

**MOLECULAR CHARACTERIZATION OF *Sclerocarya birrea* (MARULA) FIELD
GENEBANK COLLECTIONS**

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Degree of Master of Science (Biotechnology) in the School of Pure and Applied Sciences
of Kenyatta University**

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DECLARATION

I, Fridah Gechemba Machani, declare that the work presented in this thesis is my original work and has not been presented for a degree or any other award in any other University or any other institution.

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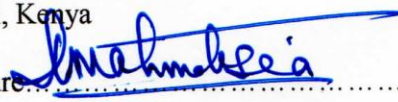
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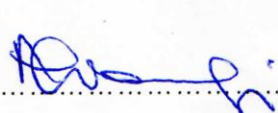
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DEDICATION

This thesis is dedicated to my mother Agnes Moraa, my twin sister Sharon Nyaboke, my nieces Purity, Yvonne and Bridgette (British), and my late father Francis Machani for their sacrifice and moral encouragement towards my education.

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ABBREVIATIONS AND ACRONYMS

µl	microliter
AFLP	Amplified Fragment length Polymorphism
AMOVA	Analysis of Molecular Variance
AOCC	African Orphan Crops Consortium
Bp	Base pair
CGIAR	Consultative Group on International Agricultural Research
CO₂	Carbon-dioxide
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic Acid
FAO	Food Agricultural Organization
g	grams
ICRAF	World Agroforestry Centre
ISSR	Inter Simple Sequence Repeats
mg	micrograms
nm	Nanometer
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TFPGA	Tools for Population Genetics Analysis
UN	United Nations
UV	Ultra-violet

ABSTRACT

Scelerocarya birrea is an important fruit tree that is widely used by the rural populations in most regions in which it is found, for its fruits, bark, timber, and even its roots. Studies have shown that the tree has the potential to boost nutrition, health and income security in Africa. However, the local communities harvest the tree products from the wild with minimal attempts to grow it on-farm. The sustainability of such wild harvests is threatened by agriculture, overgrazing, and overexploitation for other purposes. Therefore, the species needs urgent conservation measures in addition to selecting superior germplasm for on-farm tree management that will facilitate ease of species cultivation. This has prompted ICRAF and partners to collect and manage *S. birrea* germplasm in Tanzania and Malawi field genebanks as the initial step for its domestication and conservation. However, maintenance of field genebanks is very costly because trees take up a large space and they are prone to human and environmental threats. As the data gathering phase ends in the provenance trials, there is need to make an informed decision on what stands to continue being managed as a field genebank. It is important to ensure that diverse material is conserved. In order to effectively manage germplasm, genetic diversity studies are pre-requisite. The provenance trial data have shown great variability in morphological traits across populations. There no prior molecular characterization of these field genebank collections has been conducted to date. The present study was aimed at understanding the genetic diversity of *S. birrea* ICRAF field genebank collections using inter-simple sequence repeat (ISSR) markers. ISSR markers are informative, reproducible and cost-effective. Presence or absence of ISSR bands were scored manually from the gels (twice) by visual inspection of the gel images. Power Marker version 3.25, GenAlex version 6.5, Tools for Population Genetics Analysis software, and DARwin 6.0 software were used to analyze the data. Six markers yielded a total of 76 polymorphic bands across the 257 accessions. Percentage of polymorphic loci and observed heterozygosity ranged from 75% to 7.89%, and $H= 0.362$ to $H= 0.043$, respectively. The partitioning of genetic diversity found a higher (86% $P > 0.001$) intra-population variation and low inter-population variation, typical of the outcrossing nature of *S. birrea*. According to Jaccard's dissimilarity index, the highest genetic distance between accessions was 1.000 and the least genetic distance was 0.000. Neighbor-joining clustering grouped the accessions into three major clusters and twenty probable duplicates were identified, which should be eliminated to cut down the cost of conservation. The results obtained suggest that *S. birrea* ICRAF field genebank collections have a comparatively rich gene pool and, hence, valuable for conservation and domestication of the species. The twenty accessions that clustered together would be good for evaluating performance of this long-lived tree species in both locations. *Choma-M*, *Missira-M*, *Ntcheu-M*, *Magunde*, *Ohangwena-M*, *Magamba-M*, *Ngundu-M*, *Muzarabani-M*, *Matembeleland-N-M*, *Matembeleland-S-M*, and *Siavonga-M* populations should be the focus of conservation efforts and resources. There is need to cultivate the species in Kenya to improve food security, farmers' incomes and climate resilience.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Sclerocarya birrea, generally known by its trade and common name as *Marula*, forms an important component of the tradition, diet, and culture of the rural communities in Africa (Ngorima, 2006; Maroyi, 2013; Nyoka *et al.*, 2015). *Sclerocarya birrea* is widely used by the rural communities in most regions in which it is found, for its fruits, bark, timber, and even its roots (Muok *et al.*, 2011). People value the tree for its shade and beauty in addition to its valuable food and income supplements to the farmers. In South and Eastern Africa, the *Marula* tree fruits make a significant contribution to the local diets and culture, and have a substantial socio-economic importance (Hamidou *et al.*, 2014). The fruits are rich in vitamin C and can be eaten fresh, fermented to brew alcoholic drinks or used to make juice (Dlamini, 2011). *Marula* juice surpasses orange, mango and lemon juices in vitamin C content. They can also be processed to make jelly and jam (Gouwakinnou *et al.*, 2011; Muok *et al.*, 2011). The seed kernels are rich in proteins and oil that contain oleic acid and antioxidants, and they form an important food supplement. Kernels can be eaten or pressed to extract oil for cooking or for use in the cosmetic industry (Hamidou *et al.*, 2014; Hiwilepo-van *et al.*, 2014). Fruits and leaves are browsed by livestock and have a variety of medicinal uses, as do the roots and the bark (Muok *et al.*, 2011). The bark is used as both treatment and prophylaxis for malaria, while the inner bark infusion is applied to alleviate pain caused by snake and scorpion bites (AOCC, 2015).

Sclerocarya birrea may also have a considerable ecological impact. It is often a dominant species in woodlands where it occurs (Shackleton *et al.*, 2009), forming large-sized, dominant canopies that support different assemblages of sub-canopy woody plants, fords, and grasses

(Muok *et al.*, 2011). *S. birrea* is host to a collection of edible caterpillars and larvae, as well as parasitic mistletoes which produce outgrowths known as wood roses that are sold in the curio market (Shackleton *et al.* 2009). Moreover, the local communities value the tree for its potential for domestication, and its commercialization in Africa has been gaining international attention (Abdelkheir *et al.*, 2011). Furthermore, a number of national and international organizations have identified *S. birrea* as a key species for domestication as well as agroforestry promotion to boost nutrition, health and income security in Africa (AOCC, 2015).

Regardless of the many advantages of *S. birrea* for the local communities, the tree products are harvested from the wild with minimal attempts to grow it on-farm. The sustainability of such wild harvesting is threatened by long-term human pressure, such as land clearing for agriculture, overgrazing, and overexploitation for other purposes (Kando *et al.*, 2012). Therefore, there is need to select superior germplasm and establish on-farm tree management that will facilitate ease of species cultivation. At the same time, urgent conservation measures need to be put in place.

Sclerocarya birrea germplasm can be conserved using *in situ*, *circa situ* or *ex-situ* approaches. *In situ* approaches involve protecting the trees where they occur naturally or in the surroundings where they have developed their distinctive characteristics (Cruz-Cruz *et al.*, 2013). *Circa situ* conservation involves cultivating and maintaining the trees on-farm with the aim of preserving the processes of the specific genotypes and genes sampled at any particular time (Bellon and Etten, 2013). *Ex situ* conservation involve maintaining the species outside its natural habitat. It aims at the preservation of the genes and genotypes contained in the samples of the seeds used as planting materials, representing the diversity of the species

without change over a long time. *Ex situ* conservation approaches include storage of seeds in seedbanks, *in vitro* conservation of explants, cryopreservation, field genebank collections, and botanical gardens (Cruz-Cruz *et al.*, 2013).

Desiccating *Marula* seeds to low moisture content and storing them at low temperatures in a seed bank is the most convenient and cost-effective way of conserving *Marula* plant genetic resources for a long time. A field genebank is a live collection of *Marula* plants assembled from different regions that will stay in one location for many years. In order to function, on-farm conservation will depend on the knowledge, selection, preferences, and management practices of farmers. Added to this is the need for proactive management of the field genebanks to ensure that diverse germplasm material is effectively conserved. However, funding is often scarce since maintenance of a field genebank is very demanding in terms of labor and cost (FAO, 2014).

Sclerocarya birrea seeds are semi-recalcitrant and, therefore, cannot be maintained in a seed bank for a long period without losing viability and they show strong dormancy. *Marula* seeds also require a post-maturation period so as to reach maximum germination. Thus, when dried well, *S. birrea* seeds can survive up to 18 months of storage in seed bank at -18⁰C (Hamidou *et al.*, 2014). Therefore, conservation as living collections in field gene banks or botanical gardens and cultivation on-farm are the most viable ways of saving the species from extinction. In 1996 and 1998, ICRAF and partners conducted a range-wide collection of *S. birrea* germplasm as the first step for the domestication and conservation of the species. Collection of forty provenances of *Sclerocarya birrea* was done from eight countries (Tanzania, Malawi, Mali, Zambia, Swaziland, Namibia, Mozambique, Zimbabwe, and Botswana). Selection of the mother trees from which the germplasm was collected was based

on the phenotypic and superiority in fruit taste and size as recorded from the local communities. Sampling was done for trees at least 100 m apart to minimize excessive neighborhood inbreeding with each population having a bulk of nine to ten mother trees. Provenance trials to assess the germplasm, involving twenty provenances, for genotype versus environment interactions were established at Mangochi (Malawi), Masupe (Zambia) and Tumbi (Tanzania) between 1999 to 2001 (Chirwa *et al.*, 2007). World Agroforestry and partners continue undertaking management of these stands, which are now conserved as field genebanks.

The maintenance of field genebanks is very costly; trees take up a large space, and there are human and environmental threats. As the data gathering phase ends in the provenance trials, there is need to make an informed decision on what stands to continue being managed as a field genebank. It is important to ensure that diverse material is conserved. The provenance trial data have shown great variability across the three sites. Furthermore, there is variation in growth performance, fruit yield, and fruit size among the provenances planted in Malawi (Mkwezalamba *et al.*, 2015; Nyoka *et al.*, 2015; Msukwa *et al.*, 2016). The mother trees were characterized using RAPD (Kadu *et al.*, 2006) and showed significant variation within and among the populations. There is, therefore, the likelihood that even seedlings raised from this collection will also express high diversity. Therefore, there was need to genetically characterize these field genebank collections.

Molecular markers can be employed to quantify the genetic diversity of *S. birrea* ICRAF field genebank collections as they can reveal abundant differences among accessions at the DNA level. These markers provide a more direct, reliable, as well as an efficient tool for germplasm characterization, conservation, and management that is immune from the

environmental influence. One of these molecular markers is Inter-simple sequence repeats (ISSR), which are informative, reproducible and cheap, thereby lowering the cost, time and labor for diversity analysis (Ng and Tan, 2015).

1.2 Problem Statement and Justification

The multiple uses of *S. birrea* have led to an increased harvesting of the tree products largely from the wild with minimal attempts to grow it on-farm. However, sustainable supplies of such wild harvests are currently threatened by an increasing human pressure on the ecosystem. Furthermore, natural regeneration of *Marula* trees is very low as seedlings are destroyed by wild and domestic animals (Daldoum *et al.*, 2012) and farmers during land clearing. This suggests that there is an increased risk of erosion of genetic diversity and species extinction in the natural populations. It is envisioned that adequate supply of *S. birrea* products will be attained through on-farm cultivation. For these reasons, ICRAF and partners conducted range-wide collections of *Marula* germplasm, which are now conserved as field genebanks. It is vital that accession being conserved are known and described to the maximum level possible to ensure maximum use and efficient management. Therefore, characterization of the field genebank accessions is required to add value to the collections. Morphological characterization of these provenance trials has shown great variability in growth parameters and fruit production (Mkwezalamba *et al.*, 2015; Nyoka *et al.*, 2015; Msukwa *et al.*, 2016). The fruits have shown great variation in size and yield among the provenances. However, molecular characterization of these *S. birrea* field genebank accessions was yet to be conducted.

Marula seeds are semi-recalcitrant and cannot be stored in seed banks for a long time; thus, they need to be conserved in field genebanks. In order to sustainably conserve *Marula*

genetic resources, as well as select superior varieties for on-farm cultivation, effective and efficient management of the field genebank accessions is needed. However, maintaining large living collections of *S. birrea* is costly because of high costs of maintaining the accessions and large inputs of labor and land. Although ICRAF and partners continue to manage these stands as field genebanks, they still face challenges of availability of adequate resources to maintain these collections in a sustainable manner. As the data gathering phase ends in the provenance trials, there is need to make an informed decision on what stands to continue being managed as a field genebank. The effectiveness with which this decision will be made relies largely on the genetic diversity information of the germplasm in the field genebanks.

Characterization of *S. birrea* in the field genebank will, therefore, guide in the rationalization of the collections for sustainable management and use while reducing the number of redundant accessions. Molecular data helped in the identification of duplicate accessions to ensure the best use of the available resources and facilitated comparison of farmer cultivars. Molecular characterization also helped assess the genetic diversity within the collections in the context of the total available genetic diversity for the species and detect any changes in the genetic structure of the accessions. Furthermore, the management schemes to be used for conservation will require an understanding of the population dynamics and the knowledge of genetic structuring of the field genebank accessions.

1.3 Null Hypotheses

- i. There is no significant genetic variation in *Sclerocarya birrea* ICRAF field genebank collections.
- ii. There is no partitioning of genetic variation across *Sclerocarya birrea* ICRAF field genebank populations

- iii. Genetic diversity of *S. birrea* ICRAF field genebank collections does not reflect the genetic diversity of the original mother populations

1.4.1 General Objective

To determine genetic diversity and genetic structuring of *Sclerocarya birrea* ICRAF field genebank collections

1.4.2 Specific Objective

- i. To determine genetic diversity in ICRAF *Sclerocarya birrea* field genebank collections
- ii. To determine genetic structure of *Sclerocarya birrea* ICRAF field genebank collections
- iii. To determine the relationship between genetic diversity of *S. birrea* ICRAF field genebank collections and that of the original mother populations

CHAPTER TWO

LITERATURE REVIEW

2.1 *Sclerocarya birrea*

2.1.1 Taxonomy and Description

Sclerocarya birrea (A. Rich) Hochst., belongs to the genus *Sclerocarya* which falls under the family of *Anacardiaceae* together with 600 other species and 73 genera (Viljoen *et al.*, 2008). The plant family *Anacardiaceae* also consists of cashew nut (*Anacardium occidentale*), pistachio (*Pistacia vera*) and mango (*Mangifera indica*). The genus *Sclerocarya* derives from its hard seed or “nut”, that is, *Sklero* for hard and *karyon* for a nut to refer to the fruit’s hard stone (Mkwezalamba *et al.*, 2015). *Marula* is typical of the *Anacardiaceae* family because of its dioecy, resin ducts in the bark and the bearing of fleshy fruits by female trees. The species *S. birrea* has three recognized subspecies throughout its distribution, namely: *S. birrea* ssp. *caffra*, *S. birrea* ssp. *birrea*, and *S. birrea* ssp. *multifoliata* (Nyoka *et al.*, 2015). There are two species of *Sclerocarya* genus both of which are African: *Sclerocarya birrea* and *Sclerocarya gillettii*. *Sclerocarya gillettii* is the only close relative of *S. birrea* (Moganedi *et al.*, 2011; Muok *et al.*, 2011). The species name *birrea* derives from a local West African name for the tree, *birr* (Masarirambi and Nxumalo, 2012). Vernacular and common names include *Marula* (trade name), Elephant tree/jelly plum (English), *Mugongo* (Swahili), *Kyua* (Kamba Kenya), *Ngongo* (Tanzania), *Mufula* (South Africa) and *Mapfura* (Zimbabwe) (Orwa *et al.*, 2009).

Sclerocarya birrea tree is medium in size and usually grows up to 10m tall but can grow up to 20 m tall and 1.2m wide under favorable conditions (Gouwakinnou *et al.*, 2011). The bark is rough and appears pale-grey in color, (Orwa *et al.*, 2009) while the inner bark is red, pink or yellowish with darker stripes. The twigs are thick. The leaves are pinnately compound,

arranged in a spiral manner and borne terminally (Orwa *et al.*, 2009) with the sharply pointed leaflets and usually crowded near the ends of the branches (Mkwezalamba *et al.*, 2015).

Sclerocarya birrea is mainly dioecious with female and male flowers on separate trees. Trees can occasionally bear flowers of both sexes, monoecy, especially with subsp. *caffra* (Hyde and Wursten, 2010; Maroyi, 2013; Nyoka *et al.*, 2015). The flowers range from 5 to 8 cm long at the end of branches which appear pinkish-red in color. The fruits of *Marula* are round drupe, plum-sized, thick, and can range from 3 to 4 cm in diameter. They appear green when young, abscise when green and firm, and turn yellow as they ripen (Hillman *et al.*, 2008; Orwa *et al.*, 2009; Muhammad *et al.*, 2015). The ripe fruits have a characteristic turpentine flavor (Department of Agriculture, Forestry and Fisheries, 2011). The pulp is juicy and adheres tightly to the stone. The stone is hard, approximately 3cm long, with one to four cavities, and each usually contain one seed. Each cavity contains an opening covered with a cover that remains firmly attached till germination. The seeds are small and fragile, and they are covered with a thin seed coat (Orwa *et al.*, 2009).

2.1.2 Origin and Distribution

Sclerocarya birrea species has a major geographic distribution in approximately 29 African countries, stretching from Eastern Africa through South to the West of Africa and Madagascar (Muok *et al.*, 2007; 2011), Figure 2.1. The species is common and widespread throughout the semi-arid deciduous savannas of sub-Saharan Africa (Msukwa *et al.*, 2016). It is native to Botswana, Eritrea, Democratic Republic of Congo, Ethiopia, Kenya, Gambia, Malawi, Namibia, Mozambique, Niger, Somalia, Senegal, South Africa, Swaziland, Sudan, Uganda, Tanzania, Angola, Zambia and Zimbabwe, and exotic to Israel, India and Australia (Orwa *et al.*, 2009; Muok *et al.*, 2011). Its distribution range reaches 16°42'N at Abu Shendi

in Sudan, 17°15'N in Niger's Air Mountains to 31°00'S near the Port Shepstone (Nyoka *et al.*, 2015) and 50°09'E in Madagascar.

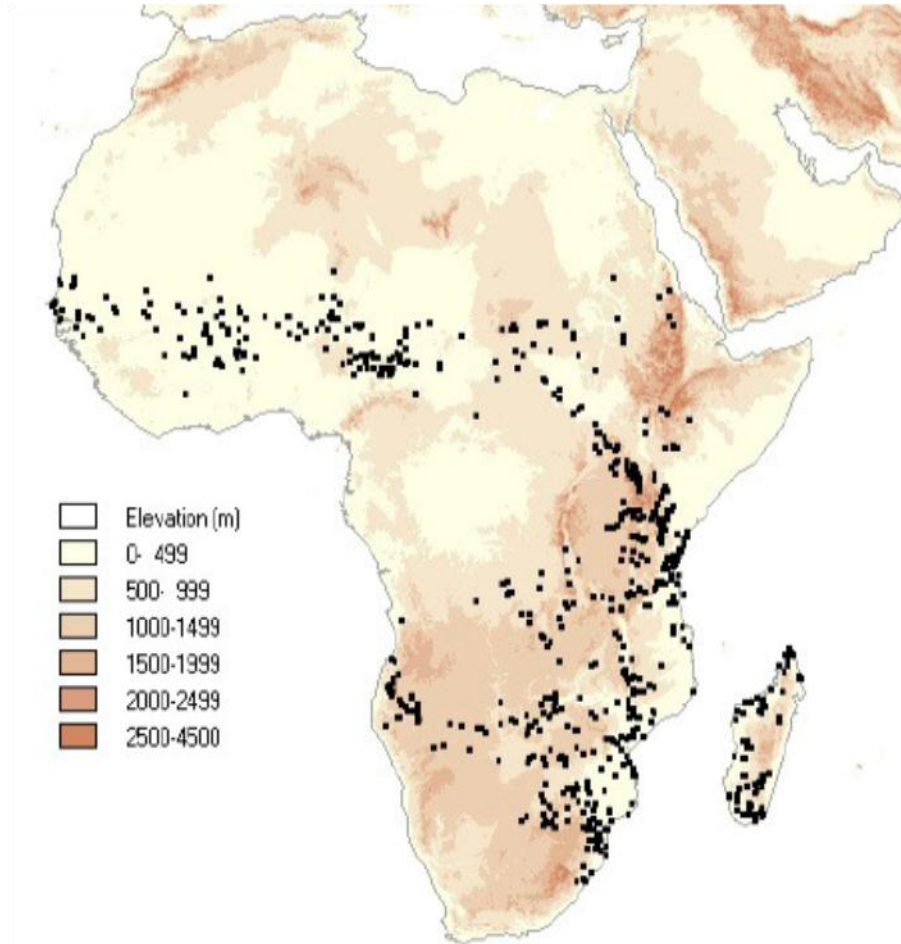


Figure 2.1 Distribution of *Sclerocarya birrea* in Africa (source: Hall *et al.*, 2002)

2.1.3 Ecological Requirements

Sclerocarya birrea thrives well in semi-arid areas. The tree is highly sensitive to frost and grows best in frost-free areas under warm conditions. If planted in regions that experience mild or occasional frost, the species needs protection at least during the first few growing seasons (Department of Agriculture, Forestry, and Fisheries, 2011). It is better adapted to dry and hot weather conditions with altitudes varying from sea level to 1800 m and a mean

annual temperature between 19-29° C (Mkwezalamba *et al.*, 2015). In Mangochi provenance trial, the species grows in 500 to 1000 m above the sea level and a temperature of 22-23° C annually.

The tree occurs naturally, is fairly drought tolerant and usually rain fed. *S. birrea* is found in arid and semiarid regions with rainfall that varies from 200-to around 1 000 mm per annum (Mkwezalamba *et al.*, 2015). In South Africa, the species grows better in a rainfall zone of 250 to 800 mm (Department of Agriculture, Forestry and Fisheries, 2011). The mean annual rainfall in Mangochi provenance trial is 900 to 1000 mm.

Sclerocarya birrea is an indigenous tree that grows in a broad range of poor soils although it prefers well-drained soil. *Marula* cannot tolerate waterlogged and flooded areas (Daldoum *et al.*, 2012). The tree thrives well on sandy soil or, occasionally, sandy loam (Mkwezalamba *et al.*, 2015). *S. birrea* tree is highly salt tolerant (Department of Agriculture, Forestry and Fisheries, 2011).

2.2.1 Cultivation

Sclerocarya birrea trees can be propagated by grafting, cuttings, and seed. The trees are easy to grow from seeds with the right treatment and handling of seeds. Seeds stored at 25⁰ to 30⁰ C moisture content germinate well when sowed. Shoot cuttings taken between September and March, the period which the trees grow actively, are suitable for vegetative propagation of *S. birrea*. *Marula* trees are also successfully propagated through grafting. Rootstocks are grown from seeds in advance and then used later for grafting when seedlings reach the height of 20 cm. Grafted trees have stronger root systems than those grown from cuttings. Grafted trees

are also shorter and bear fruits from the third year while trees from seedlings usually start fruiting at fifth to seventh year (Department of Agriculture, Forestry and Fisheries, 2011).

2.2.2 Flowering and Fruit Production of *Sclerocarya birrea*

The deciduous *Marula* trees can stand bare for many months during the dry season. In natural populations, flowering of subsp. *caffra* and subsp. *birrea* takes place at the end of the dry season just before the leaves appear and fruiting occurs during at beginning of the rainy season (Hall *et al.*, 2002). In Sudan, flowering occurs in January-April and fruiting in April-June; in Sahel, flowering takes place in January-March and fruiting in March-April; in South Africa, flowering occurs in September to November while fruiting occurs in February-June (Muok *et al.*, 2011).

Fruit production data for wild trees is scanty and often anecdotal. The average level of fruit production varies according to season and conditions. In general, most female trees produce tens of thousands of fruits per season, with a weight of above 500kgs yielded per tree (Shackleton, 2002). The fruits abscise from the tree while they are still green, firm and immature and then ripen on the ground within five days of fruit fall (Department of Agriculture, Forestry and Fisheries, 2010). Figure 2. 2 shows ripe fruits of *Marula*. Farmers often build artificial fences or thorny barriers around the trees to keep the animals from getting to the fruits first. In addition, the trees can begin to set seed as early as at the age of 5 years (Muok *et al.*, 2011).



Figure 2.2: Ripe fruits of *S. birrea*

2.3 Uses of *S. birrea*

2.3.1 Food

Sclerocarya birrea is widely used by the local populations in most of the regions where it is found, with the fruits and kernels forming an important component of the diet of the rural people. The products from *Marula* tree are used as diet supplements during the critical periods of food shortages (Msukwa *et al.*, 2016). All fruit parts are eaten, either raw or cooked (Mariod and Abdelwahab, 2012). Children mostly consume fresh fruits, providing them with a rich source of vitamin C. Fresh fruits contain high anti-ascorbic value which makes it nutritionally important in combating scurvy. The fruits are also collected and processed into alcoholic drinks, juice, jelly and jam (Dlamini, 2011; Gouwakinnou *et al.*, 2011, figure 2.3) which extends the shelf-life of the tree products and prolongs its availability and consumption beyond the fruiting season (Muok *et al.*, 2011). The juice is four times richer in vitamin C than orange juice and is boiled and used as an additive to sweeten porridge made from maize, sorghum, and millet.



Figure 2.3: Marula drinks

The kernels are rich in proteins and are crushed to remove the seeds. The seed kernels are rich in proteins (36.7%), minerals and 53% oil that contain oleic acid and anti-oxidants and they form an important food supplement (Mariod and Abdelwahab, 2012). The kernels are eaten raw or pressed to produce oil, that is used for cooking or in the cosmetic industry (Mariod and Abdelwahab, 2012; Hamidou *et al.*, 2014; Hiwilepo-van, 2014). The endocarp is oily and edible, occasionally sold on local markets, and contains up to six percent oil (Muok *et al.*, 2011). The oxidative stability of *S. birrea* oil is used by some communities to preserve meat. Other important dietary constituents include trace elements and vitamins, such as calcium, iron, copper, zinc, nicotinic acid and thiamine. Surveys have shown that over 70% of households in Southern Africa consume *Sclerocarya birrea* fruits which are a seasonal staple food for local people (Akinifesi *et al.*, 2008). Similar high rates of fruit use for food are reflected in South Africa. In West Africa, *S. birrea* is one of the ten most used species both for food and medicine. Secondary food resources include edible caterpillars, eaten in western Kenya and southern Africa, and honey from bees nesting in its bark.

2.3.2 Role in Ecosystem

Sclerocarya birrea forms an important part of the ecosystem of other plants and animals. The large tree dominates the ecological community in which it grows whose canopy supports different assemblages of sub-canopy woody plants, fords, and grasses (Shackleton *et al.*, 2009; Muok *et al.*, 2011). Mature trees in most regions are tall enough, and they contain sufficiently large crowns which serve as shade trees (Plate 1). The understory vegetation beneath the canopy (Plate 2) is modified by a blend of the shade and the associated local soil amelioration. In Mozambique and Inhaca Island; rural people exploit the modified sub-canopy environment beneath *Sclerocarya birrea* trees for growing sweet potato and other crops (Plate 2). The large crowns of mature old trees also provide a safe habitat for a community of invertebrates, arboreal and flying vertebrates and loranthaceous parasites, which help to enhance local biodiversity. The tree hosts a range of edible caterpillars, larvae and parasitic mistletoes that produce wood roses sold in curio markets (Wynberg and Laird, 2007; Shackleton *et al.*, 2009).



Plate 1: Women working under a shade of a giant *Marula* tree



Plate 2: Crops grown under the crown of giant *Marula* tree (*Marula* tree provides fertile habitat for growing additional crops)

2.3.3 Medicinal Uses

Sclerocarya birrea is used for treatment of diseases throughout its distributional range. Nearly all parts of this tree, particularly the leaves and bark, are exploited for medicinal uses in eastern, southern and western Africa and Madagascar. Traditionally, the bark extract is used to treat malaria, venereal diseases, diabetes, stomachs, liver diseases, inflammation, headache, toothache, fever, gastrointestinal disorders and dysentery/diarrhea (Akinnifesi *et al.*, 2008). The bark of *Sclerocarya birrea* is also used as traditional veterinary medicine to increase appetite for animals and to treat intestinal problems of horses. Leaves are used to treat fever, diarrhea, insect bites or skin irritations, pain, venereal diseases, including syphilis and weak veins/capillaries. The roots are crushed, mixed with water and drunk for washing scabies and to treat schistosomiasis in Tanzania. Indeed, Ojewole *et al.* (2010) and Maroyi (2013), report anti-diabetic, antiseptic, antimicrobial, anti-inflammatory, anti-diarrheal, anti-hypertensive, anti-oxidant, anti-convulsant and antiplasmodial properties of *Marula* leaf and bark extracts.

2.3.4 Fodder and Other Uses

The branches of *S. birrea* are used for animal fodder in times of drought. The wild animals, such as giraffe and kudu browse *Marula* leaves (Mkwezalamba *et al.*, 2015). Elephants eat the bark and fruits of *S. birrea*. Another contribution to fodder is that buffalo grass, *Panicum maximum*, one of the most valuable fodder grasses, grows well under *Sclerocarya birrea* canopy. The branches and trunks are used to make mortars, and pestles (Hamidou *et al.*, 2014), drums, plough wheels, decorative curios, fencing traditional bowls and for fuel wood (Maroyi, 2013). The crowns of mature trees provide shade. Dried nuts make traditional necklaces.

2.4 Management of *Sclerocarya birrea* Genetic Resources

2.4.1 *In situ* Conservation

Sustainable utilization of *Sclerocarya birrea* wild fruits is currently threatened by the high rate of deforestation for settlement and agriculture, and overgrazing. This, in turn, threatens the gene pool of this species in its natural locations (Chirwa *et al.*, 2007). Apart from the need to select superior germplasm and establish on-farm tree management that will facilitate species cultivation, urgent measures to safeguard its genetic resources need to be put in place. There are two main approaches to conservation of plant genetic resources; *in situ* and *ex situ*. Conservation of *S. birrea* genetic resources can occur *in situ*, *ex situ*, or in complementarity. *In situ* conservation of *S. birrea* aims at protecting the natural habitats within which the species occurs so that a population of this species can persist steadily (Rao and Sthapit, 2012; Cruz-Cruz *et al.*, 2013). *In situ* conservation of *Sclerocarya birrea* germplasm is the least expensive approach but alone, it is not enough to meet the challenges of saving this endangered species because its natural stands have declined rapidly. Furthermore, the strategy lacks sufficient security for long-term maintenance of tree genetic resources (Cruz-Cruz *et al.*, 2013).

2.4.2 *Ex Situ* Conservation

Ex situ conservation of *Marula* involves protecting the species outside the natural habitats (Iriondo *et al.*, 2008), by sampling and storing the species' genetic materials in centralized banks, away from the origin. The purpose of this method is to keep germplasm in secure places for future use. It aims at preservation of the genes and genotypes contained in the samples of the seeds used as planting materials representing the diversity of the species without change over a long time. *Ex-situ* approaches include storing seeds in seed banks,

botanical gardens, maintaining live collections of trees in field a genebank, cryopreservation, and *in vitro* cultures (Cruz-Cruz *et al.*, 2013).

On-farm cultivation of trees is also presently recognized as another strategy of *ex-situ* conservation. On-farm (*Circa situ*) conservation involves cultivating and maintaining the trees on farmland with the aim of preserving the processes of the specific genotypes and genes sampled at a particular time (Bellon and Etten, 2013). In order to function, on-farm conservation will depend on the knowledge, selection, preferences, and management practices of the farmers.

Different types of genome resource banking can be used as another technique to conserve germplasm based on the type of the genetic materials conserved. These include field genebanks for live plants, seed banks for storage of seeds, and *in vitro* gene banks for plant cells and tissues, among others. Seed storage in a seed bank is the most convenient and cost-effective way of conserving plant genetic resources for a long time. Seeds are convenient and easy materials to collect, and they take a small space. The technique relies on desiccation of seeds to low moisture content and storing them at low temperatures (Brutting and Wesche, 2013; Kasso and Balakrishnan, 2013). However, *Sclerocarya birrea* produce semi-recalcitrant seeds that cannot be maintained in a seed bank for a long period without losing viability. The seeds also show strong dormancy. *Marula* seeds require a post-maturation period so as to reach maximum germination (Hamidou *et al.*, 2014).

In addition, seed storage has the limitation of the need to periodically regenerate the stored samples, an approach that is expensive and difficult due to the need to maintain a representative range of the populations. *Marula* seeds remain viable in the laboratory up to a

year. Thus, when dried well, *S. birrea* seeds can survive up to 18 months of storage in seed bank at -18°C (Hamidou *et al.*, 2014). Therefore, conservation and management as living collections in field genebanks of *S. birrea* guided by its genetic diversity are the most viable ways of saving the species from extinction. A field genebank is a live collection of plants assembled from different regions that will stay in one location for many years. Field genebanks require proactive management to ensure that diverse germplasm material is effectively conserved. However, funding is often scarce since maintenance of a field genebank is very demanding in terms of labor and cost (FAO, 2014). In addition, trees take a large space and are vulnerable to environmental threats and intentional destruction by humans. Therefore, conserving the germplasm *in vitro* can easily overcome these limitations, ensuring maintenance of healthy *S. birrea* genetic resources across its distribution.

In vitro genebank approach involves conserving genetic resources using non-conventional methods by growing explants on medium. The germplasm is conserved in the form of *in vitro* maintained tissues, organs and plant cells. By doing so, the genetic resources are made available to the farmers and breeders so that new, as well as improved varieties could be developed. *In vitro* techniques can achieve medium-term conservation of *S. birrea* by allowing storage of genetic materials from several months to three years without subculture, depending on the plant material and technique used. *In vitro* cultures can allow mass propagation of the species, which will facilitate its agricultural production (Cruz-Cruz *et al.*, 2013). Another advantage is the ability to multiply the plant materials with relative ease, a low risk of introduction or re-introduction of pathogens, and a low risk of genetic instability (Engelmann, 2011).

Since *S. birrea* produces semi-recalcitrant seeds, *in vitro* field collecting can also focus on small embryos that are needed to successfully propagate the trees, given adequate handling techniques. Embryos can be isolated from *Marula* nuts in the field with minimal, but skillful aseptic precautions, surface sterilized, and then dissected at the field location. The embryos can also be transported in endosperm plugs held in sterile salt solutions for subsequent dissection in the laboratory (Withers, 1995 cited in Cruz-Cruz *et al.*, 2013). *In vitro* conservation also supports safe germplasm transfers under regulated phytosanitary control. Storage of tissue cultures also allows the germplasm conservation of in protected environments, aseptic plant production, easy and safe international exchange of plant materials, as well as lower conservation costs. It is most appropriate for rapid multiplication purposes, dissemination and active collections.

However, maintaining genetic materials in cultures is labor intensive and prone to loss of valuable germplasm through human error and accidental contamination of cultures. Tissue storage can also give rise to genetic instability through somaclonal variation (Engelmann, 2011). In genetic conservation, culture systems that are more prone to instability, such as cell and protoplast cultures, should be avoided in favor of highly organized systems like shoot cultures (Ashmore, 1997 cited in Cruz-Cruz *et al.*, 2013). For effective *in vitro* germplasm conservation, suitable species regeneration protocols are also needed.

2.4.3 Germplasm Banking in South and East Africa

In southern and east Africa, conservation of *Sclerocarya birrea* is carried out in genebanks. ICRAF and partners have collected *Marula* germplasm across the species' range and seeds stored in a seed bank for medium term. Trees have also been planted as provenance trials in Masupe (Zambia), Mangochi (Malawi) and Tumbi (Tanzania) to assess the germplasm for

various environments. ICRAF and partners continue managing these accessions as conserved living collections in field genebanks. At the same time, farmers across Africa continue to retain and nurture semi-domesticates of *Marula* trees that occur in homesteads and fields. A number of national and international organizations have recognized *Sclerocarya birrea* as a key species for agroforestry promotion and domestication to support health, nutritional and income security in Africa (Jama *et al.*, 2008). In southern Africa, participatory domestication of *Sclerocarya birrea* has been going on since 1996 in Malawi, Zimbabwe, Zambia, and Tanzania (Akinnifesi *et al.*, 2006, 2007).

2.4.4 Domestication and Management in Agroforestry

The tree-based enterprises of rural people help in ensuring food and nutritional security, upsurge their income and assets, and aid in solving their land management problems (Garrity *et al.*, 2006). A number of national and international organizations have recognized *Sclerocarya birrea* as a key species for agroforestry promotion and domestication to support health, nutritional and income security in Africa (Jama *et al.*, 2008). Priority setting activities within East and Central Africa region have also identified a number of species including *Sclerocarya birrea* for domestication in the dry lands. The species has a great potential for further domestication through the development of cultivars. The variation in fruit characteristics and vitamin C content indicates the opportunities for capturing various multiple trait combinations appropriate for tree selections directed towards various commercialization opportunities or marketable products, such as kernels and fruits. Besides, *Marula* tree is suitable for introduction into dryland agroforestry systems and is highly valued by the local communities for its domestication and commercialization potential in Africa.

Introduction of *S. birrea* into agroforestry systems will enhance its performance through improved yield and quality of fruits and other products. *Marula* trees managed in farmland have shown a 12-fold increase in the yield with larger fruits in size compared to trees in natural woodland (Nyoka *et al.*, 2015). Cultivated trees also grow more rapidly than those in woodlands. Besides, introduction of *Marula* trees into farmland will ensure that every household plants and manages the trees, which will ease wild harvest of the tree products and make the farming systems more sustainable and beneficial (Awodoyin *et al.*, 2015). Incorporating *S. birrea* in agroforestry would promote its genetic diversity and match this intraspecific diversity to the requirements of the farmers, markets and diverse environments (Simons and Leakey, 2004). Different farmers could use different provenances, and thus, based on landscape, one could experience great variation among the species. Because of the outcrossing nature of this species, gene mixing is expected to take place during seed production, resulting in a continuous creation of novel hybrids and complexes. These new hybrids would add to and broaden the genetic base of the species (Atta-Krah *et al.*, 2004).

Selecting superior varieties and planting *Marula* trees on-farm would help to restore the resources of this important tree. In southern Africa, participatory domestication of *Sclerocarya birrea* has been going on since 1996 in Malawi, Zimbabwe, Zambia, and Tanzania (Akinnifesi *et al.*, 2006, 2007). Again, through domestication of the species, the rural people could improve their trees as well as produce best fruits in their local areas. This would reduce the labor involved in fruit juicing and increase the quantity of the pulp extracted. Moreover, with appropriate selection, farmers could undoubtedly improve the flavors of *Marula* products (Leakey *et al.*, 2005a, b).

2.4.5 Conservation Policies

Policies and laws that govern the management and use of *Sclerocarya birrea* vary greatly across the various countries where the tree is found. In Namibia and South Africa, customary laws and strong cultural taboos govern the use of *Marula* and protect the wild fruit trees on communal lands. The rules control the harvesting of *Marula* fruits and cutting of *Marula* trees. Government laws, on the other hand, do not specifically protect wild fruit trees in protected areas. In South Africa, the National Forests Act of 1998 protects a list of tree species including *Marula* from being cut, disturbed or damaged (Wynberg *et al.*, 2002). The tree is highly valued traditionally and female trees, in particular, are never cut down in most rural communities where the species occurs. There is also a growing national and international policy-level interest in the domestication of *Marula*. There are no specific government laws that govern harvesting of *Marula* products, but removal of bark from any tree species without permission is prohibited in areas of conservation. These bans are however not practical across many communities since the trees are cut down for firewood and to obtain caterpillars. Tree cutting has increased over the past twenty years especially those closer to villages due to reduced control from the authorities responsible for conserving nature.

In the countries where *S. birrea* is found, a few forest policies that touch on species conservation help to protect and manage all reserved forests and support *in-situ* and *ex-situ* conservation of forest genetic resources. The policies also supports increased on-farm tree planting in partnership with the farmers. Tanzania policy on forest conservation, for example, designates full ownership and management responsibilities for a forest area to the local community surrounding it. Forests are either managed jointly with the central government in

cases where a government forest reserve pre-exists or solely by the village government for trees outside the government forest reserve (Abdallah and Monela, 2007).

2.5 Establishment of Field Genebanks

The trials were established between 1999 to 2001. The original germplasm planted in Malawi came from 30 geographic populations of the three *S. birrea* subspecies. Selection of the mother trees from which the germplasm was collected was based on the phenotypic and superiority in fruit taste and size as recorded from the local communities. Sampling was done for trees at least 100 m apart to minimize excessive neighborhood inbreeding with each population having a bulk of nine to ten mother trees. Nine populations that were collected from Tanzania and planted in Malawi germinated poorly in the nursery and the resulting seedlings were bulked.

2.6 Genetic Variation and its Measurements

2.6.1 Nature of Genetic Variation

Genetic diversity is a representation of all of the genetically established differences that occur between individuals of a species in relation to the expression of a certain trait or set of traits. There are three major levels of genetic variation, namely; heterozygosity or gene diversity (genetic variation within individuals), genetic difference among individuals within a population, and genetic differences between populations. Genetic variation in populations arises through mutations that can change the DNA sequences and is maintained by a complex blend of factors (Atta-Krah *et al.*, 2004). In agroforestry systems, these factors have a tendency of operating differently in large and small populations because many tree species in agroforestry systems occur at very low densities on farm. A study by Kindt (2002) on germplasm sources and tree densities in agroforestry systems in central and western Kenya,

Cameroon, and Central Uganda revealed 75% of all observed tree species on farms to be represented at a density of one or less individual per hectare.

2.6.2 Importance of Genetic Variation in Tree Species

Genetic variation ensures long-term survival of species. As a fundamental biodiversity component, genetic variation forms the basis of both the species and ecosystem diversity. Therefore, understanding the extent, as well as the distribution of genetic differences within and among populations of trees is fundamental for the determination of the appropriate genetic management strategies for utilization and conservation. Three main biological reasons explain the importance of genetic diversity in both agroforestry systems and natural ecosystems.

First is to safeguard against the instability that can arise from its absence. High levels of genetic diversity are essential because they provide trees with the ability to evolve and adjust to new environments, such as the fluctuating climatic and weather conditions, caused by global warming and elevated CO₂ levels. This allows local adaptation and migration of better-suited provenances along ecological gradient and changing environments both on farm and in natural ecosystems (Williams *et al.*, 2007; Aitken *et al.*, 2008; Dawson *et al.*, 2009). The central theory of natural selection states that the rate of a population's evolutionary change is proportional to the amount of the genetic differences available within that population. It is the differences between individuals of the same species that warrants that the entire species can acclimatize and adjust in response to artificial and natural selection pressures (Hawkes *et al.*, 2000). Genetic variation is also essential to respond to new diseases and pests. As a result, it is a good strategy to ensure a broad genetic base in agroforestry

systems to mitigate the uncertainty effects, such as those associated with disease outbreaks, changing environments or changing climates.

Secondly, heterozygosity is positively linked to fitness, that is, the relative contribution of an individual tree's genotype to the next generation population's gene pool (Hansson and Westerberg, 2002). According to Boshier (2000), trees often carry a hefty load of deleterious recessive alleles, and thus, avoidance of inbreeding is of particular significance for these organisms. A wide genetic variation within an individual tree species is vital to prevent inbreeding depression. Many tree species found on farms in the tropics are outcrossing; 'incipient' or semi-domesticates (Jamnadass *et al.*, 2009). Therefore, unless a wide gene pool is maintained within species, trees are vulnerable to inbreeding depression, the process of selfing or related-matings that result in loss of heterozygote superiority and exposure to deleterious mutations (Boshier, 2000; Lowe *et al.*, 2005; Dawson *et al.*, 2009).

Inbreeding depression reduces individual fitness and raises the possibilities of a species or population extinction (Hansson and Westerberg, 2002; Reed and Frankham, 2003 cited in Jamnadass *et al.*, 2009). Indeed, the negative effects of inbreeding in trees are well documented and include embryo abortion, lower germination rates, limited fruit set, and reduced overall fruit or seed yield. Furthermore, selfed or inbred progenies can suffer from lower seedling vigour and poor growth form, and less productivity at maturity (Stacy, 2001 cited in Dawson *et al.*, 2009).

Third, the blueprint of life that represents all the information for all biological processes on the planet is locked in all the gene diversity available. So, loss of this difference would also mean the loss of the potential for any future improvement to meet the changing human needs,

as well as end-user requirements (Hawkes *et al.*, 2000). SGRP, (2000 as cited in Atta-Krah *et al.*, 2004) notes that the interactive and additive effects of intra- and inter-specific genetic variation determine both the evolutionary potential of species and the resilience of the agroecosystems. Convention on Biological Diversity deems this intra- and interspecific genetic diversity has become essential in the currently ever changing environment with agricultural developments, pollution, global warming and desertification (Atta-Krah *et al.*, 2004).

In agricultural context, selection pressures are not just a function of ecology, but depend upon the changing requirements of the markets that producers serve. Genetic variability already present in the farm landscape allows farmers to more easily respond to new markets that are looking for different characteristics in tree products (Lengkeek, 2003). Genetic diversity in agricultural landscapes also helps farmers to manage their inputs in more efficient ways. For instance, a collection of varieties of *Marula* fruit species that ripen at different times might be beneficial for farmers because this allows the more efficient use of capital and labor. It also prevents the surpluses that occur when perishable tree products, such as fruits are brought to market over a short period (Dawson *et al.*, 2007).

2.6.3 Phenotypic and Genetic Diversity of *S. birrea*

Phenotypic diversity studies using morphological traits (Chirwa *et al.*, 2007; Muok *et al.*, 2007; Dlamini, 2011; Gouwakinnou *et al.*, 2011; Mkwezalamba *et al.*, 2015; Nyoka *et al.*, 2015; Msukwa *et al.*, 2016) have revealed extensive variation in growth and fruit traits in *S. birrea*. Muok *et al.* (2007) observed high morphological variation within and among *S. birrea* populations in Kenya. This variation includes leaf size and shape, fruit size, kernel and shell mass, color and taste and general form. Chirwa *et al.* (2007) also found significant

provenance differences in height, root collar diameter, as well as branching in three-year old *S. birrea* trees in Malawi. Dlamini (2011) found significant differences in seed mass and fruit composition of families and provenances of *S. birrea* subspecies *caffra*. Mkwezalamba *et al.* (2015) and Nyoka *et al.* (2015) also observed considerable between provenance and tree-to-tree variation in morphological traits of (15-year and 7-year old, respectively) *Marula* trees planted in Malawi. According to Leakey *et al.* (2005a, b) and Leakey (2005), genetic variation studies of trees growing in their native environment revealed substantial tree-to-tree variation in fruit characteristics. The species exhibits a high phenotypic variability, particularly in fruit quality and size, traits that are currently exploited by aboriginal communities for commercial gain.

Genetic diversity studies of *S. birrea* using molecular markers (Kadu *et al.*, 2006; Abdelkheir *et al.*, 2011; Kando *et al.*, 2011; Moganedi *et al.*, 2011) have revealed significant genetic variation. Abdelkheir *et al.* (2011) reported significant genetic diversity within and among five natural populations of *S. birrea* in Sudan whose conservation will help to maintain the species. Muok *et al.* (2007) found higher genetic diversity among trees within two *S. birrea* populations in Kenya and deduced that Kenya could be host to all the three *S. birrea* subspecies. According to Kadu *et al.* (2006), genetic differences between *S. birrea* populations are greater in Kenya and Tanzania than in other countries, which may be considered centers of genetic diversity for this species. No information is available on the genetic diversity of *S. birrea* collections conserved by World Agroforestry as field genebanks.

2.7 Use of Molecular Markers in Genetic Variation Studies

Molecular markers, also known as DNA markers are powerful tools for the analysis of genetic diversity since they are based on DNA polymorphisms. Since DNA sequences determine the diversity of organisms, any techniques used to evaluate polymorphism in DNA directly measure the genetic diversity. Because molecular markers display Mendelian inheritance, it is possible to trace the fingerprint of each organism and determine the evolutionary history of the species by phylogenetics, population genetic structures and genetic mapping (Hoshino *et al.*, 2012). Currently, genetic markers have become an indispensable tool for the comprehension, management, and improvement of natural as well as planted populations of tree species. The discriminatory power these markers provide is useful in resolving and understanding hybridization and species differentiation (Jhariya *et al.*, 2014). DNA markers are insensitive to the environment and are practically unlimited in number (Mahajan and Gupta, 2012). These markers are divided into hybridization-based, PCR-based and sequence-based markers. The PCR-based markers include random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), sequence tagged sites, single nucleotide polymorphism, and inter-simple sequence repeat.

2.7.1 Inter-Simple Sequence Repeat (ISSR) Marker System

Inter-simple sequence repeats (ISSR) are PCR-based markers used for rapid screening of genetic diversity and intraspecific variation. ISSR technique involves amplifying the DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in the opposite direction. The technique uses microsatellites, usually 16–25 bp long, as primers in a single primer PCR reaction to target multiple genomic loci. Amplification of these regions yields many amplicons of different sizes that can serve as a

dominant multilocus marker system for detection of genetic variation (Reddy *et al.*, 2002; Ng and Tan, 2015). Since the primer sequences are longer, (16-25 bp), there is higher stringency. The amplified products usually range from 200-2000 bp long and are amenable to agarose and polyacrylamide gel electrophoresis detection. The microsatellite repeats used as primers are dinucleotide, trinucleotide, tetra-nucleotide or pentanucleotide. The primers are either unanchored or more usually anchored at 5' or 3' end with one to four degenerate bases extended to the flanking sequences (Ng and Tan, 2015).

The technique combines most of the AFLP benefits and microsatellite analysis with the universality of RAPD. In addition, ISSRs have high reproducibility due to the longer primers (16–25 bps) as compared to RAPD primers (10-bps) and costs less than AFLP. The primers anneal at high temperatures (45– 60⁰C) leading to higher stringency (Ardeh, 2013). The studies on reproducibility have shown that it is only the faint bands are not reproducible. About 92–95% of the scored fragments could be repeated across DNA samples of the same cultivar, and across separate PCR runs when detected using polyacrylamide gels. Furthermore, 1 ng template DNA can yield the same amplification products as does 25 or 5 ng per 2µl PCR reaction (Reddy *et al.*, 2002). ISSRs segregate mostly as dominant markers following simple Mendelian inheritance, and so they do not permit distinction of heterozygotes from homozygotes. However, they can also be used to develop co-dominant markers, thus, enabling distinction between homozygotes and heterozygotes (Adibah *et al.*, 2012; Ng and Tan, 2015). SSRs are distributed throughout the genome, making ISSR primers more suitable than other arbitrary primers (Ardeh, 2013). These markers produce DNA fragments across the genome, and each of them is considered a locus.

According to Reddy *et al.* (2002), the source of ISSR variability can be attributed to many reasons either in single or in combination including mutations in template DNA. Particularly, mutations at the priming site could prevent amplification of a fragment giving rise to a presence or absence of polymorphism. A deletion or an insertion event within the microsatellite region or the amplified region can result in length polymorphism or the absence of a product depending on the amplifiability of the resulting fragment. The extent of ISSR polymorphism also varies with the nature of the primer used, unanchored, 5'-anchored, or 3'-anchored. Usually, di-nucleotide repeats anchored either at the 5' or 3' end reveals high polymorphism (Reddy *et al.*, 2002). Extending the primers with 1 to 4 degenerate bases at the 3' end gives clearer banding patterns compared to those anchored at the 5' end.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

This study was conducted at the Molecular Genetics Laboratory of World Agroforestry Center (ICRAF) headquarters in Nairobi, Kenya. World Agroforestry Center is located along the United Nations (UN) Avenue Gigiri, Nairobi, off Limuru Road. It is one of the Consultative Group on International Agricultural Research (CGIAR) Centers (<http://www.worldagroforestry.org/>).

3.2 Plant Materials

Healthy mature leaf materials of *Sclerocarya birrea* were collected from Tanzania and Malawi provenance trials. Sampling took place between January and March 2016. Leaf materials were collected randomly from five different trees in each row of the layout. About five leaves were collected from each sampled tree. A total of 161 and 96 samples were collected from Malawi and Tanzania, respectively across nine populations and 44 sub-populations. Appendix 1 provides details of the plant materials used for this study. All the leaf samples were placed in sealable plastic bags, dried and preserved using silica gel. The samples were then shipped to the World Agroforestry Center headquarters, Molecular Laboratory, Nairobi and stored in -20⁰C until DNA isolation.

3.3 Extraction and Purification of DNA

Total genomic DNA was extracted from each of silica-dried leaf samples using MN NucleoSpin Plant 11 kit (Macherey-Nagel, Germany), following the instructions of the manufacturer, with minor modifications. About 20 mg of dry weight plant material were ground using a mortar and pestle. The mortar and pestle were pre-cooled using liquid

nitrogen. The plant materials were frozen in liquid nitrogen and ground thoroughly to a fine powder, occasionally refilling the mortar with liquid nitrogen to keep the samples frozen. The samples were transferred into precooled 2 ml tubes using precooled spatulas, and 1000 µl of the Lysis buffer (3% CTAB) was added into the tubes. The mixture was vortexed thoroughly, and 20 µl of RNase A was added to the samples and mixed thoroughly. The mixture was then incubated at 65°C for 45 minutes with intermittent shaking after every 15 minutes.

The crude lysate was centrifuged at 12,000g for 10 minutes. The supernatant was passed through the NucleoSpin filters by spinning at 11,000g for 2 minutes and the clear flow-through collected. About 900 µl of DNA binding buffer was added into the flow-through and mixed thoroughly by pipetting up and down. The mixture was let to settle for 5 minutes, and 700 µl of each sample was loaded into NucleoSpin Plant II Columns in new 2 ml collection tubes. The tubes were centrifuged at 11,000g for 1 minute, the flow-through discarded, and the step repeated once. The silica membranes were washed using 400 µl of wash buffer 1 (PW1) and centrifuged at 11,000g for 1 minute, followed by 700 µl of wash buffer 2 (PW2) and centrifugation at 11,000g for 1 minute. Finally, 200 µl of PW2 was added to the silica membranes and the tubes centrifuged at 11,000g for 2 minutes to remove the wash buffer and completely dry the membrane. The NucleoSpin columns were placed into new 1.5 ml microcentrifuge tubes for elution of the DNA. DNA elution was done twice with 50 µl of the elution buffer (TE), preheated at 65°C and pipetted directly into the membranes. The columns were then incubated at 65°C for five minutes and centrifuged at 11,000g for 1 minute to elute the DNA.

3.4 Estimation of DNA Quality and Quantity

The extracted genomic DNA was quantified on 0.8 percent agarose gel electrophoresis to determine its quantity and intactness. Precisely 0.8 g of agarose was mixed with 100 ml of 0.5X TBE, heated in a microwave for 2 minutes and then cast on the gel casting trays after adding 5 µl of gelred (Bioline, USA). The gels were allowed to dry for about 1 hour before loading the DNA. Approximately 5 µl of genomic DNA from individual extractions was mixed with 3 µl of gel loading dye. The sample mixtures were then loaded into the gels, one sample per well and the gels were run for 45 minutes, at 100 V. Gels were then visualized under UV illumination at 312nm and photographed using Gel Doc trans-illuminator (Vilber Lourmat, France). A standard molecular weight marker (100 ng/µl lambda DNA, NEBS, USA) was run against the DNA extracts and was used to determine the intensities and integrity of the bands from the UV electrograph.

3.5. Inter-Simple Sequence Repeat (ISSR) Primer Selection

Twenty-five ISSR primers (Thermo Fisher Scientific) were pre-screened, and those that produced good amplification products were selected for further analysis of *S. birrea* accessions. Each of the 25 ISSR marker primers was run against five selected DNA samples for their suitability to characterize the species. A step-down PCR reaction consisting of four stages (as shown in 3.6) was conducted. Six of the 25 primers were chosen for the study from those that gave clear banding patterns in the order of the number of amplification products generated. The six primers chosen were those that yielded more amplicons with clear bands.

3.6. Inter-Simple Sequence Repeat (ISSR) PCR Amplification

Of the 25 ISSR primers that were initially screened, six primers were chosen for further genotyping of *S. birrea* accessions. The sequences of the six selected primers are shown in

Table 3.1. The PCR amplification reactions were carried out in a total volume of 20 µl. The reaction mixture consisted of 10.6 µl of deionized water, 4 µl of *Taq* buffer, 0.4 units of *MyTaq* polymerase (Bioline, USA), 2 µl of primer, and 3 µl of template DNA. A touchdown PCR reaction was conducted using Veriti96 PCR system (Applied Biosystems). The thermal cycling program was as follows: Stage one consisted of 1 cycle of initial denaturation at 95⁰ C for 3 minutes. Stage two consisted of 12 cycles of 95⁰ C for 1 minute, 58 to 47⁰ C for 30 seconds at a decreasing rate of 1⁰ C per cycle, and 72⁰ C for 1 minute. The third stage consisted of 23 cycles of 95⁰ C for 1 minute, 47⁰ C for 30 seconds, and 72⁰ C for 1 minute. Stage four consisted of 1 cycle of final extension at 72⁰ C for 7 minutes and a holding step at 15⁰ C.

Table 3.1 Sequences of the six ISSR markers used for characterization of *S. birrea*

MARKER	SEQUENCE (5'-3')	ANCHOR
U4576C07	CACGACGTTGTAAAACGACCACACACACACACAA	(AC)7, 3' AA
U4576C08	CACGACGTTGTAAAACGACACACACACACACACT	(AC)9, 3' T
U4576C09	CACGACGTTGTAAAACGACAGAGAGAGAGAGAGAGYT	(AG)8, 3' YT
U4576D04	CACGACGTTGTAAAACGACCACACACACACACACARG	(CA)8, 3' RG
U4576D05	CACGACGTTGTAAAACGACCACACACACACACACACAYG	(CA)9, 3' YG
U4576D07	CACGACGTTGTAAAACGACACACACACACACACACYA	(AC)8, 3' YA

Key: R – A or G, Y – C or T

3.7. Separation and Resolution of PCR products

The PCR products of 257 accessions from the six ISSR markers were resolved using 2% agarose gel electrophoresis in 1X TBE buffer. The gels were run at 100V for 3 hours using 7 µl of gel loading dye and 15 µl of PCR products pre-stained with gelred. The PCR products

were visualized under UV illumination following 20 minutes of post-staining and a photo taken using Gel Doc trans-illuminator (Vilber Lourmat, France). The PCR products were sized against 100 bp plus DNA ladder (O-Gene ruler, Thermo Fisher Scientific).

3.8 Scoring of PCR Products and Data Management

The ISSR bands were scored manually from the gels (twice) by visual inspection of the gel images. Each polymorphic band was interpreted as a locus and scored as ‘1’ for product presence, and ‘0’ for product absence in a binary matrix and the data tabulated in Microsoft Excel 2010 spreadsheet. A standard band scoring size was set at a range of 100 bp to 2000 pb. Appendix 2 shows raw scores.

3.9 Analysis of Molecular Data

Power marker version 3.25 (Liu and Muse, 2005) software was used to analyze four parameters of genetic diversity; allele number, major allele frequencies, gene diversity, and polymorphic information content. GenAlex version 6.5 (Peakall and Smouse, 2012) statistical software was then used to compute the number of different alleles and effective alleles across the 44 populations studied based on six ISSR markers. DARwin 6.0 software (Perrier and Jacquemoud-Collet, 2006) was used to calculate the dissimilarity index between individual accessions and construct a phylogenetic tree. Tools for Population Genetics Analysis (TFPGA) software version 1.3 (Miller, 1997) used to compute observed heterozygosity. Nei’s genetic diversity, percent polymorphic loci and principal coordinate analysis for all populations were also computed on GenAlex based on six ISSR markers.

Un-Weighted Neighbor-Joining dendrogram was constructed using DARwin 6.0 software to visualize the relationships among individual accessions based on Jaccard’s dissimilarity index

and 1,000 bootstraps. Principal coordinate analysis (PCoA) based on Nei's (1972/1987) unbiased genetic distance was also used to visualize the relationships among 257 accessions and between the different populations of *S. birrea* on a 2-dimensional space. Analysis of Molecular variance was used to reveal the partitioning of the genetic variation among and within the 44 populations of *S. birrea* with the help of GenAlex software.

CHAPTER FOUR

RESULTS

4.1 Quantification and Estimation of DNA Quality

Upon gel electrophoresis, it was observed that the MN NucleoSpin Plant 11 (Macherey-Nagel) DNA extraction kit gave high molecular weight, pure DNA from *S. birrea* leaves. The samples DNA were quantified on 0.8 percent agarose gel electrophoresis using Lambda DNA of 100 ng/μl size standard. DNA quantification using gel red fluorescence revealed the concentration ranged from 10 to 30 ng/μl of isolated DNA (Figure 4.1).

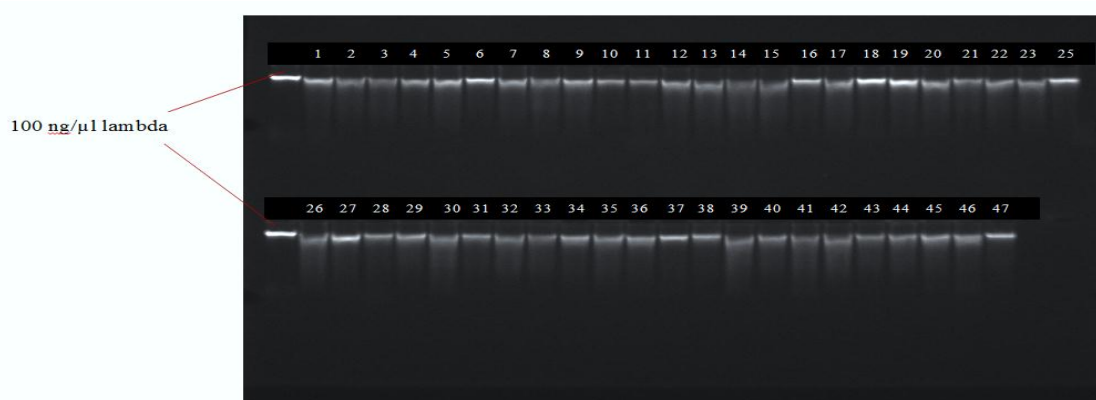


Figure 4.1: 0.8% agarose gel image showing high molecular weight, genomic DNA of 47 samples. Samples labeled as 1-6 represent Malawi accessions; 7-11 Zimbabwe accessions; 12-16 Mozambique accessions; 17-21 Mali accessions; 22-26 Zambia accessions; 27-31 Tanzania-M accessions; 32-36 Namibia accessions; 37-41 Swaziland accessions; 42-47 Tanzania accessions

4.2 Polymorphism of Inter-Simple Sequence Repeat Markers

Of the 25 markers initially screened, six of them, anchored at the 3' end with one or two degenerate bases produced clear polymorphic amplification products. The six markers selected for further characterization of the 257 accessions under the study included U4576C07, U4576C08, U4576C09, U4576D04, U4576D05 and U4576D07. The six markers

yielded a total of 76 polymorphic bands across the 257 accessions. The scored fragment sizes ranged from 100 base pairs to 2000 base pairs in length while the number of amplification products ranged from one to ten products per accession. Figure 4.2 shows the amplification profile of U4576C08 primer for 24 accessions from Tanzania. Primer generated the highest number of polymorphic loci (16 markers across 257 accessions) while U4576C07, and U4576D04 yielded the lowest number of polymorphic loci (11 markers each). Some accessions did not yield any amplicons with one primer but yielded amplification products with another primer. For example, accessions *T5-R1-25*, *T2-R1-49*, and *T10-R2-89* did not amplify with U4576C08 even after repeating the PCR, but they yielded six, seven, and six amplicons with U4576C09, respectively.

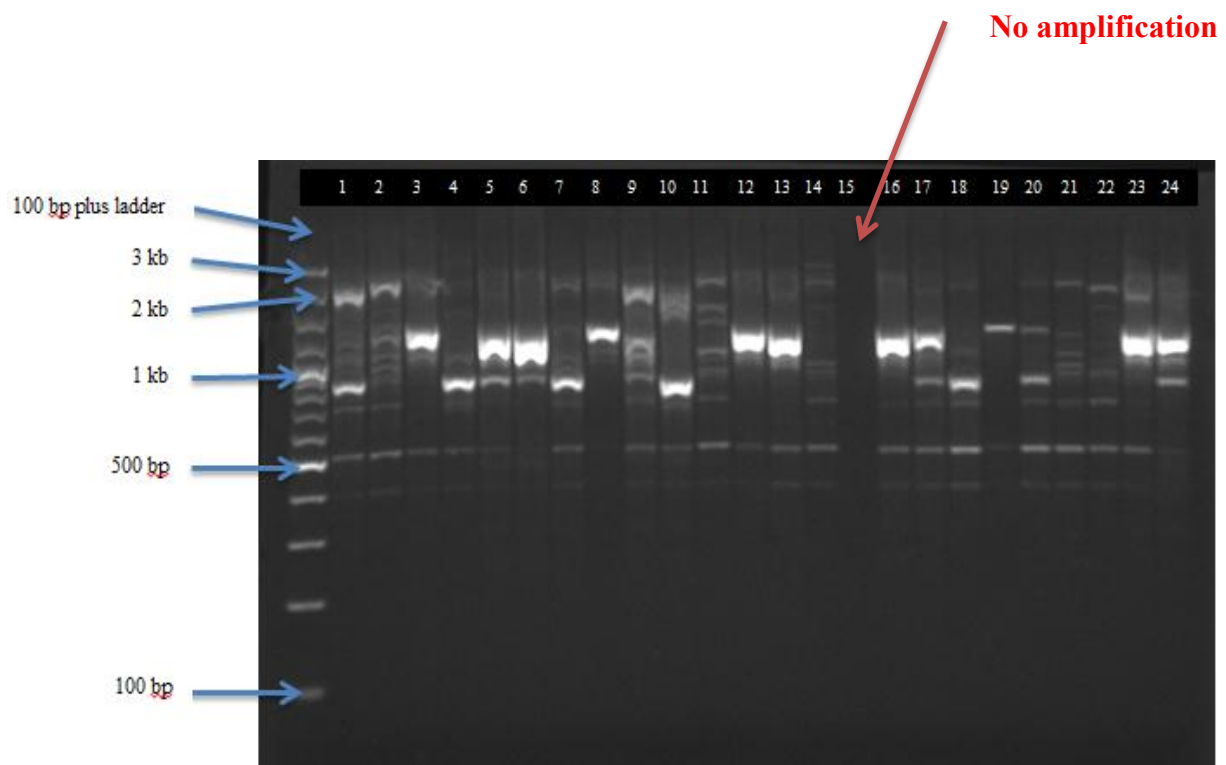


Figure 4.2 a

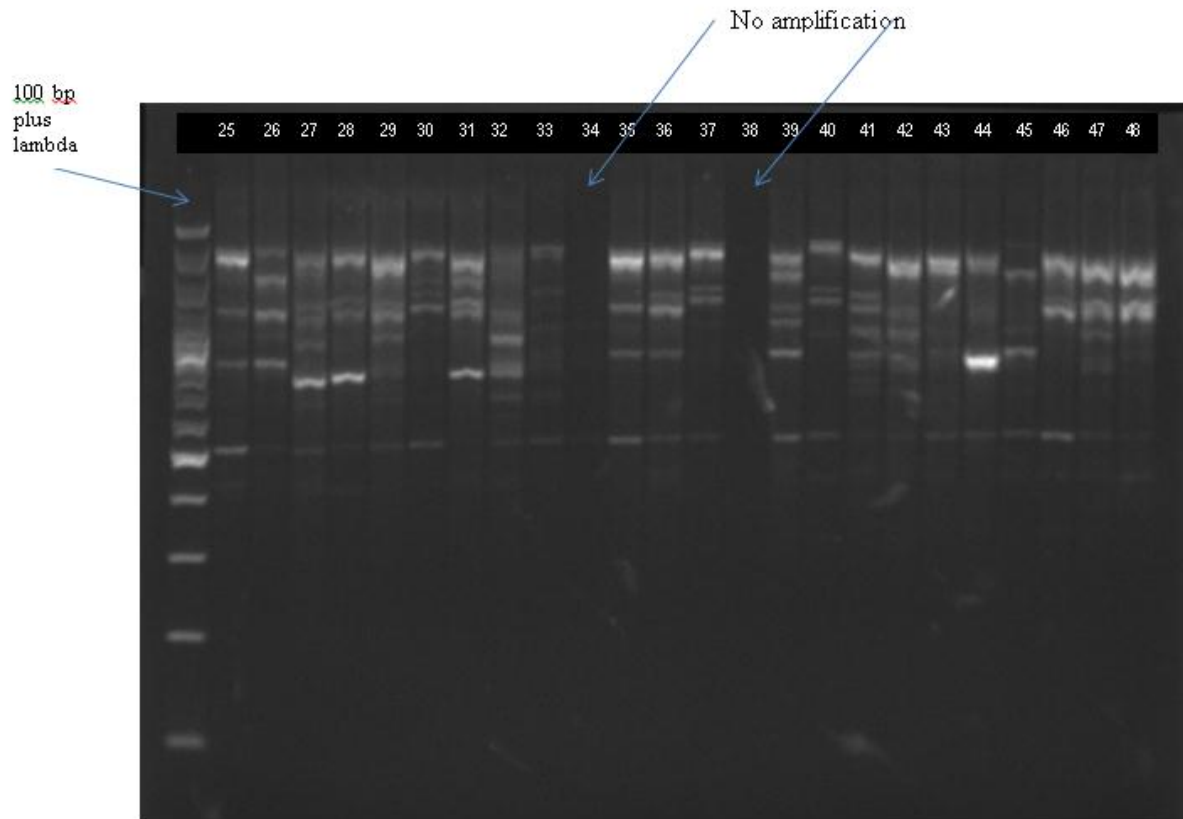


Figure 4.2 b

Figure 4.2a, b: 2% agarose gel image showing PCR amplification products of 48 samples from Tanzania using primer U4576C08.

The overall mean number of alleles was 2.066 per locus (Table 4.1). Major allele frequencies ranged from 0.730 to 0.882 with a mean of 0.793 (Table 4.1). Marker U4576C07 showed the highest allele frequency (0.882), while marker U4576C09 showed the lowest allele frequency (0.730) across the 257 accessions studied. Markers U4576D04 and U4576D05 had allele frequencies slightly higher than the mean (0.816 and 0.793, respectively; Table 4.1). The informativeness of the six genetic markers for linkage analyses as revealed by measures of polymorphic information content (PIC; Table 4.1) shows an average PIC of 0.236.

Table 4.1: Major allele frequencies, allele number and Polymorphic information content (PIC) of 257 *S. birrea* genotypes as revealed by six ISSR markers

Marker Type	Major Allele Frequency	Allele No	PIC
U4576C07	0.882	2.000	0.154
U4576C08	0.783	2.063	0.244
U4576C09	0.730	2.083	0.271
U4576D04	0.816	2.091	0.222
U4576D05	0.793	2.071	0.239
U4576D07	0.765	2.083	0.273
Mean	0.793	2.066	0.236

The mean number of different alleles was 1.078 per population (Table 4.2). The mean number of effective alleles was 1.252 per population (Table 4.2), as revealed by the six markers. The number of different alleles ranged from 0.158 ($N = 3$) in *Mialo-Kondoa-M* accessions to 1.500 ($N = 7$) in *Choma-M* accessions (Table 4.2). *Mialo-Kondoa-M* accessions had the least mean number of effective alleles ($N_e = 1.041$), and *Mataka-T* had the highest mean number of effective alleles ($N_e = 1.380$) with $N = 6$ (Table 4.2). The highest percentage of polymorphic loci was observed in *Choma-M* accessions (75%), and the lowest polymorphic loci occurred in *Mialo-Kondoa-M* (7.89%) population of accessions studied (Table 4.2), supporting the allelic richness data. The mean percentage of polymorphic loci across populations was 51.67% (Table 4.2).

Table 4.2: Number of different alleles, number of effective alleles and percent polymorphic loci for 44 *S. birrea* populations in Malawi and Tanzania provenance trials

Populations	Sample Size (N)	Mean No. of different alleles (Na)	SE	Mean No. of effective alleles (Ne)	SE	(99% criterion) Polymorphic Loci
<i>Tutume-T</i>	5.000	1.066	0.113	1.269	0.040	51.32
<i>Marulamantsi-T</i>	5.000	0.855	0.110	1.215	0.038	39.47
<i>Kalimbenza-T</i>	7.000	1.368	0.101	1.356	0.043	63.16
<i>Oshikondilogo-T</i>	6.000	0.934	0.111	1.221	0.037	43.42
<i>Ohangwena-T</i>	7.000	1.303	0.108	1.301	0.039	63.16
<i>Manyonyaneni-T</i>	6.000	1.316	0.105	1.339	0.041	61.84
<i>Makata-T</i>	6.000	1.303	0.104	1.380	0.045	60.53
<i>Wami-T</i>	5.000	1.342	0.102	1.332	0.041	61.84
<i>Zimmasv-T</i>	7.000	0.961	0.112	1.230	0.039	44.74
<i>Zimmase-T</i>	7.000	0.908	0.110	1.204	0.037	40.79
<i>Zimman-T</i>	6.000	0.974	0.108	1.223	0.039	42.11
<i>Zimmatn-T</i>	5.000	0.645	0.097	1.149	0.036	23.68
<i>Zimmants-T</i>	7.000	1.303	0.108	1.366	0.042	63.16
<i>Chikawawa-T</i>	2.000	0.724	0.104	1.214	0.038	30.26
<i>Mangochi-T</i>	6.000	0.816	0.112	1.227	0.040	39.47
<i>Ntcheu-T</i>	4.000	0.618	0.094	1.131	0.033	21.05
<i>Mpandamatonga-T</i>	5.000	0.816	0.109	1.235	0.042	36.84
<i>Missira-M</i>	9.000	1.421	0.105	1.292	0.033	71.05
<i>Chikwawa-M</i>	6.000	0.895	0.115	1.210	0.035	44.74
<i>Mangochi-M</i>	8.000	1.211	0.113	1.244	0.033	60.53
<i>Ntcheu-M</i>	7.000	1.237	0.112	1.195	0.028	61.84
<i>Rumphi-M</i>	5.000	1.224	0.112	1.283	0.036	60.53
<i>Marracuene-M</i>	7.000	1.132	0.114	1.193	0.025	56.59
<i>Magunde-M</i>	11.000	1.447	0.103	1.266	0.031	72.37
<i>Moamba-M</i>	6.000	1.211	0.110	1.321	0.041	57.89
<i>Oshikondilongo-M</i>	6.000	1.184	0.113	1.234	0.031	59.21

<i>Ohangwena-M</i>	5.000	1.368	0.104	1.365	0.040	65.79
<i>Kalimbeza-M</i>	5.000	1.053	0.114	1.275	0.038	51.32
<i>Kalanga-M</i>	8.000	1.289	0.111	1.337	0.040	64.47
<i>Mkata Morogoro-M</i>	2.000	0.632	0.099	1.177	0.035	25
<i>Ubeni Morogoro-M</i>	3.000	0.434	0.094	1.097	0.023	21.053
<i>Wami Coast Region-M</i>	3.000	0.658	0.109	1.189	0.035	32.89
<i>Magamba Handeni-M</i>	8.000	1.263	0.111	1.260	0.033	63.16
<i>Chigongwe Dodoma-M</i>	3.000	0.908	0.113	1.262	0.039	43.42
<i>Mialo Kondoa-M</i>	3.000	0.158	0.062	1.041	0.017	7.89
<i>Mandimu Singida-M</i>	2.000	0.500	0.095	1.149	0.033	21.05
<i>Ngundu-M</i>	7.000	1.487	0.100	1.305	0.035	73.68
<i>Mudzi-M</i>	6.000	1.184	0.113	1.238	0.031	59.21
<i>Biriwiri-M</i>	6.000	1.263	0.111	1.296	0.037	63.16
<i>Muzarabani-M</i>	7.000	1.461	0.102	1.307	0.033	72.37
<i>Matebeleleland N-M</i>	8.000	1.316	0.110	1.284	0.035	65.79
<i>Matebeleleland S-M</i>	6.000	1.303	0.109	1.274	0.033	64.47
<i>Siavonga-M</i>	7.000	1.447	0.103	1.275	0.030	72.37
<i>Choma-M</i>	7.000	1.500	0.100	1.309	0.034	75
Mean	5.841±0.033	1.078	0.106	1.252	0.036	51.67±2.60%

NB: T- Tanzania provenance trial; M- Malawi provenance trial

4.3 Gene Diversity Estimates of *S. birrea* Populations

Nei's gene diversity estimates showed significant differences within the studied *S. birrea* accessions. Heterozygosity is the presence of different forms of alleles at a given locus. A significantly high level of heterozygosity was observed over all loci with an overall average of $H = 0.242$ per population (Table 4.3). However, the mean average heterozygosity per population ranged from $H = 0.362$, in *Choma-M* accessions to $H = 0.043$, in *Mialo-Kondoa-M* accessions (Table 4.3). Comparatively, high diversity levels were observed in *Missira-M*, *Ntcheu-M*, *Magunde-M*, *Ohangwena-M*, *Magamba-M*, *Ngundu-M*, *Muzarabani-M*, *MatembelelandN-M*, *MatembelelandS-M*, and *Siavonga-M* populations with heterozygosity levels significantly higher than the mean diversity estimate value (Table 4.3). Samples from *Makata-T* ($H = 0.240$); *Tutume-T* ($H = 0.236$); *Chigongwe-M* ($H = 0.222$); and *Chikwawa-M* ($H = 0.214$); showed lower diversity estimates values, slightly lower compared to the mean diversity estimates of the forty-four populations (Table 4.3).

Expected heterozygosity (Nei's gene diversity) shows the probability that an accession taken at random from a population will be heterozygous over the assayed loci. The average expected heterozygosity ranged from $H_E = 0.246$ in *Ohangwena-M* to $H_E = 0.031$ in *Mialo-Kondoa-M* accessions (Table 4.3). Of particular importance were also accessions from *Zimmants-T*, *Manyonyaneni-T*, *Makata-T*, *Kalimbenza-T*, *Choma-M*, *Siavonga-M*, *Muzarabani-M*, *Biriwiri-M*, *Ngundu-M*, *Kalanga-M*, *Moamba-M*, and *Missira-M* which had the average expected heterozygosities significantly above the mean value (Table 4.3). *Mangochi-M* ($H_E = 0.171$) and *Oshikondilongo-M* ($H_E = 0.170$) accessions had the average expected heterozygosities slightly below but closer to the mean value over all loci (Table 4.3).

Table 4.3: Summaries of measures of heterozygosity for *S. birrea* accession in Tanzania and Malawi provenance trials

Populations	Sample size (N)	Observed Heterozygosity (H)	Expected Heterozygosity (He)	SE
<i>Tutume-T</i>	5.000	0.236	0.181	0.024
<i>Marulamantsi-T</i>	5.000	0.179	0.143	0.023
<i>Kalimbenza-T</i>	7.000	0.268	0.226	0.024
<i>Oshikondilogo-T</i>	6.000	0.194	0.147	0.022
<i>Ohangwena-T</i>	7.000	0.271	0.198	0.023
<i>Manyonyaneni-T</i>	6.000	0.268	0.222	0.024
<i>Makata-T</i>	6.000	0.24	0.238	0.026
<i>Wami-T</i>	5.000	0.278	0.222	0.024
<i>Zimmasv-T</i>	7.000	0.194	0.148	0.023
<i>Zimmase-T</i>	7.000	0.178	0.134	0.022
<i>Zimman-T</i>	6.000	0.178	0.144	0.023
<i>Zimmatn-T</i>	5.000	0.099	0.094	0.021
<i>Zimmants-T</i>	7.000	0.264	0.234	0.024
<i>Chikawawa-T</i>	2.000	0.167	0.167	0.029
<i>Mangochi-T</i>	6.000	0.166	0.145	0.024
<i>Ntcheu-T</i>	4.000	0.095	0.087	0.021
<i>Mpandamatonga-T</i>	5.000	0.151	0.148	0.025
<i>Missira-M</i>	9.000	0.332	0.201	0.020
<i>Chikwawa-M</i>	6.000	0.214	0.144	0.021
<i>Mangochi-M</i>	8.000	0.291	0.171	0.020
<i>Ntcheu-M</i>	7.000	0.303	0.146	0.017
<i>Rumphi-M</i>	5.000	0.295	0.199	0.022
<i>Marracuene-M</i>	7.000	0.289	0.147	0.017
<i>Magunde-M</i>	11.000	0.343	0.187	0.018

<i>Moamba-M</i>	6.000	0.252	0.210	0.024
<i>Oshikondilongo-M</i>	6.000	0.297	0.170	0.020
<i>Ohangwena-M</i>	5.000	0.309	0.246	0.024
<i>Kalimbeza-M</i>	5.000	0.245	0.187	0.024
<i>Kalanga-M</i>	8.000	0.282	0.218	0.023
<i>Mkata Morogoro-M</i>	2.000	0.138	0.138	0.028
<i>Ubeni Morogoro-M</i>	3.000	0.12	0.079	0.018
<i>Wami Coast Region-M</i>	3.000	0.172	0.139	0.024
<i>Magamba Handeni-M</i>	8.000	0.306	0.182	0.020
<i>Chigongwe Dodoma-M</i>	3.000	0.222	0.189	0.026
<i>Mialo Kondoa-M</i>	3.000	0.043	0.031	0.013
<i>Mandimu Singida-M</i>	2.000	0.116	0.116	0.026
<i>Ngundu-M</i>	7.000	0.354	0.212	0.020
<i>Mudzi-M</i>	6.000	0.297	0.173	0.020
<i>Biriwiri-M</i>	6.000	0.304	0.203	0.022
<i>Muzarabani-M</i>	7.000	0.353	0.215	0.020
<i>Matebeleland N-M</i>	8.000	0.312	0.195	0.021
<i>Matebeleland S-M</i>	6.000	0.318	0.196	0.020
<i>Siavonga-M</i>	7.000	0.35	0.200	0.019
<i>Choma-M</i>	7.000	0.362	0.216	0.020
Mean	5.841±0.033	0.242±0.010	0.172	0.022

NB: T- Tanzania provenance trial; M- Malawi provenance trial

4.4 Phylogenetic Relationships of *S. birrea* Accessions

Dissimilarity matrices based on Jaccard's dissimilarity index were used to determine the levels of relatedness of the 257 ICRAF *S. birrea* field genebank accessions as revealed by six ISSR markers. The genetic distance as measured by Jaccard's dissimilarity index ranged from 1.000 to 0.000 (data is not shown because the distance matrix is too large but is available on request).

Neighbor-joining dendrogram based on Jaccard's dissimilarity index was constructed to reveal the phylogenetic relationships among individual accessions contained in ICRAF field genebanks (Figure 4.3). The accessions clustered into three major clusters. The first cluster consisted of 224 accessions. This cluster split into many groups with one of them consisting of only accessions planted in Tanzania. All the accessions in Tanzania except *T14-R4-282*, *T5-R3-146* and *T7-R1-43* clustered separately within this major cluster. *T7-R1-43* occurred farther away from the rest of Tanzania accessions followed by *T14-R4-282* and *T5-R3-146*.

The second cluster contained thirty accessions mainly in Malawi provenance trial. Eighteen accessions; *ML2-7*, *ML2-10*, *ML3-7*, *MZ1-2*, *MZ1-9a*, *MZ1-9b*, *MZ2-6*, *N1-1*, *N1-4*, *T4-4*, *T8-1*, *T8-6*, *Z2-25774*, *Z3-25792*, *Z5-25829*, *ZA2-9*, *ZA3-1* and *ZA3-5* clustered together, (Figure 4.3). The reliability of this tree branch was supported by 12 percent bootstrap value. Another set of two accessions; *M1-13* and *ML3-22*, clustered together and the branch was supported by 52 percent bootstrap value (Figure 4.3). The third cluster had only three accessions, *T5-1a*, *T4-2* and *Z5-25824*, all planted in Malawi, and the bootstrap value of this branch was 4 percent. The nodes of the phylogenetic tree branches, constructed from 1,000 bootstraps, were supported by values ranging from zero to 97 percent as shown in (Figure

4.3). The relationships revealed by the dendrogram correspond to those shown by PCoA and Jaccard's dissimilarity indices between the studied accessions.

Figure 4.3: Dendrogram showing phylogenetic relationships among 257 *S. birrea* accessions studies as revealed by six ISSR primers

4.5 Principal Coordinate Analysis (PCoA)

The genetic relationship among accessions and between the forty-four populations of *S. birrea* was visualized using the principal coordinate analysis (PCoA). A scatter plot was generated based on Nei's 1987 genetic distances (Figure 4.4). The relationships of *S. birrea* accessions on the PCoA support the UPGMA clustering. The first three axes show 17.88%, 13.39% and 7.26% of the variance, respectively. The PC1 coordinates contained clusters of accessions mainly in Malawi while the PC2 coordinates consist of predominantly accessions from *Tanzania*, revealing the major split between accessions conserved in Tanzania and those in Malawi provenances.

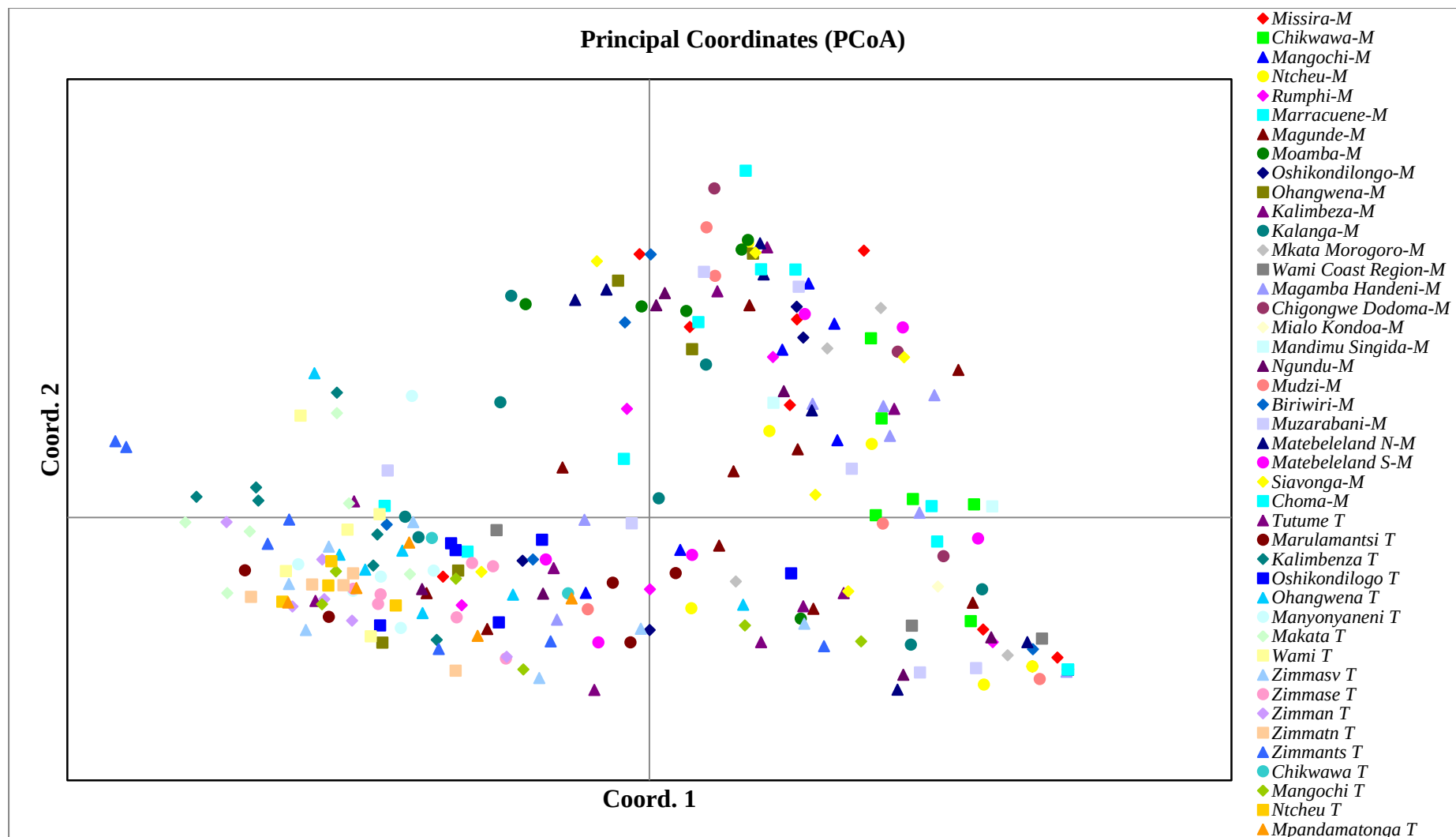


Figure 4.4: Principal coordinates analysis of 257 *Marula* accessions as revealed by six ISSR markers

4.6 Partitioning of Genetic Variation

Analysis of molecular variance (AMOVA) was used to obtain the patterns and the level of differentiation among and between *S. birrea* populations revealed by UPGMA and Neighbor-joining clustering and multidimensional scaling (PCoA). The population structure summaries obtained from AMOVA are presented in Table 4.4, and Figure 4.5. Unstructured analysis of molecular variance based on forty-four populations indicated more variation (86%, $P > 0.001$) within populations and little variation (14%, $P > 0.001$) among the populations.

Table 4.4: Summary AMOVA for 44 *Marula* populations as revealed by six ISSR markers

Summary AMOVA Table						
Source of variation	df	SS	MS	Est. Var	%	P-Value
Among Pops	43	791.32	18.40	1.52	14%	0.001
Within Pops	213	2034.85	9.55	9.55	86%	0.001
Total	256	2826.16		11.07	100%	<0.001

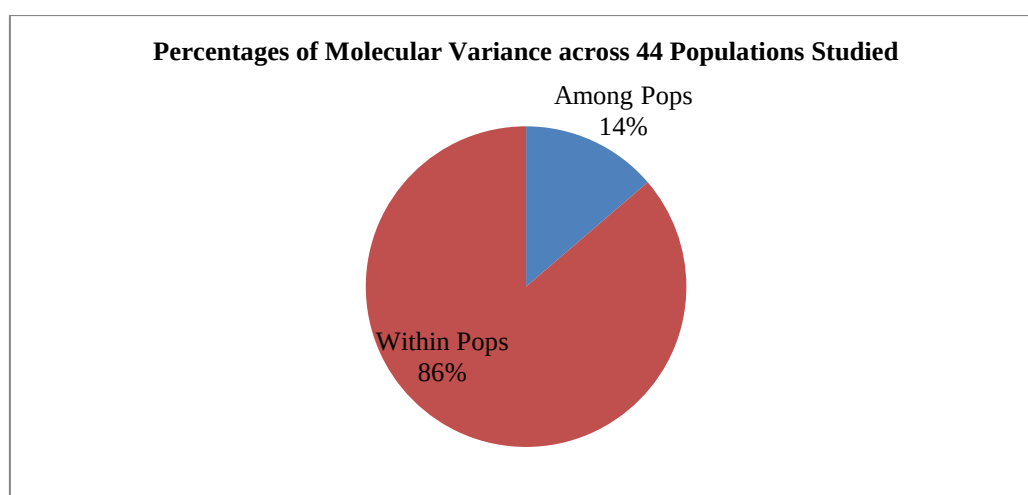


Figure 4.5: Percent partitioning among and within 44 *S. birrea* populations as revealed by six ISSR markers

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The amount of genetic diversity available in a population is positively correlated with its fitness (A'vila-di'az and Oyama, 2007). Szulkin *et al.* (2010) have also shown that a decline in the species' observed heterozygosity can induce a reduction in the average fitness of the accessions. Thus, genetic variation is a key requirement for adaptation, evolution, and tree survival (Hughes *et al.*, 2008; Jump *et al.*, 2009; Wiehle *et al.*, 2014). The 76 loci amplified in the present study on *S. birrea*, were found to be highly polymorphic and displayed up to 10 alleles per sample, which is in agreement with the dominant multilocus nature of Inter-simple sequence repeat markers (Reddy *et al.*, 2002; Ng and Tan, 2015). Lack of amplification with primer U4576C08 in some accessions can be attributed to lack of the AC repeat regions or mutation in the AC repeat regions in those accessions. Besides, lack of the T anchoring nucleotide at the end of AC repeat regions in those accessions could also have prevented any amplification.

The percentage of polymorphic loci is a genetic diversity parameter commonly used to assess genetic richness. The current study found higher percentage of polymorphism in Malawi (56.06%) compared to Tanzania provenance whose percent polymorphic loci was 46.28%. This suggests that accessions conserved in Malawi have a richer gene pool, making them potential seed sources for introduction into agroforestry and domestication of *S. birrea*. Abdelkheir *et al.* (2011) and Kando *et al.* (2012) reported higher levels of polymorphism (58.8% and 53.03%, respectively, on natural populations of *Marula* in Sudan and Burkina Faso, respectively). Tomar *et al.* (2011) and Dasmohapatra *et al.* (2014) also reported higher polymorphism for *Mangifera indica*, and *Anacardium occidentale* respectively, which are

close relatives of *Marula*, using the same type of primers. Higher levels of polymorphism suggest high genetic differentiation within populations (Sreekumar and Renuka, 2006). However, the results of the present study show low levels of genetic diversity. This suggests a possibility of genetic erosion due to habitat loss that is ascribed to anthropogenic factors acting on the mother populations long before the time of collections. Selection of superior mother trees might have also reduced polymorphism as the field genebanks were established by a very small number of seeds from the mother trees.

Although the proportion of polymorphism is an estimation of genetic variation, a more specific and suitable measure of gene diversity is obtained using Nei's 1987 gene diversity statistics. A trend similar to the proportion of polymorphism was observed with average heterozygosity estimates with significantly high level of heterozygosity over all loci ($H=0.241$). High levels of heterozygosity infer high genetic variability. The observed heterozygosity is often compared to what is expected under Hardy-Weinberg Equilibrium. The current study obtained expected heterozygosity (Nei's gene diversity) ranging from 0.246 to 0.03 with a mean of 0.172 ± 0.022 . Results of this study show that the proportion of heterozygous trees is higher than the probability that trees taken randomly will be heterozygous over the assayed loci. This could be attributed to an isolate-breaking effect (Sole-Cava *et al.*, 2013) since *Marula* provenance trials were established by mixing accessions from previously isolated populations. Observed heterozygosity was also higher than the expected heterozygosity because *S. birrea* is outcrossing.

Results of this study show higher genetic diversity than those reported for *S. birrea* in southern Africa by Kadu *et al.* (2006), where some populations in the current study came from. In Kadu *et al.* (2006) and Kando *et al.* (2012), RAPDs were used to characterize natural

populations of *S. birrea* and reported heterozygosity values of $H = 0.131$ and 0.20 , respectively. These differences in genetic variation between the present study and the previous studies could be attributed the differences in the type of primers used. RAPDs rely on amplification of random segments of genomic DNA and are performed under low stringency. ISSRs amplify regions of genomic DNA that are flanked by two microsatellites and are performed under high stringency. Therefore, the results may be because low reproducibility of RAPDs compared to ISSRs due to non-specific amplification.

Yan *et al.* (2015) performed a comparative analysis of cultivated and wild Chinese *Prunus mira* and found higher polymorphism (77.80%) with ISSR when compared to 72.73% RAPD polymorphism. Yan *et al.* (2015) attributed this discrepancy to the better discrimination ability of ISSRs. Dasmohapatra *et al.* (2014) also found higher ISSR polymorphism (89.63%) than RAPD (81.55%) polymorphism on Indian Cashew. The current higher heterozygosity compared to those reported for RAPD data suggest that ISSR markers are more informative and reproducible. However, the results of the present study were in range with those reported for *S. birrea* in South Africa by Moganedi *et al.* (2012) using AFLP analysis. AFLPs are comparable to ISSRs for their reproducibility, reliability, resolutions and stringency. High genetic diversity implies that continued management and conservation of *S. birrea* ICRAF field genebank collections can provide almost all the genetic polymorphism of this endangered species and ensure that it is still conserved. Germplasm from these accessions can serve as high quality genetic materials for cultivation of the species as the best genetic resource conservation strategy while providing an alternative source of supply of *S. birrea* products. The conserved germplasm can also be utilized for future tree breeding and improvement programs.

The results of the present study, however, show low genetic diversity compared to that reported by Diallo *et al.* (2007) ($H=0.350$) on *Tamarindus indica*, a tropical woody species with the same ecology as *S. birrea*. Ansari *et al.* (2012) also found high Nei's genetic diversity ($H=0.36$) on *Tectona grandis* using similar primers as those used in this study. Values of genetic diversity in the current study were extremely variable with *Mialo Kondoa-M* having the least value. This difference in genetic diversity among populations is due to varying sample sizes and different mother populations. The mother populations were sampled on the basis of superior phenotypes, fruit size and fruit taste. Some populations were established using seeds from the same fruit while others were established using seeds from different fruits, accounting for this among provenance variation in genetic diversity. Accessions in Malawi and Tanzania were established under similar local climatic conditions, suggesting that the variation is attributable to sampling bias.

The findings of this study support the variation reported for *S. birrea* tree growth, fruit yield and fruit size by Mkwezalamba *et al.* (2015), Nyoka *et al.* (2015) and Msukwa *et al.* (2016). These researchers found substantial provenance differences in morphological traits of *S. birrea* planted in Malawi, reflecting the wide genetic variation observed in the germplasm. The current study found that accessions in Malawi were more diverse compared to those in Tanzania which can be attributed to the selection of planting materials from the nurseries. Differences may have been compounded by biased sampling of planting materials from the nurseries and also due to the small sample sizes used in the study. It would be important to include all samples in the stands, as well as the replicates to conclude on the genetic variation among the mother plants and the plants raised from their seeds. Most of the populations from southern African and planted in both Tanzania and Malawi, such as *Ntcheu*, *Oshikondilongo*, and *Ohangwena* were more diverse in Malawi provenance compared to Tanzania.

Populations from Tanzania planted in both Tanzania and Malawi, for example, *Wami* was more diverse in Tanzania stand compared to the Malawi stand. Populations from the same mother source had varying genetic diversity from one another despite being half-sib families. Seeds from the same mother tree had different pollen sources and those from different mother trees shared the pollen source. Half-siblings only share genetic information from one parent and the other genetic information is different, therefore, they are genetically diverse.

In comparison to that of the mother population, Kadu *et al.* (2006) reported low genetic diversity ($H = 0.131$) of the original mother populations. *Sclerocarya birrea* ICRAF field genebank collection were more diverse ($H = 0.242$) than the mother populations, which is expected for high outcrossing levels of tropical trees, such as *S. birrea*. Incomplete sampling of the mother populations due to a small geographical sampling range employed and small sample size in mother populations can also explain the difference. A different marker system, which produces different band patterns from ISSRs, was used to characterize the mother populations. ICRAF field genebank collections were also established from gemplasm selected based on superiority in morphological traits, a biased sampling that is expected to produce a different pattern of DNA polymorphism. The difference in genetic diversity is also expected because the current populations consist of half-sib families that share half of the genetic information with the mother trees and half with the pollen sources.

The results of present study suggest that the collections have a rich gene pool. Seeds harvested from these populations will retain the fitness superiority of the inter-population outbreeding, a positive effect on the aggregate measure of the species' fitness to climate change. The seeds will contain a systematized representation of genetic diversity of the populations and can be cryopreserved for future regeneration and use as and when needed.

However, the subset of populations that hold very little genetic diversity are insufficient reservoirs of genetic diversity and would be inappropriate as sole sources of germplasm for introduction of *S. birrea* into agroforestry. Therefore, keeping seeds across multiple trees and populations will be required for any future regeneration, tree breeding and improvement programs to maintain genetically viable *ex situ* collections.

Management schemes of ICRAF field genebank *S. birrea* collections and domestication programs will depend on the amount and the structuring of its genetic diversity. The current partitioning of genetic diversity found higher within population variation ($P > 0.001$), which is expected because of the outbreeding nature of *S. birrea*. High within population genetic diversity indicates that ICRAF field genebank *S. birrea* accessions have high genetic variation. This structuring of genetic diversity is consistent with those reported for *S. birrea* by Abdelkheir *et al.* (2011), Kadu *et al.* (2006), Kando *et al.* (2012), and Moganedi *et al.* (2011). Abdelkheir *et al.* (2011) argued that high intra-population diversity is expected for *S. birrea* because it a dioecious species with very little or no selfing. According to Yeh, 2000 (cited in Kando *et al.*, 2012), the reproductive system of *S. birrea*, as well as its population density might impact on the species' intra-population diversity. The mode of pollination of *S. birrea* is mainly outcrossing, explaining the relatively high levels of intra-population genetic variation found in the studied *S. birrea* populations. Given its pollination system, one would expect a significant genetic differentiation between populations. However, according to Hall *et al.* (2002, cited in Kando *et al.*, 2012), the main pollinator of *S. birrea* is *Apis mellifera*, which can only move pollen over short distances. Dipterans, which move less frequently from one tree to tree, can also ensure pollination of this species. This mode of foraging, therefore, focus on mixing of genes within populations instead of between populations and its

consequences could be the low gene flow between populations and increased gene flow within populations.

Jaccard's dissimilarity index revealed that the highest genetic distance was one while the least genetic distance was zero. Most accessions were genetically distinct, reflecting the tree-to-tree variation in morphological traits reported for *S. birrea* by Mkwezalamba *et al.* (2015), Nyoka *et al.* (2015) and Msukwa *et al.* (2016). This high genetic variation among the studied accessions is because they are half-sibs that share genetic information from only one parent. Very low genetic distances indicate a recent divergence. Accessions with a zero genetic distance between them were considered probable duplicates. Results of this study suggest that *S. birrea* accessions are genetically diverse. Neighbor-joining phylogenetic tree revealed that accessions planted in Tanzania are more similar to each other despite the geographical distance of the mother populations. Accessions from *Manyonyaneni* and *Tutume* share a common ancestor, in agreement with the clustering of mother populations by Kadu *et al.* (2006), in which these populations clustered together. However, the number of populations analyzed by Kadu *et al.* was fewer than those characterized in the present study. Kadu *et al.* also clustered populations rather than individual trees. The current study, therefore, provides the first evidence for the relationship among *S. birrea* accessions in ICRAF field genebank.

Twenty probable duplicates were identified in the second cluster, and all of them are planted in Malawi. Although only three accessions were more similar in the third cluster, it was supported by the highest probability of being a true branch (4%) compared to the first and second clusters (3% and 1%, respectively). The bootstrap values were very low suggesting that only a few accessions support those nodes. The large dataset could have made

bootstrapping to fall victim of bias and give low values. The observed branches could, therefore, be due to a single extreme datapoint.

One of the options to improve the composition of *S. birrea* collections and improve conservation of its germplasm is the identification and elimination of duplicate materials. Germplasm collections invariably contain duplicate accessions, both within and between genebanks. The results of this study identified twenty probable duplicates within Malawi field genebank which should be eliminated because they do not contribute to the genetic diversity of the collection, but require a budget for maintenance. Duplicates might occur within collections because of various reasons. For example, transfer of information and common errors may give rise to presence of identical material registered under different identifiers. The mother populations from which seeds were collected for the establishment of *S. birrea* ICRAF field genebanks had not been genetically characterized by the time of those collections. There is a chance that populations were genetically identical, hence the duplicates identified in this study. High numbers of duplicates were identified, hence, there is need to eliminate these redundancies so as to cut down the cost of conservation. However, the twenty samples showing duplicates would be good for evaluating performance of this long-lived tree species in both locations.

5.2 Conclusions

From this study, the following conclusions can be made. *Sclerocarya birrea* accessions in ICRAF field genebank have a comparatively rich gene pool, hence, valuable for conservation and potential seed sources for introduction into agroforestry and domestication of *S. birrea*. Inter-population genetic differentiation was low and consistent with the outcrossing nature of the species. There were twenty probable duplicates identified within the collections

conserved in Malawi field genebank. From this study, the three null hypotheses were found to be true and, therefore, accepted.

5.3 Recommendations

- i. *Choma-M, Missira-M, Ntcheu-M, Magunde, Ohangwena-M, Magamba-M, Ngundu-M, Muzarabani-M, Matembeleland-N-M, Matembeleland-S-M, and Siavonga-M* populations should be the focus of conservation efforts and resources.
- ii. There is also a need to monitor the identified duplicate accessions for production to assess the influence of locations on production.
- iii. Future tree breeding, domestication and improvement programs should make use of the most diverse populations that offer a rich gene pool.
- iv. There is a need to domesticate the species in Kenya as it has the potential to boost food and income security, as well as address the issue of climate change.

5.4 Suggestions for Future Studies

- i. Because of high levels of intra-species genetic differentiation reported in the field genebank material, it would be worthwhile to have a continuous production evaluation of the stands in Malawi and Tanzania. Through this analysis, better performing trees can identify the source of clonal selection for species improvement.
- ii. Future studies should use of highly sensitive and more informative primers, such as DArT, SNPs, and microsatellites.
- iii. Future studies on the relationship between genetic diversity and geographical distribution, the environment, genetics and specific phenotypic traits will identify important attributes to be preserved and utilized for commercial selection in *S. birrea*.

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APPENDICES

Appendix 1: Details of the plant materials used for this study

COUNTRY	POPULATION	CODE	COUNTRY	POPULATION	CODE	COUNTRY	POPULATION	CODE
Mali	Missira-M	M1-13	Malawi	Rumphu-M	ML4-18	Namibia	Oshikondilongo-M	N1-13
Mali	Missira-M	M1-17	Malawi	Rumphu-M	ML4-2	Namibia	Oshikondilongo-M	N1-3
Mali	Missira-M	M1-19	Malawi	Rumphu-M	ML4-21	Namibia	Oshikondilongo-M	N1-4
Mali	Missira-M	M1-2	Malawi	Rumphu-M	ML4-6	Namibia	Oshikondilongo-M	N1-7
Mali	Missira-M	M1-22	Malawi	Rumphu-M	ML4-7	Namibia	Oshikondilongo-M	N1-9
Mali	Missira-M	M1-3a	Mozambique	Marracuene-M	MZ1-1	Namibia	Ohangwena-M	N2-1
Mali	Missira-M	M1-3b	Mozambique	Marracuene-M	MZ1-12	Namibia	Ohangwena-M	N2-12
Mali	Missira-M	M1-8	Mozambique	Marracuene-M	MZ1-15	Namibia	Ohangwena-M	N2-3
Mali	Missira-M	M1-9	Mozambique	Marracuene-M	MZ1-2	Namibia	Ohangwena-M	N2-5
Malawi	Chikwawa-M	ML1-12	Mozambique	Marracuene-M	MZ1-3	Namibia	Ohangwena-M	N2-9
Malawi	Chikwawa-M	ML1-13	Mozambique	Marracuene-M	MZ1-9a	Namibia	Kalimbeza-M	N3-1
Malawi	Chikwawa-M	ML1-18	Mozambique	Marracuene-M	MZ1-9b	Namibia	Kalimbeza-M	N3-14
Malawi	Chikwawa-M	ML1-22	Mozambique	Magunde-M	MZ2-10	Namibia	Kalimbeza-M	N3-3
Malawi	Chikwawa-M	ML1-24	Mozambique	Magunde-M	MZ2-13a	Namibia	Kalimbeza-M	N3-5
Malawi	Chikwawa-M	ML1-4	Mozambique	Magunde-M	MZ2-13b	Namibia	Kalimbeza-M	N3-7
Malawi	Mangochi-M	ML2-1	Mozambique	Magunde-M	MZ2-16	Swaziland	Kalanga-M	S1-1
Malawi	Mangochi-M	ML2-10	Mozambique	Magunde-M	MZ2-19	Swaziland	Kalanga-M	S1-10
Malawi	Mangochi-M	ML2-12	Mozambique	Magunde-M	MZ2-1a	Swaziland	Kalanga-M	S1-14
Malawi	Mangochi-M	ML2-16	Mozambique	Magunde-M	MZ2-1b	Swaziland	Kalanga-M	S1-16
Malawi	Mangochi-M	ML2-22	Mozambique	Magunde-M	MZ2-1c	Swaziland	Kalanga-M	S1-2
Malawi	Mangochi-M	ML2-2a	Mozambique	Magunde-M	MZ2-3	Swaziland	Kalanga-M	S1-3
Malawi	Mangochi-M	ML2-2b	Mozambique	Magunde-M	MZ2-4	Swaziland	Kalanga-M	S1-4
Malawi	Mangochi-M	ML2-7	Mozambique	Magunde-M	MZ2-6	Swaziland	Kalanga-M	S1-50
Malawi	Ntcheu-M	ML3-1	Mozambique	Moamba-M	MZ3-14	Tanzania	Mkata-M	T3-2
Malawi	Ntcheu-M	ML3-14	Mozambique	Moamba-M	MZ3-19	Tanzania	Mkata-M	T3-5
Malawi	Ntcheu-M	ML3-18	Mozambique	Moamba-M	MZ3-2	Tanzania	Ubena-M	T4-2
Malawi	Ntcheu-M	ML3-2	Mozambique	Moamba-M	MZ3-4	Tanzania	Ubena-M	T4-4
Malawi	Ntcheu-M	ML3-22	Mozambique	Moamba-M	MZ3-6	Tanzania	Ubena-M	T4-7
Malawi	Ntcheu-M	ML3-5	Mozambique	Moamba-M	MZ3-9	Tanzania	Wami-M	T5-1a
Malawi	Ntcheu-M	ML3-7	Namibia	Oshikondilongo-M	N1-1	Tanzania	Wami-M	T5-1b
Tanzania	Wami-M	T5-3	Zimbabwe	Biriwiri-M	Z3-25791	Zambia	Siavonga-M	ZA2-15b
Tanzania	Magamba-M	T6-1	Zimbabwe	Biriwiri-M	Z3-25792	Zambia	Siavonga-M	ZA2-4
Tanzania	Magamba-M	T6-10	Zimbabwe	Biriwiri-M	Z3-25793	Zambia	Siavonga-M	ZA2-6
Tanzania	Magamba-M	T6-2	Zimbabwe	Biriwiri-M	Z3-25794	Zambia	Siavonga-M	ZA2-9
Tanzania	Magamba-M	T6-3	Zimbabwe	Biriwiri-M	Z3-25797	Zambia	Choma-M	ZA3-1
Tanzania	Magamba-M	T6-4	Zimbabwe	Biriwiri-M	Z3-25798	Zambia	Choma-M	ZA3-17
Tanzania	Magamba-M	T6-8a	Zimbabwe	Muzarabani-M	Z4-25821	Zambia	Choma-M	ZA3-19
Tanzania	Magamba-M	T6-8b	Zimbabwe	Muzarabani-M	Z4-25822	Zambia	Choma-M	ZA3-2
Tanzania	Magamba-M	T6-9	Zimbabwe	Muzarabani-M	Z4-25826	Zambia	Choma-M	ZA3-5
Tanzania	Chigongwe-M	T7-1	Zimbabwe	Muzarabani-M	Z4-25831	Zambia	Choma-M	ZA3-7
Tanzania	Chigongwe-M	T7-3a	Zimbabwe	Muzarabani-M	Z4-25982a	Zambia	Choma-M	ZA3--9
Tanzania	Chigongwe-M	T7-3b	Zimbabwe	Muzarabani-M	Z4-25982b	Botswana	Tutume T	T10-R1-67
Tanzania	Mialo Kondoa-M	T8-1	Zimbabwe	Muzarabani-M	Z4-25988	Botswana	Tutume T	T10-R1-68a
Tanzania	Mialo Kondoa-M	T8-6	Zimbabwe	Matebeleland N-M	Z5-25818	Botswana	Tutume T	T10-R1-68b
Tanzania	Mialo Kondoa-M	T8-8	Zimbabwe	Matebeleland N-M	Z5-25823	Botswana	Tutume T	T10-R2-89
Tanzania	Mandimu-M	T9-3	Zimbabwe	Matebeleland N-M	Z5-25824	Botswana	Tutume T	T10-R2-90
Tanzania	Mandimu-M	T9-5	Zimbabwe	Matebeleland N-M	Z5-25825	Botswana	Marulamantsi T	T11-R1-133
Zimbabwe	Ngundu-M	Z1-25741	Zimbabwe	Matebeleland N-M	Z5-25828	Botswana	Marulamantsi T	T11-R1-34
Zimbabwe	Ngundu-M	Z1-25745a	Zimbabwe	Matebeleland N-M	Z5-25829	Botswana	Marulamantsi T	T11-R1-35
Zimbabwe	Ngundu-M	Z1-25745b	Zimbabwe	Matebeleland N-M	Z5-25975a	Botswana	Marulamantsi T	T11-R1-36

Zimbabwe	Ngundu-M	Z1-25746	Zimbabwe	Matebeleland N-M	Z5-25975b	Botswana	Marulamantsi T	T11-R2-126
Zimbabwe	Ngundu-M	Z1-25747	Zimbabwe	Matebeleland S-M	Z6-25834	Namibia	Kalimbenza T	T12-R1-12
Zimbabwe	Ngundu-M	Z1-25749	Zimbabwe	Matebeleland S-M	Z6-25836	Namibia	Kalimbenza T	T12-R1-29
Zimbabwe	Ngundu-M	Z1-25750	Zimbabwe	Matebeleland S-M	Z6-25838	Namibia	Kalimbenza T	T12-R1-30
Zimbabwe	Mudzi-M	Z2-25766	Zimbabwe	Matebeleland S-M	Z6-25841	Namibia	Kalimbenza T	T12-R1-31
Zimbabwe	Mudzi-M	Z2-25767a	Zimbabwe	Matebeleland S-M	Z6-25844	Namibia	Kalimbenza T	T12-R1-32
Zimbabwe	Mudzi-M	Z2-25767b	Zimbabwe	Matebeleland S-M	Z6-25851	Namibia	Kalimbenza T	T12-R2-22
Zimbabwe	Mudzi-M	Z2-25768	Zambia	Siavonga-M	ZA2-1	Namibia	Kalimbenza T	T12-R2-81
Zimbabwe	Mudzi-M	Z2-25769	Zambia	Siavonga-M	ZA2-12	Namibia	Oshikondilogo T	T13-R1-01
Zimbabwe	Mudzi-M	Z2-25774	Zambia	Siavonga-M	ZA2-15a	Namibia	Oshikondilogo T	T13-R1-3
Namibia	Oshikondilogo T	T13-R2-85	Tanzania	Wami T	T17-R1-63	Tanzania	Zimmatn T	T4-R4-254
Namibia	Oshikondilogo T	T13-R2-86	Tanzania	Wami T	T17-R1-64	Tanzania	Zimmants T	T5-R1-25
Namibia	Oshikondilogo T	TT13-R1-4	Tanzania	Zimmasv T	T1-R1-10	Tanzania	Zimmants T	T5-R1-26
Namibia	Oshikondilogo T	T13-T2	Tanzania	Zimmasv T	T1-R1-11	Tanzania	Zimmants T	T5-R1-27
Namibia	Ohangwena T	T14-R1-21	Tanzania	Zimmasv T	T1-R1-12	Tanzania	Zimmants T	T5-R2-97
Namibia	Ohangwena T	T14-R1-22	Tanzania	Zimmasv T	T1-R1-9	Tanzania	Zimmants T	T5-R2-99
Namibia	Ohangwena T	T14-R1-23	Tanzania	Zimmasv T	T1-R2-117	Tanzania	Zimmants T	T5-R3-146
Namibia	Ohangwena T	T14-R1-24	Tanzania	Zimmasv T	T1-R2-118	Tanzania	Zimmants T	T5-R3-149
Namibia	Ohangwena T	T14-R2-109	Tanzania	Zimmasv T	T1-R2-119	Malawi	Chikwawa T	T6-R1-55
Namibia	Ohangwena T	T14-R2-110	Tanzania	Zimmase T	T2-R1-49	Malawi	Chikwawa T	T6-R1-56
Namibia	Ohangwena T	T14-R4-282	Tanzania	Zimmase T	T2-R1-50	Malawi	Mangochi T	T7-R1-41
Swaziland	Manyonyaneni T	T15-R1-115	Tanzania	Zimmase T	T2-R1-51	Malawi	Mangochi T	T7-R1-42
Swaziland	Manyonyaneni T	T15-R1-37	Tanzania	Zimmase T	T2-R1-52	Malawi	Mangochi T	T7-R1-43
Swaziland	Manyonyaneni T	T15-R1-38	Tanzania	Zimmase T	T2-R1-56	Malawi	Mangochi T	T7-R1-44
Swaziland	Manyonyaneni T	T15-R1-40	Tanzania	Zimmase T	T2-R2-77	Malawi	Mangochi T	T7-R2-105
Swaziland	Manyonyaneni T	T15-R2-113	Tanzania	Zimmase T	T2-R2-78	Malawi	Mangochi T	T7-R2-106
Swaziland	Manyoyaneni T	T15-R2-114	Tanzania	Zimman T	T3-R1-6	Malawi	Ntcheu T	T8-R1-17
Malawi	Makata T	T16-R1-13	Tanzania	Zimman T	T3-R1-7	Malawi	Ntcheu T	T8-R1-19
Malawi	Makata T	T16-R1-14	Tanzania	Zimman T	T3-R1-85	Malawi	Ntcheu T	T8-R1-20
Malawi	Makata T	T16-R1-15	Tanzania	Zimman T	T3-R2--121	Malawi	Ntcheu T	T8-R2-101
Malawi	Makata T	T16-R1-16	Tanzania	Zimman T	T3-R2-122	Malawi	Mpandamatonga T	T9-R1-45
Malawi	Makata T	T16-R2-93	Tanzania	Zimman T	T3-R2-123	Malawi	Mpandamatonga T	T9-R1-46
Malawi	Makata T	T16-R2-94	Tanzania	Zimmatn T	T4--R1-57	Malawi	Mpandamatonga T	T9-R1-47
Tanzania	Wami T	T17-R1-6	Tanzania	Zimmatn T	T4-R1-59	Malawi	Mpandamatonga T	T9-R1-48
Tanzania	Wami T	T17-R1-61	Tanzania	Zimmatn T	T4-R1-60	Malawi	Mpandamatonga T	T9-R2-74
Tanzania	Wami T	T17-R1-62	Tanzania	Zimmatn T	T4-R2-70			

Appendix 2: Raw Scores from the Gels