

4.4.1 Morphological characteristics of pollen and mother parents

In this study, 23 parents (21 mother and 2 pollen) were used to help determine if morphology of the genotypes may affect the eventual hybrids produced and the following results were reported:

4.4.1.1. Morphological characteristics of mother parents

There were significant differences ($p<0.05$) among the heights of *E. urophylla* maternal genotypes where EU9 had the highest height, 6.3 m while EU17, EU12, EU1 and EU2 were the lowest (Table 4.2). The DBH also showed significant differences ($p<0.05$) where EU9 recorded the highest DBH of 81cm while the lowest were EU8, EU17 and EU2 (Table 4.2). There were also variations in flower numbers which showed significant differences ($p<0.05$) where EU9 recorded the highest flower number of 195 while the lowest were EU4, EU17 and EU14 (Table 4.2). The initial CV for number of flowers at anthesis was initially 108.6% but transformation was done using Log_{10} to normalize the data (Table 4.2).

Table 4.22. Morphological characteristics of mother parents

Mother parents	Height (m)	DBH (cm)	No. of flowers
EU_1	4.9	55	20
EU_2	4.7	47	23
EU_3	5.3	49	49
EU_4	5.8	53	8
EU_5	5.7	56	33
EU_6	5.5	59	28
EU_7	5.7	53	31
EU_8	6.1	45	10
EU_9	6.3	81	195
EU_10	5.9	72	10
EU_11	5.7	65	70
EU_12	4.9	58	34
EU_13	5.7	55	119
EU_14	6.1	58	3
EU_15	6.2	71	37
EU_16	5.7	54	71
EU_17	4.9	46	7
EU_18	5.6	52	53
EU_19	5.8	55	30
EU_20	5.8	54	23
EU_21	5.5	57	10
Mean	5.6	57	41.1
p-value	<0.05	<0.05	<0.05
LSD	0.9	18.3	74.8
CV (%)	7.9	15.6	30.8

CV: Coefficient of Variation; Least Significant Difference; *= significant at p-value threshold of ($p<0.05$), respectively. The figures show maternal parents' height, DBH and number of flowers at anthesis relationships.

4.4.1.2. Height, DBH and number of flowers relationships between *E. urophylla* maternal genotypes

Moderate positive correlation ($R=0.526$, $p<0.05$) was observed between height and DBH (Table 4.3). There was also a positive correlation ($R=0.527$, $p<0.05$) between DBH and number of flowers (Table 4.3). However, no ($R=0.307$, $p>0.05$) correlation was observed between height and number of flowers (Table 4.3).

Table 4.23. Correlation matrix between height, DBH and number of flowers of *E. urophylla* maternal genotypes

		Correlation Matrix		
		Height (m)	DBH (cm)	No of flowers
Correlation	Height (m)	-		
	DBH (cm)	0.5255	-	
	No.of flowers	0.3070	0.5269	-
Sig $_{0.05}$	Height (m)	-		
	DBH (cm)	0.0144	-	
	No.of flowers	0.1758	0.0141	-

4.4.1.3 Pollen parents

There were no significant differences ($p>0.05$) for height of *E. grandis* pollen parents and also flowers used for pollen extraction (Table 4.4). The DBH showed significant differences ($p<0.05$) where EG2 recorded the highest DBH of 50cm while the lowest was 40cm in EG1 (Table 4.4). The CV for the number of flowers at anthesis was initially 40.1% but transformation was done using Log_{10} to normalize the data (Table 4.4).

Table 4.24. Morphological characteristics of the pollen parents

Pollen parents	**Height**	**DBH**	**No.of flowers**
EG_1	8.5	40	428
EG_2	9.1	50	239
Mean	8.8	45	334
p-value	<0.05	>0.05	>0.05
CV (%)	4.8	15.7	7.2

CV: Coefficient of Variation;*, ^{ns}= significant, not significant at p-value threshold of ($p<0.05$) and ($p>0.05$), respectively. The figures show pollen parents' height, DBH and number of flowers at anthesis relationships

4.4.2 Hybridization of the Eucalyptus species

4.4.2.1 Pollen collection and characteristics

Pollen collection was done only on two *E. grandis* trees due to limited availability of mature flowers at anthesis during off-season period from superior *E. grandis* stand in Muguga. Variations were evident for the number of flowers collected, where 428 and 239 flowers were collected for EG1 and EG2 (Table 4.3), respectively. About 428 flowers (Table 4.4) from EG1 were collected from 17 branches and weighed 11.52 g. Pollen from EG1 was fine in texture and bright-yellow in colour (Image. 4.3). The second pollen parent, EG2, had about 239 flowers (Table 4.4) from 10 branches and weighed 8.53 g. Pollen from EG2 was coarse and dark-yellow in colour (Image 4.3). Parent EG2 had fewer branches hence the fewer number of flowers. The extracted pollen was also examined for general appearance (Image 4.4) in order to determine its suitability for being trapped by sticky exudate during pollination.

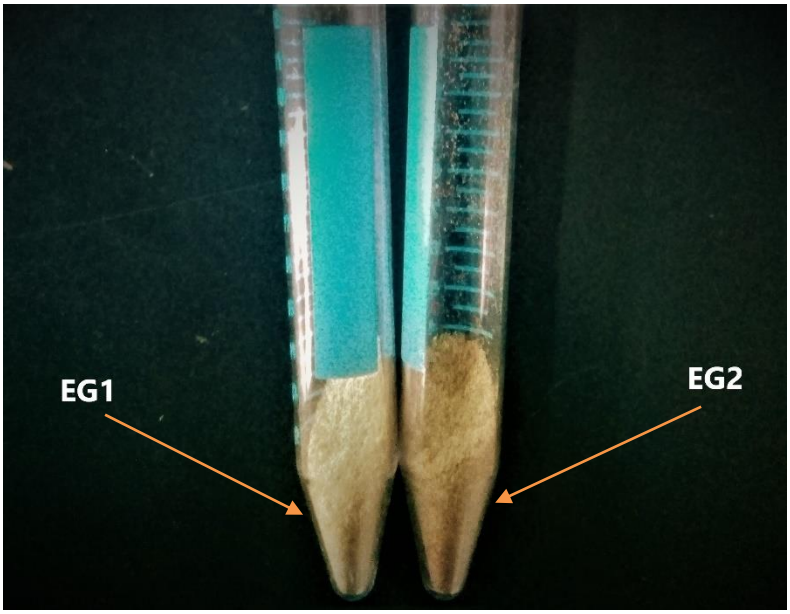

Image 4.3. *E. grandis* pollen from EG1 and EG2.

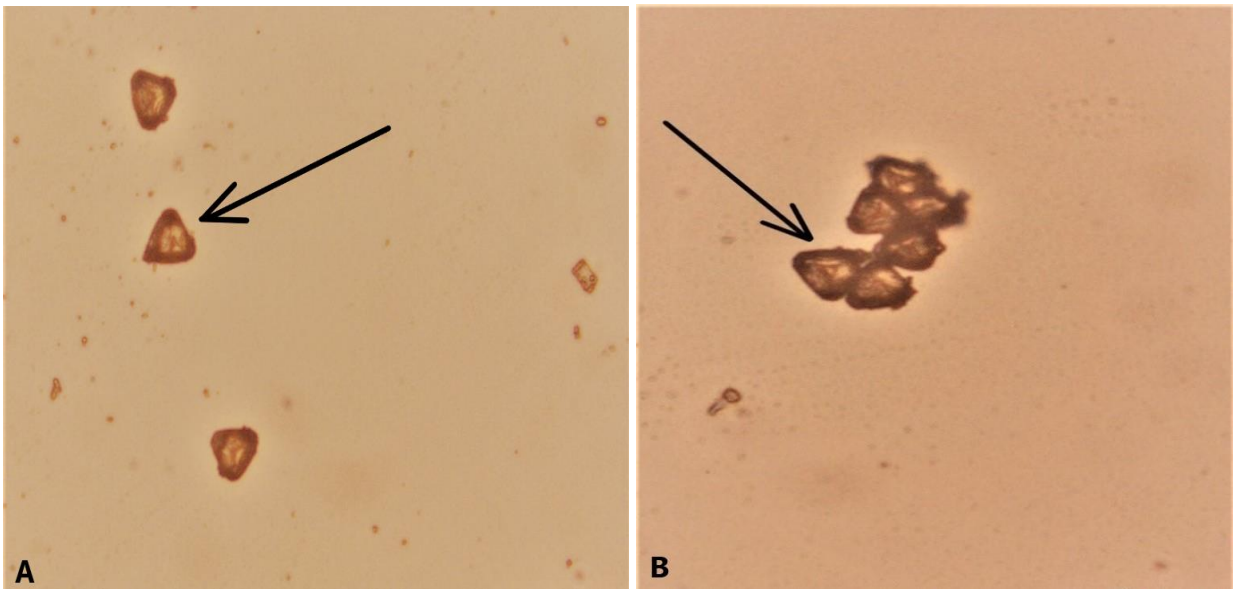

Image 4.4. Optimus light microscope (model D21-CB) image. A. Pentagonal structure of *E. grandis* pollen (EG1). B. showing (EG2) under 40X magnification.

4.4.2.2 Pollen germination on liquid media

The pollen germination percentage was done on EG1 and EG2 (Image 4.5), to determine viability of pollen to carry out successful pollination and hence fertilization. The mean germination rates were 73.7 and 75.7% but showed no significant ($p>0.05$) differences (Table 4.5).

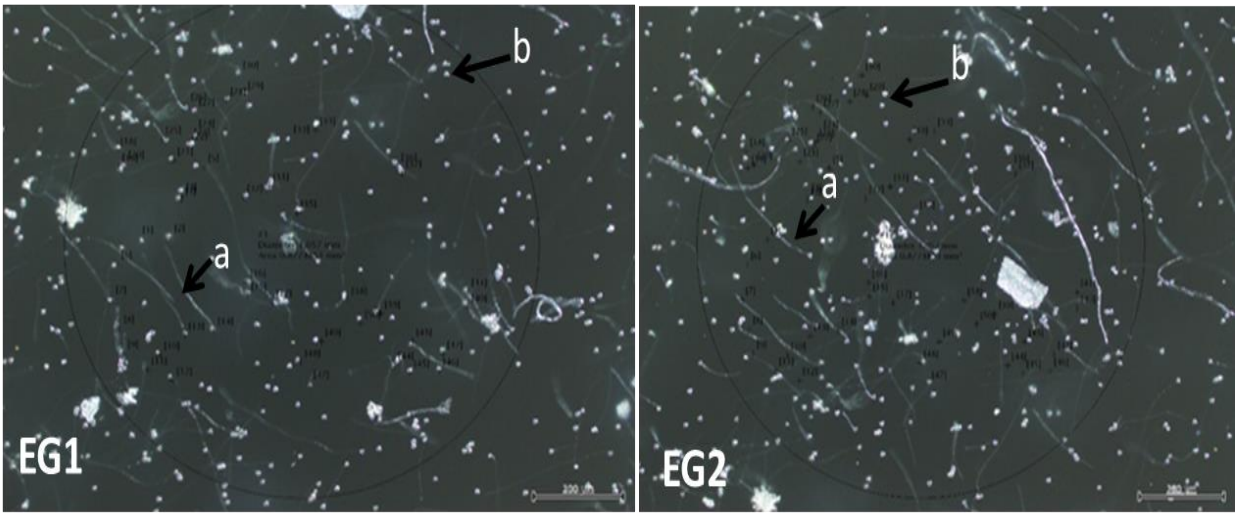

Image 4.5. Optimus light microscope (model D21-C) images. a) Germinated b) Ungerminated pollen grains of EG1 and EG2 under 40X magnification after 24 hours.

Table 425. Pollen parents' germination rate on liquid media

Pollen parents	Germination Rate (%)
EG_1	73.7
EG_2	75.7
Grand Mean	74.7
p-value	>0.05
CV (%)	1.9

4.4.3 Hybridization of Eucalyptus species using one-stop and conventional pollination techniques

Two trees were selected for one-stop pollination method due to fragility of the styles that snap when a slight cut is made on the stigma to release a sticky exudate and create opening for pollen application. This technique did not produce any hybrid since the two trees with 5 branches and 43 flowers involved dried up hence no further reporting was done using this technique (Appendix 1).

The conventional technique involved application of pollen on 810 flowers on 43 branches from 19 EU trees (Appendix 1). Eleven trees, which included 468 number of flowers on 25 branches, were hybridized using EG1 resulting to only four with seed set, while eight trees with 342 flowers from 18 branches were hybridized using EG2 resulting to two with seed set (Appendix 1). The naturally hybridized flowers on 6 EU trees were considered as controls for control pollinated flowers (Table 4.6; 4.7; 4.8). Following harvesting of the hybrids as a result of the two pollen parents, 6 trees produced hybrids (Table 4.8). A total of four trees were as a result of EG1 (Table 4.11) and two from EG2 (Table 4.11) and they were significantly ($p<0.05$) different.

4.4.4 Flower morphology

Eucalypts are naturally outcrossing species but since controlled pollination was conducted on different species, there was a need to determine the morphology of naturally- and control-pollinated flowers to find out if affected by the hybridization method used and its impact on future breeding programs. Control pollinated flowers had mean diameters ranging from 4.94 to 13.99mm from week 5 to 17 and they were significantly ($p<0.01$) different (Table 4.8), with the closest similarity observed between week 9 and week 13 (Table 4.6; 4.8). The flower lengths also showed varied performances ranging from 4.4mm to 16.77mm and they were significantly ($p<0.01$) different (Table 4.8), with the closest similarity observed between week 5 and week 9 (Table 4.6; 4.8). On the other hand, naturally pollinated flowers had diameters ranging between 5.38 mm and 16.37mm and they were significantly ($p<0.01$) different (Table 4.8), with the closest similarity observed between week 5 and week 9 (Table 4.7; 4.8). The flower lengths also showed varied performances ranging from 5.31mm to 14.95mm and they were significantly ($p<0.01$) different (Table 4.8), with the closest similarity observed between week 5 and week 9 (Table 4.7; 4.8).

Table 4.26. Flower diameter and length of controlled pollinated flowers

Time	Flower Diameters	Flower Lengths
Week 5	7.4	6.4
Week 9	8.6	7.3
Week 13	8.8	7.9
Week 17	9.1	8.7
Grand mean	8.5	7.6
p-value	>0.05	>0.05
CV (%)	7.3	7.9

Table 4.27. Flower diameter and length of naturally pollinated flowers

Time	Flower Diameters	Flower Lengths
Week 5	7.5	7.2
Week 9	8.0	7.8
Week 13	9.3	8.8
Week 17	9.9	9.7
Grand mean	8.7	8.4
p-value	<0.05	>0.05
LSD	1.7	*
CV (%)	4.5	6.5

Table 4.28. Flower diameter and length of controlled and naturally pollinated flowers

Pollination Method	Time	Flower Diameters	Flower Lengths
Control Pollination	Week 5	7.4	6.4
	Week 9	8.6	7.3
	Week 13	8.8	7.9
	Week 17	9.1	8.7
Natural Pollination	Week 5	7.5	7.2
	Week 9	8.0	7.8
	Week 13	9.3	8.8
	Week 17	9.9	9.7
	Grand mean	8.6	8.0
	p-value	<0.01	<0.01
	LSD	0.9	1.1
	CV (%)	5.9	8.3

4.4.5 Stigma and style health performance during seed set period

Stigma and style health is important since they serve as indicators of successful fertilizations after full seed set.

4.4.5.1 Stigma performance

Following removal of the pollination bags, a total of 110 stigmas were examined during seed setting period but they showed no significant ($p>0.05$) differences (Table 4.9). The closest similarity was observed between week 9 and 13 where no stigma dried out (Table 4.9).

Table 4.29. Stigma numbers of controlled pollinated flowers

Time	No. of stigmas
Week 5	110
Week 9	50
Week 13	50
Week 17	32
Mean	60.5
p-value	>0.05
CV (%)	11.3

4.4.5.2 Style health performance

After debagging, 22 branches with 226 flower styles were examined when green and healthy but 111 flower styles from 14 branches completely withered, but they showed no significant ($p>0.05$) differences (Table 4.10). The closest similarity was observed between week 5 and 9 (Table 4.10). The initial CV was 21.5% but transformation was done using Log_{10} to normalize the data (Table 4.10).

Table 4.30. Health performance of flower styles for controlled pollinated flowers

Time	Status of style health	Number of branches holding flowers over time
Week 5	Green/Green-Yellow	22
Week 9	Yellow and shriveled	16
Week 13	Brown and shriveled	15
Week 17	Complete withering	14
	Mean	16.8
	p-value	>0.05
	CV (%)	5.8

The figures show flower style performance during seed setting period by examination of branches

4.4.6 Effect of controlled pollination on fruit/seed set

Controlled pollination between *E. grandis* and *E. urophylla* resulted to total production of 2869 hybrid seeds from 82 fruits. There were significant differences ($p<0.05$) among the number of fruits and hybrid seeds at the near and mid sections from the branch tips but insignificantly ($p>0.05$) different at the farthest sections (Table 4.11). GU13 recorded the highest seed numbers of 1526 from 29 fruits while the lowest was 13 from 2 fruits with closest similarities observed between genotypes GU2 and GU4 (Table 4.11).

For variation in mature fruits, the near tip (1-4cm) had fruit numbers ranging from 3 (GU4) to 10 (GU2) and they were significantly ($p<0.05$) different (Table 4.11), the mid tip (5-9cm) from 3 (GU7) to 29 (GU16) and they were significantly($p<0.05$) different (Table 4.11) and far tip (beyond 10cm) from 2 (GU2 and GU4) to 17 (GU16) but they showed no significant ($p>0.05$) differences (Table 4.11).

For variation in seeds, the near tip (1-4cm) had seed numbers ranging from 51 (GU4) to 361 (GU2) and they were significantly ($p<0.05$) different (Table 4.11), the mid tip (5-9cm) from 102 (GU4) to 1526 (GU13) and they were significantly ($p<0.05$) different (Table 4.11) and the far tip (beyond

10cm) from 13 (GU4) to 630 (GU16) but they showed no significant ($p>0.05$) differences (Table 4.11).

The initial CV at near tip (1-4cm) for the number of fruits and seeds was 49.7 and 56.6%, mid-tip was (5-9cm) 83.9 and 113.9% and far tip (beyond 10cm) was 123.7 and 162.5%, respectively but transformation was done using Log_{10} (Table 4.11).

Table 4.31. Mature fruits and hybrid seeds obtained after hybridization

			Near tip (1-4cm)		**Mid tip (5-9cm)**		**Far tip (beyond 10cm)**	
Pollen Parent	**Mother Parent**	**Hybrid**	**No. of fruits**	**No. of seeds**	**No. of fruits**	**No. of seeds**	**No. of fruits**	**No. of seeds**
EG1	EU2	GU 2	10	361	7	315	2	14
EG1	EU3	GU 3	7	213	8	300	-	-
EG1	EU4	GU 4	3	51	4	102	2	13
EG1	EU7	GU 7	5	329	3	134	-	-
EG2	EU13	GU 13	-	-	29	1526	-	-
EG2	EU16	GU 16	-	-	16	408	17	630
		Mean	6.3	238.5	11.2	464.2	7.0	219.1
		p-value	<0.05	<0.01	<0.01	<0.01	>0.05	>0.05
		LSD	2.6	67.5	3.6	242.9	*	*
		CV (%)	20.2	5.9	22.1	10.7	87.9	57.2

The dashes indicate no fruit and hybrid seeds harvested at the respective tip points.

4.4.7 Relationship between emasculation and fruit set

Emasculated flowers showed significant differences ($p<0.05$) where GU13 recorded the highest number of 85 while the lowest was 8 in GU4 with the closest similarities observed between genotypes GU2 and GU7 (Table 4.12). Mature fruits also showed significant differences ($p<0.05$) where GU13 recorded the highest number of 29 while the lowest was 8 in GU4 and GU7 with the closest similarities still observed between the latter genotypes (Table 4.12). The initial CV for emasculated flowers, mature fruits and survival rate was 79.3, 53.3 and 60.7% respectively, but transformation was done using Log_{10} to normalize the data (Table 4.12).

Table 4.32. Performance of healthy emasculated flowers on transit to fruit development

Hybrids	Emasculated flowers	Mature fruits	Survival Rate (%)
GU2	23	19	82.6
GU3	49	15	30.6
GU4	9	9	100
GU7	31	9	29.1
GU13	119	29	24.4
GU16	71	33	46.5
Mean	50.3	18	52.2
p-value	<0.05	<0.05	<0.05
LSD	53.6	5.91	60.8
CV (%)	15.1	12.8	15.4

4.4.8 Effect of controlled and natural pollination on seed emergence rate of GU hybrids

Seed emergence rates ranged from 4.4 (GU4) to 88.1% (GU16) for controlled pollination and they were significantly ($p<0.05$) different (Table 4.13). Natural pollination had the highest seed emergence rate of 93.3% (GU7) while the lowest was 77.8% (GU4) and they were significantly ($p<0.05$) different (Table 4.13). Artificial and naturally pollinated seed emergence rates ranged from 41.1% (GU4) to 86.6% (GU16) but they showed no significant ($p>0.05$) differences (Table 4.13). The initial CV for hybrid seed emergence and hybrid/naturally pollinated emergence rates was 69.0 and 27.3% but transformation was done using Log_{10} to normalize the data (Table 4.13).

Table 4.33. Seed emergence rate comparison between controlled and natural pollinated seeds

Hybrids	Hybrid seed emergence Rate (%)	Naturally pollinated seed Rate (%)	Hybrid /Naturally pollinated seeds
GU16	88.1	85.1	86.6
GU2	66.5	87.3	76.9
GU13	18.2	83.7	50.9
GU4	4.4	77.8	41.1
GU3	28.5	88.1	58.3
GU7	62.9	93.3	78.1
Grand Mean	44.8	85.8	65.3
p- value	<0.01	<0.01	>0.05
LSD	18.1	3.1	*
CV (%)	24.5	6.0	7.0

4.5 Discussion

Height and DBH of *E. grandis* and *E. urophylla* genotypes had no relationship with number of flowers

There was no relationship between height and the number of flowers where tall trees like, EU9 and EU14 with similar height had varying flower numbers of 195 and 3 respectively, while smaller trees, EU1 and EU17 with similar heights varied in flower numbers of 20 and 7 respectively. The variations in the flower numbers could be attributed to biotic and abiotic influences including flower physiology, interference by birds and insects or environmental stresses such strong winds and rain (Jurskis, 2005). Nonetheless, DBH showed a positive relationship with heights, indicating that trees which have high DBH tend to be tall compared to the ones with low DBH, probably due to efficient water uptake and soil nutrients which facilitated diametre expansion. Bernardo, et al., (1998) on grafted *E. urophylla*, *E. camaldulensis* and *E. pellita* similarly reported that increase in DBH is directly proportional to increase in tree height. The DBH likewise showed a positive relationship with the number of flowers indicating that trees with high DBH tend to have many flowers, probably because more food reserves were channelled towards reproductive development.

Wilson and Bennett, (1999) on grafted *E. tricarpa* also reported that bigger trees (>40cm DBH) flowered more intensely and regularly for a longer period of time than smaller trees (≤ 40 cm) and attributed it to its provision of abundant floral resources compared to smaller ones, since they can robustly support more flowers per unit area of canopy.

High pollen germination percentage showed effectiveness for controlled pollination

Eucalypts have limited flowering frequency (Birtchnell and Gibson, 2006) and due to this, pollen harvest and storage would be effective for hybridization programmes (Potts and Gore, 1995). Pollen must be viable during pollination time for seed set to take place (Heslop, 1992). In the study, pollen viability was high at 74.7% indicating effectiveness for controlled pollination. High pollen viability is very necessary in improving chances of success in a given breeding programme, since it will facilitate growth of pollen tubes for the fertilization process (Trindade, 2001). Similar high pollen viability of above 65% has been reported on *E. marginata, E. pellita, E. camaldulensis, E. globulus* and *E. urophylla* (Girijashankar, 2010; Margaret and Jen, 2006). In addition, EG2 pollen was coarse and it resulted to production of more hybrid seeds than EG1 which was fine in texture probably because EG2 had higher grip to the sticky exudate during pollination than EG1. Also, pollen parent EG2 was dark-yellow while EG1 was bright-yellow in colour indicating that coarse pollen tend to appear darker visually compared to fine pollen. Variation in pollen colour and texture has been reported on eucalyptus by Gonçalves et al. (2016). The extracted pollen was used on conventional technique which was fairly successful due to the many hybrid seeds which were harvested, and also on one-stop pollination which did not produce any hybrid, but the latter has been documented by Habard et al. (2000) to be successful in United States of America, Republic of South Africa, Uruguay and Chile therefore indicating the need to have more of its trial.

Flower size influenced performance of *grandis-urophylla* seeds

The highest mean diameters and lengths of controlled and naturally pollinated flowers were recorded on week 17 while the lowest recorded on week 5, indicating that there was a progressive increase in flower size during the seed setting period. The flower sizes in both controlled and natural pollination showed a positive relationship with seed set suggesting that flower size influenced the capacity of carrying hybrid seeds. Bawa et al. (2018) reported that large flowers tend to produce highly seeded fruits while small flowers are likely to produce few seeded fruits after attainment of maturity, since large flowers have enough room to allow fertilization and seed set compared to smaller ones.

Stigma and style health transitions indicated successful fertilizations

There was a gradual reduction of stigmas by 70.9% during the seed setting period, which could be attributed to increased fertilization, though the decrease also be as result of other factors such as poor stigma receptivity, inadequate pollen application or extreme stigma sensitivity (Shelbourne, 2019). However, 29.1% still remained green and healthy despite attaining maturity hence indicating unsuccessful fertilizations, probably due to excessive pollen application which hindered hydration, very little pollen application which caused mentor effect in the sense that several pollen grains must clamp together before they germinate or even rough handling of stigmas during pollination which interfered with the receptivity (Heslop, 1992). In addition, 63.6% of flower styles during the seed setting period experienced transition from being green and healthy to total withering, indicating that the flowers were undergoing fertilization. Despite this, 36.4% of flowers still had their styles intact at maturity indicating unsuccessful fertilizations, probably due to poor development of pollen tubes which were supposed to deliver viable nuclei to the flower ovule for the purposes of seed formation (Pound, 2002). Similarly, Assis et al. (2005) and Randall et al.

(2014) reported that death of stigmas after successful fertilizations is followed up by production of seeds.

Mature fruits had a positive relationship with hybrid seeds obtained after hybridization

Among the successful crosses, GU13 produced the highest number (1526) of seeds from 29 fruits while GU4 produced the least (13) from only 2 fruits suggesting that the number of fruits had a positive relationship with the number of seeds, because fruits are directly responsible for creating an environment for fertilization and seed formation (Potts and Gore, 1995). Fantinatti and Usberti, (2007) on *E. grandis* and Suitor, (2007) on *E. globulus* similarly reported that the number of fruits have a positive relationship with the number of seeds to be produced after attainment of maturity. However, the mid-section of the branches showed the best performance compared to other sections probably due to physiological influence or minimal interference by animals or humans, but further investigations should be conducted to ascertain this scenario.

Low emasculation on ripe *E. urophylla* flowers ensured high survival rate to maturity

Highest survival rate (100%) occurred on GU4 which had the least number of flowers while the lowest survival rate (24.4%) was on GU13, indicating that emasculation on fewer flowers was much more efficient due to maximum concentration involved. This is in contrast with emasculation done on many flowers which involved a lot of pistil breakages. Dickinson et al. (2010) and Horsley et al., (2010) on *Corymbia, E. macarthurii*, *E. grandis* and *E. smithii* reported that physical damage to the pistils causes reduced seed set.

High rate of seed emergence was influenced by maturity

Hybrid seed emergence rate highly varied such that it ranged from 4.4 (GU4) to 88.1% (GU16), indicating that some seeds may have not been mature at harvest time or other factors like seed abnormalities affecting certain genotypes but it could not be explained in this study contrary to

naturally pollinated seeds which had low variations ranging from 77.8 to 93.3% suggesting that most seeds were mature and hardly affected by other factors. However, the two types of seeds showed no significant difference suggesting that either can be used in a breeding program depending on the objectives. In other studies, varying seed emergence rates have also been reported on *E. globulus* and *E. urograndis* (José, et al., 2016; Rix et al., 2011). The differences in seed emergences rate could also be due to other factors such as environmental influences or seed oddities such as failure of seed coat shedding or radicle emergence (Rix et al., 2015; ISTA 2006).

4.6 Conclusion

The findings in this study have revealed that *grandis-urophylla* hybrids can be produced using Kenyan germplasm through the conventional technique especially if emasculation is done precisely and efficiently with maximum concentration because it greatly influences seed setting. On the other hand, more expertise and further training is needed on one-stop pollination technique since it has been documented to be successful in other countries amidst other merits including cost effectiveness, time saving and less labour intensive.

CHAPTER FIVE: GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 General Discussion

In the assessment of genetic structure of *E. grandis* genotypes and their progenies from varied seed collection centers, the sites incurred a significant change in allele frequency variations within and among populations. This has a significant evolutionary importance in the sense that discriminating pressure tend to get rid of deleterious alleles, by selectively choosing novel genetic variants which can bring about adaptive advantages (Pojskić, 2018), as a result of genetic drift, natural selection, gene flow, and other causes including maintenance breeding and germplasm renewal (Chen, 2020; Andrews, 2010). Notably, when one or several of these forces are taking place in a given population, then that population disrupts the Hardy-Weinberg assumptions, and evolution takes place (Andrews, 2010). The gene diversity index was generally high hence providing an opportunity to generate hybrids with other species such as *E. grandis* x *E. camaldulensis* or *E. grandis* x *E. globulus* (Griffin et al., 2000; Shen, 2000), with desired characteristics while taking into consideration farmer-preferred traits (Govindaraj, 2015). Eucalypts are basically an outcrossing species with late acting post zygotic self-incompatibility resulting to very high rate of outcrossing that can even surpass 90% genetic load accumulation (Silva et al., 2011; Bryne et al., 2008) and very high levels of variation among nucleotides (Novaes et al., 2008). The high PIC among the *E. grandis* genotypes indicated a greater allelic number and this agrees with report by Hildebrand, (1992) who suggested that genotypes with a couple of alleles has 0.375 as the maximum PIC whereas the ones with many alleles incline towards having higher PIC values, hence the allelic richness indicates that the genotypes have increased fitness over successive generations, high survival chances in different dynamic environmental changes as well as enhanced population growth potential (Andrews, 2010). Genetic distances among the parents and progenies were

generally low signifying a close relationship (Ngugi and Onyango, 2012) among the Nyeri Kiandanguro, Nyeri Kabarage, Londiani Kamara and Turbo genotypes while James Finlay showed distant relationship (Ngugi and Onyango, 2012) among its genotypes. Analysis of Molecular variance across the five sites showed a distinct narrow genetic differentiation on among individuals, among populations and within individuals. The very low broad sense heritability among the *E. grandis* genotypes signified minimal genetic contributions to the population's phenotypic variance inclusive of dominant, additive and multi-genic interactions, as well as paternal and maternal effects (Monir and Zhu, 2018).

In controlled pollination between *E. grandis* and *E. urophylla* genotypes, the conventional technique was much more successful compared to one-stop pollination (OSP) but these results contradict Habard et al. (1999) and Williams et al. (1999) who found OSP to be much more successful compared to the conventional technique. The poor success rate of OSP could be attributed to high exposure of physical damage due to the small cuts which were made on the styles, as compared to the conventional technique which did not involve cutting. OSP has been documented by Assis, (2005) to save on labour and therefore minimizing the cost of producing hybrids. OSP has also been used in other countries including Portugal, Australia and Chile (Espejo et al., 2001; D. Boomsma, seed Energy, *pers.comm*. 2004).

5.2 Conclusion

To satisfy the Hardy Weinberg law; Fst=Fit and Fis=0 (Wright, 1965) but the populations did not obey this, therefore indicating that the genetic variations has not been remaining constant from one generation to another, hence signifying presence of genetic diversity. In addition high level of gene flow was reported (Nm>1), therefore indicating that the pure stands have high genetic diversity which help in preventing genetic drift but it also showed a high likelihood that the seed collection centres experienced contamination over time due to the introduction of foreign genetic materials to the respective populations. The latter phenomenon could be attributed to the presence of other eucalyptus plantations.

On the other hand, further trials on controlled pollination using one-stop technique should be carried out on subsequent studies in order to assess its performance using the Kenyan germplasm. Artificial Induced Protogyny (AIP) should also be incorporated in future studies since it has been documented to also have good success rate, and its major advantage is that it is less costly in terms of production (Assis, 2005).

5.3 Recommendations

- Further trials using one-stop pollination and artificial induced protogyny techniques is necessary to confirm its effect on hybrid seed production.
- Future studies should focus on the factors that lead to rapid decrease of pollinated flowers during the seed set period.
- Molecular techniques should be used to assess the genetic structure of *grandis-urophylla* hybrids to broaden knowledge for the sake of future breeding programs.
- James Finlay genotypes should be used for heterosis while Nyeri Kabarage, Londiani Kamara, Nyeri Kiandanguro and Turbo genotypes should be used for targeting specific traits in future hybridization programs.

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APPENDICES

Appendix 1: Specific genotype crosses made using EG1 and EG2 and the resultant hybrids

Mother Parents ♂		Pollen Parents ♀	Resultant Hybrids	Pollination Technique	Emasculated flowers	Number of branches
EU_1	x	EG1	-	conventional	20	1
EU_2	x	EG1	GU2	conventional	23	3
EU_3	x	EG1	GU3	conventional	49	2
EU_4	x	EG1	GU4	conventional	8	1
EU_5	x	EG1	-	conventional	33	2
EU_6	x	EG1	-	conventional	28	2
EU_7	x	EG1	GU7	conventional	31	2
EU_8	x	EG1	-	conventional	10	1
EU_9	x	EG1	-	conventional	195	7
EU_10	x	EG1	-	conventional	10	1
EU_11	x	EG1	-	conventional	70	3
EU_12	x	EG2	-	One-stop	34	2
EU_13	x	EG2	GU13	conventional	119	5
EU_14	x	EG2	-	conventional	3	1
EU_15	x	EG2	-	conventional	37	2
EU_16	x	EG2	GU16	conventional	71	3
EU_17	x	EG2	-	conventional	7	1
EU_18	x	EG2	-	conventional	53	3
EU_19	x	EG2	-	conventional	30	2
EU_20	x	EG2	-	conventional	23	2
EU_21	x	EG2	-	One-stop	10	1

The table shows specific crosses made during the controlled pollination. The dashes indicate unsuccessful crosses.

Appendix 2. Morphological characteristics of *E. urophylla* maternal parents

Analysis of variance

Variate: Height_m

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mother_Parents	6	0.3981	0.0663	0.26	0.048
Residual	14	3.6000	0.2571		
Total	20	3.9981			

Standard errors of differences of means

Table	Mother_Parents
rep.	3
d.f.	14
s.e.d.	0.414
cv (%)	7.9

Least significant differences of means (5% level)

Table	Mother_Parents
rep.	3
d.f.	14
l.s.d.	0.888

Analysis of variance

Variate: DBH_cm

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mother_Parents	6	53.1	8.9	0.08	0.043
Residual	14	1530.7	109.3		
Total	20	1583.8			

Standard errors of differences of means

Table	Mother_Parents
rep.	3
d.f.	14
s.e.d.	8.54
cv (%)	15.6

Least significant differences of means (5% level)

Table	Mother_Parents
rep.	3
d.f.	14
l.s.d.	18.31

Analysis of variance

Variate: No_of_flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Mother_Parents	6	14377.	2396.	1.31	0.024
Residual	14	25552.	1825.		
Total	20	39929.			

Standard errors of differences of means

Table	Mother_Parents
rep.	3
d.f.	14
s.e.d.	34.88
cv (%)	30.8

Least significant differences of means (5% level)

Table	Mother_Parents
rep.	3
d.f.	14
l.s.d.	74.81

Appendix 3. Morphological characteristics of *E. grandis* pollen parents

One-sample t-test

Variate: Height_cm

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
Height_m	2	8.800	0.1800	0.4243	0.3000

95% confidence interval for mean: (4.987, 12.61)

Test statistic t = 29.33 on 1 d.f.

Probability = 0.022

CV (%) =4.8

One-sample t-test

Variate: DBH_cm.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
DBH_cm	2	45.00	50.00	7.071	5.000

95% confidence interval for mean: (-18.55, 108.6)

Test of null hypothesis that mean of DBH_cm is equal to 0

Test statistic t = 9.00 on 1 d.f.

Probability = 0.070

CV (%) =15.7

One-sample t-test

Variate: No_of_flowers.

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
No_of_flowers	2	333.5	17860	133.6	94.50

95% confidence interval for mean: (-867.6, 1535)

Test of null hypothesis that mean of No_of_flowers is equal to 0

Test statistic t = 3.53 on 1 d.f.

Probability = 0.176

CV (%) =7.2

Appendix 4. Pollen parents' germination rate on nutrient agar medium

Analysis of variance

Variate: Germination_Rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Pollen_Parents	1	6.000	6.000	1.38	0.305
Residual	4	17.333	4.333		
Total	5	23.333			

Stratum standard errors and coefficients of variation

Variate: Germination_rate

d.f.	s.e.	cv%
4	2.082	1.9

Appendix 5. Flower diameter and length of artificially and naturally pollinated flowers

Analysis of variance

Variate: Flower_Diametre

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Period	1	0.72250	0.72250	22.23	0.042
Residual	2	0.06500	0.03250		
Total	3	0.78750			

Variate: Flower_Diametre

Grand mean 8.375

Period	Phase 1	Phase 2
	7.950	8.800

Standard errors of differences of means

Table	Period
rep.	2
d.f.	2
s.e.d.	0.1803

Least significant differences of means (5% level)

Table	Period
rep.	2
d.f.	2
l.s.d.	0.7757

Stratum standard errors and coefficients of variation

Variate: Flower_Diametre

d.f.	s.e.	cv%
2	0.1803	2.2

Length Artificial
Analysis of variance

Variate: Flower_Length

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Period	1	2.5600	2.5600	19.69	0.047
Residual	2	0.2600	0.1300		
Total	3	2.8200			

Variate: Flower_Length

Grand mean 7.40

Period	Phase 1	Phase 2
	6.60	8.20

Standard errors of differences of means

Table	Period
rep.	2
d.f.	2
s.e.d.	0.361

Least significant differences of means (5% level)

Table	Period
rep.	2
d.f.	2
l.s.d.	1.551

Stratum standard errors and coefficients of variation

Variate: Flower_Length

d.f.	s.e.	cv%
2	0.361	4.9

Flower Diametre Natural
Analysis of variance

Variate: Flower_Diameter_Controlled

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	0.9025	0.9025	2.36	0.264
Residual	2	0.7650	0.3825		
Total	3	1.6675			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.618

Least significant differences of means (5% level)

Table	Time
rep.	2
d.f.	2
l.s.d.	2.661

Stratum standard errors and coefficients of variation

Variate: Flower_Diameter_Controlled

d.f.	s.e.	cv%
2	0.618	7.3

Analysis of variance

Variate: Flower_Lengths_Controlled

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	2.1025	2.1025	5.80	0.138
Residual	2	0.7250	0.3625		
Total	3	2.8275			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.602

Least significant differences of means (5% level)

Table	Time
rep.	2
d.f.	2
l.s.d.	2.591

Stratum standard errors and coefficients of variation

Variate: Flower_Lengths_Controlled

d.f.	s.e.	cv%
2	0.602	7.9

Analysis of variance

Variate: Flower_Diameter_Natural

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	3.4225	3.4225	22.44	0.042
Residual	2	0.3050	0.1525		
Total	3	3.7275			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.391

Least significant differences of means (5% level)

Table	Time
rep.	2
d.f.	2
l.s.d.	1.680

Stratum standard errors and coefficients of variation

Variate: Flower_Diameter_Natural

d.f.	s.e.	cv%
2	0.391	4.5

Analysis of variance

Variate: Flower_Diameter_Natural

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	3.4225	3.4225	22.44	0.042
Residual	2	0.3050	0.1525		
Total	3	3.7275			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.391

Least significant differences of means (5% level)

Table	Time
rep.	2
d.f.	2
l.s.d.	1.680

Stratum standard errors and coefficients of variation

Variate: Flower_Diameter_Natural

d.f.	s.e.	cv%
2	0.391	4.5

Analysis of variance

Variate: Flower_Lengths_Natural

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	3.0625	3.0625	10.47	0.084
Residual	2	0.5850	0.2925		
Total	3	3.6475			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.541

Least significant differences of means (5% level)

Table	Time
rep.	2
d.f.	2
l.s.d.	2.327

Stratum standard errors and coefficients of variation

Variate: Flower_Lengths_Natural

d.f.	s.e.	cv%
2	0.541	6.5

Analysis of variance

Variate: Flower_Diameter of controlled/natural pollinated flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	3.9200	3.9200	15.13	0.008
Residual	6	1.5550	0.2592		
Total	7	5.4750			

Standard errors of differences of means

Table	Time
rep.	4
d.f.	6
s.e.d.	0.360

Least significant differences of means (5% level)

Table	Time
rep.	4
d.f.	6
l.s.d.	0.881

Stratum standard errors and coefficients of variation

Variate: Flower_Diameter

d.f.	s.e.	cv%
6	0.509	5.9

Analysis of variance

Variate: Flower_Lengths of contolled/natural pollinated flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	5.1200	5.1200	11.66	0.014
Residual	6	2.6350	0.4392		
Total	7	7.7550			

Standard errors of differences of means

Table	Time
rep.	4
d.f.	6
s.e.d.	0.469

Least significant differences of means (5% level)

Table	Time
rep.	4
d.f.	6
l.s.d.	1.147

Stratum standard errors and coefficients of variation

Variate: Flower_Lengths

d.f.	s.e.	cv%
6	0.663	8.3

Appendix 6. Stigma performance of controlled pollinated flowers

Analysis of variance

Variate: Stigmas

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	0.07189	0.07189	1.86	0.306
Residual	2	0.07741	0.03870		
Total	3	0.14930			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	0.1967

Stratum standard errors and coefficients of variation

Variate: Stigmas

d.f.	s.e.	cv%
2	0.1967	11.3

Appendix 7. Health performance of flower styles for controlled pollinated

Analysis of variance

Variate: Stigmas

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time	1	20.250	20.250	2.19	0.277
Residual	2	18.500	9.250		
Total	3	38.750			

Standard errors of differences of means

Table	Time
rep.	2
d.f.	2
s.e.d.	3.04

Stratum standard errors and coefficients of variation

Variate: Stigmas

d.f.	s.e.	cv%
2	3.04	5.8

Appendix 8. Mature fruits and hybrid seeds obtained after hybridization

Near Tip (1-4cm)

Variate: No_of_fruits_Near tip

Analysis of variance

Variate: No_of_fruits

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrids	3	0.175986	0.058662	6.05	0.05
Residual	4	0.038813	0.009703		
Total	7	0.214799			

Least significant differences of means (5% level)

Table	Hybrids
rep.	2
d.f.	4
l.s.d.	2.594

Stratum standard errors and coefficients of variation

Variate: No_of_fruits

d.f.	s.e.	cv%
4	0.0985	20.2

Variate: No_of_Seeds_Near tip

Analysis of variance

Variate: No_of_seeds Near tip

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrids	3	0.96403	0.32134	23.79	0.005
Residual	4	0.05403	0.01351		
Total	7	1.01805			

Least significant differences of means (5% level)

Table	Hybrids
rep.	2
d.f.	4
l.s.d.	67.52

Stratum standard errors and coefficients of variation

Variate: No_of_seeds

d.f.	s.e.	cv%
4	0.1162	5.9

Mid tip (5-9cm)

Analysis of variance

Variate: Number_of_fruits

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrids	5	1.17507	0.23501	12.39	0.004
Residual	6	0.11381	0.01897		
Total	11	1.28888			

Least significant differences of means (5% level)

Table	Hybrids
rep.	2
d.f.	6
l.s.d.	3.61

Stratum standard errors and coefficients of variation

Variate: Number_of_fruits

d.f.	s.e.	cv%
6	0.1377	22.1

Analysis of variance

Variate: Number_of_seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrids	5	1.87745	0.37549	7.03	0.017
Residual	6	0.32047	0.05341		
Total	11	2.19792			

Least significant differences of means (5% level)

Table	Hybrids
rep.	2
d.f.	6
l.s.d.	242.9

Stratum standard errors and coefficients of variation

Variate: Number_of_seeds

d.f.	s.e.	cv%
6	0.2311	10.7

Far tip (beyond 10cm)
One-sample t-test

Variate: No_of_fruits

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
No_of_fruits	3	7.000	75.00	8.660	5.000

95% confidence interval for mean: (-14.51, 28.51)

Test statistic t = 1.40 on 2 d.f.

Probability = 0.296

CV (%) =87.9%

One-sample t-test

Variate: No_of_seeds

Summary

Sample	Size	Mean	Variance	Standard deviation	Standard error of mean
No_of_seeds	3	219.0	126691	355.9	205.5

95% confidence interval for mean: (-665.2, 1103)

Test statistic t = 1.07 on 2 d.f.

Probability = 0.398

CV (%) =57.2%

Appendix 9. Performance of emasculated flowers on transit to fruit development

Analysis of variance

Variate: Emasculated_flowers

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrid	5	1.45976	0.29195	6.02	0.025
Residual	6	0.29104	0.04851		
Total	11	1.75080			

Least significant differences of means (5% level)

Table	Hybrid
rep.	2
d.f.	6
l.s.d.	53.60

Stratum standard errors and coefficients of variation

Variate: Emasculated_flowers

d.f.	s.e.	cv%
6	0.2202	15.1

Analysis of variance

Variate: Mature_Fruits

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrids	5	0.67714	0.13543	10.34	0.007
Residual	6	0.07855	0.01309		
Total	11	0.75569			

Least significant differences of means (5% level)

Table	Hybrids
rep.	2
d.f.	6
l.s.d.	5.91

Stratum standard errors and coefficients of variation

Variate: Mature_Fruits

d.f.	s.e.	cv%
6	0.1144	12.8

Appendix 10. Seed emergence rate comparison between controlled and natural pollination

Analysis of variance

Variate: Controlled_Pollination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrid	5	12716.1	2543.2	24.60	<.01
Residual	12	1240.4	103.4		
Total	17	13956.5			

Least significant differences of means (5% level)

Table	Hybrid
rep.	3
d.f.	12
l.s.d.	18.09

Variate: Controlled_Pollination

d.f.	s.e.	cv%
12	10.17	24.5

Analysis of variance

Variate: Natural_Pollination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrid	5	417.778	83.556	26.86	<.01
Residual	12	37.333	3.111		
Total	17	455.111			

Least significant differences of means (5% level)

Table	Hybrid
rep.	3
d.f.	12
l.s.d.	3.138

Stratum standard errors and coefficients of variation

Variate: Natural_Pollination

d.f.	s.e.	cv%
12	1.764	6.0

Analysis of variance

Variate: Controlled/Natural Pollination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Hybrid	5	8356.8	1671.4	2.09	0.095
Residual	30	24014.9	800.5		
Total	35	32371.7			

Standard errors of differences of mean and coefficient of variation

Table	Hybrid
rep.	6
d.f.	30
s.e.d.	16.33
cv_%	7.0

Appendix 11. State of SSR primers and their mix constitution

SSR primers	State of screened SSR primers	Fluorescent colors	Mixes
EMBRA 6	FP	Green	Mix 1
EMBRA 19	NFP	Blue	
EMBRA 28	FP	Red	
EMBRA 75	NFP	Yellow	
EMBRA 114	FP	Green	Mix 2
EMBRA 8	NFP	Blue	
EMBRA 3	FP	Red	
EMBRA 26	FP	Yellow	
EMBRA 12	FP	Green	Mix 3
EMBRA 36	FP	Blue	
EMBRA 7	FP	Red	
EMBRA 204	FP	Yellow	
EMBRA 34	NFP	Green	Mix 4
EMBRA 41	FP	Blue	
EMBRA 46	NFP	Red	
EMBRA 23	FP	Yellow	
EMBRA 158	FP	Green	Mix 5
EMBRA 45	FP	Blue	
EMBRA 1	FP	Red	
EMBRA 2	FP	Yellow	
EMBRA 194	FP	Green	Mix 6
EMBRA 43	FP	Blue	

FP-Functional Primer **NFP-**Non Functional Primer