PHENOTYPIC CHARACTERIZATION AND MOLECULAR STUDIES
OF KENYAN PASSION FRUIT (Passiflora edulis (Sims)) GENOTYPES
BASED ON SSR MARKERS

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

October, 2016
DECLARATION

I, Felix Matheri, 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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DEDICATION

This thesis is dedicated to my mother Regina ‘Simba’ Mumbi without whose financial and moral support, it would not be possible.
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I would like to begin by acknowledging the Almighty God for the grace, care and protection that He has extended in my life since the day I began this academic journey and my life at large.

I also wish to thank my mother Regina Mumbi for her selfless assistance and determination to have me succeed. I can’t forget to thank my brother George Weru for his unwavering support during my studies. To my sister, Mercy Mumbi, thank you.

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ABSTRACT

Passion fruit (*Passiflora edulis*) was introduced to Kenya in the 1920s by European farmers. The fruit is a significant income earner for the country from both local and international markets. There are increased research activities on variety improvement and development of new varieties with the hallmark of this research being the KPFs 4, 11, 12 by KALRO. The KPFs are hybrids developed through natural crosses of the coastal yellow varieties and purple variety. Despite these gains in breeding, there is little information of both phenotypic and molecular variability of these new cultivars as well as the existing ones. The present study aimed at evaluating the molecular and phenotypic variation in the hybrid and parent varieties. Phenotypic characterization was done by targeting 7 morpho-agronomic descriptors with three biological replicates. Analysis was done using MINITAB 16 software, for description principle components and construction of a dendrogram using the Euclidean distance tool. The first principal component alone accounted for 51.3% variance, while the first two components accounted for cumulative variance of 74.3%. Molecular characterization was done using SSR markers. Eleven (11) markers were polymorphic while one marker; PE 20 was monomorphic. Calculation of genetic distance, PIC, allele number as well as gene diversity was done using Powermarker® version 3.25 while phylogeny reconstruction was done using DARWIN version 6. On the other hand, AMOVA was calculated using GenAlex 6.502. The PIC ranged from 0.2929 (PE 04) to 0.6179 (PE 90) with a mean of 0.3909 indicating that marker PE 90 was highly informative and other markers were reasonably informative. The mean PIC of 0.3909 indicated that generally, all the 11 polymorphic markers were informative and as such can be utilized for further analysis of *Passiflora edulis* genome. The dissimilarity matrix indicated that KRC-2 and KRP-2 had the highest level of dissimilarity, confirming the known molecular variation that exists between the yellow and purple varieties where the respective genotypes belong to. Several genotypes had a dissimilarity of 0% and indication that they shared closer ancestry compared to other genotypes. The phylogenetic tree discriminated the samples into three main clusters with several sub-clusters carrying different fruit populations, indicating genetic relatedness among members of the same population. From the phylogenetic tree, genetic variability was highest between genotype KRC-2 and KRP-4 which are coastal and purple varieties respectively. This corroborates the findings of dissimilarity matrix where they had a dissimilarity of 76%. The AMOVA indicated intra-population variation of 91% while inter-population variation was 9%. From the results, Molecular and phenotypic characterization proved to be useful tools for discrimination of various genotypes of *Passiflora edulis*. The results from this study will enable better detection and management of genetic variability that could hinder adoption of the KPF as an alternative crop for the semi-arid agro-ecologies.
## ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>%</td>
<td>Percent</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celcius</td>
</tr>
<tr>
<td>µl</td>
<td>Micro litre</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified Fragment length Polymorphism</td>
</tr>
<tr>
<td>AMOVA</td>
<td>Analysis of Molecular Variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP-PCR</td>
<td>Arbitrarily primed Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cleaved Amplified Polymorphic Sequence</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>DAF</td>
<td>DNA Amplification Fingerprinting</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed Sequence Tag</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agricultural Organization</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GoK</td>
<td>Government of Kenya</td>
</tr>
<tr>
<td>Ha</td>
<td>Hectare</td>
</tr>
<tr>
<td>HCDA</td>
<td>Horticultural Crops Development Authority</td>
</tr>
<tr>
<td>HDC</td>
<td>Horticulture Development Centre</td>
</tr>
<tr>
<td>ISSR</td>
<td>Inter Simple Sequence Repeats</td>
</tr>
<tr>
<td>KALRO</td>
<td>Kenya Agricultural and Livestock Research Organization</td>
</tr>
<tr>
<td>KAPAP</td>
<td>Kenya Agricultural Productivity and Agribusiness Project.</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KHCP</td>
<td>Kenya Horticultural Competitiveness Program</td>
</tr>
<tr>
<td>KPF</td>
<td>Kenya Passion Fruit</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>MoA</td>
<td>Ministry of Agriculture</td>
</tr>
<tr>
<td>NAFIS</td>
<td>National Farmers Information Services</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>PCA</td>
<td>Principal Component Analysis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative Trait Loci</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotation per Minute</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SSR</td>
<td>Simple Sequence Repeats</td>
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<tr>
<td>STS</td>
<td>Sequence-Tagged Sites</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<tr>
<td>TE</td>
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CHAPTER ONE

INTRODUCTION

1.1 Background information

Kenya relies mainly on agriculture, which accounts for 24% of the GDP and hence invests in this sector (MoA, 2010). According to GoK (2007), approximately 5 million smallholders in rural areas rely on farming as a source of livelihood; through production of food, cash and horticultural crops both for subsistence and commercial purposes. Kenyan horticultural exports sustain the livelihoods of approximately 1.20 million people and are an important source of foreign exchange. The horticultural exports account for 14% of total export earnings (HCDA, 2011). In 2010, Kenya earned Kshs. 77.70 billion from horticultural exports with passion fruit contributing Kshs. 1.9 billion which translated to 1.19% of the domestic value of the total horticultural produce (Republic of Kenya, 2010; HCDA, 2011). Approximately 80% of horticultural producers are small holder farmers, with a large portion (96%) of horticultural produce being consumed locally, thus sold in the local markets (Namu, 2007; Kibet et al., 2011a).

In 2010, passion fruit was ranked third (8%) after avocado (62%) and mango (26%) in Kenya in terms of foreign exchange earnings for the country (HCDA, 2011). Kenya is considered the market leader of fruit juice exports in East Africa and is also listed among the large producers of passion fruit globally (KHCP, 2011). The major regional market for passion fruit produced
in Kenya is Uganda (Sebstad and Snodgrass, 2008). If production is carried out efficiently, passion fruit enterprises have good returns, with a gross margin of Ksh. 629,850 per hectare (Kibet, 2011). Production is expected to increase subsequently from the first to the third year (Fintrac, 2009). Therefore, the passion fruit is a high value crop with potential for poverty alleviation since it is mainly grown by farmers owning 0.5 - 2 acres of land (Otipa et al., 2008; Kibet et al., 2011b). Passion fruit farming is preferred due to the fast maturity period of 9 months and the minimal labor and land space requirements (Kahinga et al., 2006).

The passion fruit is documented to be native to the Southern Brazil, Paraguay and North Argentina, thus making this region considered as the main center of genetic diversity of the Passiflora species (Oliveira et al., 2008; Kilalo et al., 2013). Passion fruit was introduced to Kenya by the white settlers in the early 20th century (Morton, 1987). However, the plant only gained significant economic importance as an income generating crop in the 1990s when Kenya started bulk export of fruits and vegetables to the international markets (Muigai, 2002).

In the passion fruit production system, lack of improved varieties impedes the production of homogeneous, high-quality fruit. Thus, research on the genetic improvement of passion fruit such as development of new cultivars or improvement of the existing ones raises hope that this can be mitigated (Reis et al., 2011). Despite being crucial to production systems, development of new
varieties of passion fruit is faced with immense challenges. These include irregular production, low yields and high disease incidence, a scenario similar to the existing and well established varieties. Therefore, variability of the plant material to be improved is essential in order to maintain and increase the accompanying genetic gains throughout the various subsequent selection cycles of the variety (Costa et al., 2012).

Genetic diversity is narrowed by low variability in the base population and high selection intensity. This consequently reduces genetic gains in subsequent cycles that would have otherwise been achieved through expansion of variability in the base population (Costa et al., 2012). There is increased inbreeding, slight loss of variability and small changes in allele frequencies in two recurrent cycles of yellow passion fruit (Reis et al., 2011).

No significant genetic variability has so far been observed in different accessions of yellow passion fruit, assessed with Random Amplified Polymorphic DNA (Viana et al., 2003), Inter Simple Sequence Repeat (Santos et al., 2010) and morpho-agronomic descriptors (Crochemore et al., 2003). This indicates possible narrowing of the genetic basis of some commercial cultivars. Maintenance of genetic variability in breeding populations should be monitored at various stages of improvement, in order to ensure a successful and uninterrupted development of new varieties with improved traits such as increased yield, fruit quality and disease resistance. Advances in biotechnology and genomics offer a gradual change from
phenotypic to genotypic selection, thus the possibilities of using tools of biotechnology and genomics in breeding are numerous. Such tools include use of molecular markers (Reis et al., 2011).

A molecular marker is a sequence of a gene or DNA that is situated on a chromosome (Collard et al., 2005; Schulmann, 2007). Markers make it possible to detect differences between individuals by showing polymorphism, and therefore detecting genetic differences between individual organisms or species (Collard et al., 2005). Molecular markers are superior to techniques based on morpho- agronomic traits or geographic origin since they provide a means for direct measurement of genetic diversity and also go beyond the indirect diversity measures. Despite the significant ecological and economic importance of passion fruit, molecular markers have not been extensively utilized in genetic studies of this genus. In addition, basic genetic researches related to both population studies and pre-breeding programs of passion fruit remain scarce for most Passiflora species to date (Cerqueira-Silva et al., 2014a).

Of all the available molecular markers, microsatellites are considered superior because they are abundant, spread throughout the genome and also produce higher levels of polymorphism than other markers. These features, together with their ease of detection, have made the SSR useful markers. Their potential for automation as well as co-dominant manner of inheritance are additional advantages over other types of molecular markers (Islam et al.,
2012). Since the SSR markers started being applied to passion fruit recently, and is least explored compared to other equally important crops, this necessitates a study on new varieties such as the KPFs is crucial (Reis et al., 2011).

1.2 Problem statement and justification

Despite being under cultivation in Kenya since its introduction in the 1920s, there is scanty information on the genetic basis underlying passion fruit breeding (Wangungu, 2012). This is despite the fact that many researchers have engaged in development of new varieties with the hallmark of this research being development of KPF-4, KPF-11 and KPF-12 varieties. Their development was done through conventional breeding by crossing the yellow passion fruit varieties with the purple variety in order to pool the superior traits from both forms (Hortinews, 2015). Despite introgression of preferred genes being evaluated, physically, there does not exist documentation on the phenotypic variation in these hybrids as well as their phenotypic comparison with their parent genotypes. Past studies have largely focused on pest and diseases management as well as provision of planting materials of the passion fruit; (Mbaka et al., 2006; Otipa et al., 2008; Amata et al., 2009; Gachanja and Ochieng’ 2011; Gaturuku and Isutsa, 2011; Wangungu, 2012).

Compared to other agricultural crops, relatively little information is known about the genetic diversity within and among the species of Passiflora (Santos et al., 2011). Selection of superior genotypes is normally based on the
phenotypic evaluation of progenies. The findings of this study will be used to supplement the current body of knowledge of plant breeding specifically of Kenyan passion fruit.

1.3 Null hypotheses

i) There is no phenotypic variability among the selected Kenyan Passiflora edulis genotypes.

ii) There is no molecular variability among the selected Kenya Passiflora edulis genotypes.

1.4 Objectives

1.4.1 General objective

To determine the phenotypic and genetic variability of Kenyan Passiflora edulis genotypes.

1.4.2 Specific objectives

i) To determine the phenotypic variability in Kenyan Passiflora edulis genotypes using morpho-agronomic descriptors.

ii) To determine molecular variability in Kenyan Passiflora edulis genotypes using SSR markers.
CHAPTER TWO
LITERATURE REVIEW

2.1 *Passiflora edulis* (Sims) *flavicarpa* (Deg)

2.1.1 Origin and description

Passion fruit (*Passiflora edulis*) is considered to have originated from southern Brazil through Paraguay to northern Argentina (Acland, 1971; Morton, 1987). Brazil is therefore considered as the center of genetic diversity of passion fruit, with a total of one hundred and fifty species in the family of *passifloracea* being found in Brazil alone. Eighty two of these species are endemic to Brazil (Fajardo *et al.*, 1998; Viana *et al.*, 2003; Ocampo *et al.*, 2010; Bernacci *et al.*, 2013). Phylogenetic studies, assisted by molecular techniques, have shown that the genus *Passiflora* can be organized into four subgenera namely: *Astrophaea, Decaloba, Deidamiodes* and *Passiflora* (Muschner *et al.*, 2003; Feuillet and MacDougal, 2004; Pádua, 2004; Muschner *et al.*, 2012). The family *Passifloracea*, to which *Passiflora edulis* belongs, falls under the subgenera *passiflora*. These studies contradict previous ones that hypothesized that *Passiflora* can be organized into 22 (Killip, 1938) and 23 (Escobar, 1989) subgenera.

Recent studies based on the analysis of genomic sequences, mitochondrial regions and plastidial genomes indicate that historically, the ancestors of *Passiflora* arrived in Central America and diversified quickly from there, with numerous dispersion events (Muschner *et al.*, 2012). Despite these
uncertainties facing taxonomy, *Passiflora* is highly diverse, with approximately 520 species (Feuillet and MacDougal, 2004). Other authors place the estimate of species at more than 630 species (Santos *et al.*, 2010).

Passion fruit plant may be described as a perennial woody vine that produces edible round to ovoid fruit with numerous small seeds (Lipmann, 1978). Passion fruit varieties cultivated in Kenya have two main forms, purple passion (*Passiflora edulis* (Sims)) and the yellow passion (*Passiflora edulis var flavicarpa*) (DeVillers and Fraser, 2000; MoA, 2003). Several varieties fall under the yellow form. These include; C5, Brazil which are cultivated in the coastal areas and the KPFs 4, 11 and 12 which were developed by KALRO after more than 20 years of research. The purple variety is more preferred due to its high sugar contents (Fushimi and Gitonga, 2001; Erbaugh *et al.*, 2010; Hortinews, 2015).

The passion fruit is an allogamous plant since it presents self-incompatibility that prevents self-fertilization and crossing of different plants containing similar incompatibility alleles (Bruckner *et al.*, 2005). Passion fruits present the sporophytic type of self-incompatibility having a gene with gametophytic effect associated with the sporophytic system (Bruckner *et al.*, 1995; Suassuna *et al.*, 2003).
2.1.2 Distribution and agro-ecological requirements

The more than 630 species of passion fruit are distributed in the tropical regions of Africa, America and Asia (Vanderplank, 1996). Majority of passion fruit accessions are found in South American with four countries in this region, representing approximately 84% of total global accessions. Brazil has the highest number of accessions, representing 32% (Ferreira, 2005). Other countries with large accessions include Ecuador, Peru and Colombia with 30%, 14%, and 8% respectively (Cerqueira- Silva et al., 2014a).

In Kenya, cultivation of passion fruit is done in three broad agro-ecological zones including lowlands which cover coastal areas such as Matuga and Taita Taveta. Another agro-ecological zone where cultivation of passion fruit is practiced is the mid zones covering areas such as Thika and Kitui in Central and Eastern regions (MoA, 2003).

The third main agro-ecological zone where passion fruit is grown is the highlands which covers areas such as Sotik, Laikipia, Mathioya, Meru and Embu in Central Eastern and Rift Valley regions. The yellow variety thrives well in the lower agro-ecological zones, while the purple passion is suited to the highlands which have relatively cool climates; with both forms performing relatively well in the mid agro-ecological zones (Morton, 1987; MoA, 2003; Wangungu, 2012).
2.1.3 Economic importance of *Passiflora edulis*

The economic importance of passion fruit plant comes mainly from its fruit. *P. edulis f. flavicarpa* (passion fruit) together with its closely related species; *P. alata* (sweet passion fruit) are the most commercialized species in *Passifloracea*, genus and are generally directed at the food industry (Dhawan *et al.*, 2004). Other related species such as *P. caerulea, P. incarnata*, as well as several interspecific hybrids cultivated mainly in Europe and the USA find great application in the ornamental market (Rushing, 2003).

The *Passiflora* species at large has been documented to bear medicinal value. The following species are notable among the various species of passion fruit with potential medicinal properties: *P. alata, P. caerulea, P. edulis, P. foetida, P. incarnata, P. laurifolia, and P. maliformis* (Cerqueira-Silva *et al.*, 2014a). The fruits, leaves, roots, and flowers of both wild and commercial species have been documented as possessing the capability to fight diseases, such as gastric tumors, helminth infestations, as well as stress, and are an integral element of the cultural tradition of various populations in the world (Costa *et al.*, 2005). According to Amata *et al.* (2009), passion fruit is a rich source of minerals as well as vitamins A, C, and D. The passion fruit is also known to contain alkaloids, carotenoids, and flavonoids that are beneficial to human health (Dhawan *et al.*, 2004). Passion fruit seeds are sources of essential fatty acids (55%–66% linoleic acid, 18%–20% oleic acid, and 10%–14% palmitic acid), which are useful in the food and cosmetic industries (Zeraik *et al.*, 2010). The commercial potential of passion fruit extends to include the
extraction of oils for the manufacture of soaps, creams, shampoos, and other cosmetic products (Faleiro et al., 2011). Compounds in passion fruit plants with pharmaceutical value such as anxiolytic, antihypertensive, sedative, and analgesic properties are well studied and documented (Ngan and Conduit, 2011; Konta et al., 2014).

In Kenya, passion fruit’s economic value has only been exploited from its fruit and not aesthetic value. The fruit has been a significant income earner both in local and international markets. The price was ranging between Kshs 70-80 per kilo in 2012, (NAFIS-Kenya, 2012). According to HCDA (2011), horticultural exports which include passion fruit, sustain the livelihoods of approximately 1.20 million people in Kenya, and are an important source of foreign exchange, accounting for 14% of total export earnings. In 2010, Kenya earned Kshs. 77.70 billion from horticultural exports (HCDA, 2011). Aside from exports, a large percentage (96%) of horticultural produce is consumed locally (Kibe, 2009). Approximately 80% of horticultural producers are small holder farmers (Namu, 2007). The yellow passion also offers a source of revenue to nurseries operators since their vegetatively propagated seedlings cost higher than those of parent varieties. They cost about Ksh 30 per seedling (Hortinews, 2015).

Passion fruit production in Kenya had been increasing gradually from the beginning of the 21st century until 2007 when it started to decline. There was notable increase in production between 2005 and 2007 when production
doubled after which it declined in 2008 with fluctuations in production through the subsequent years (Table 2.1). This decline is attributed to perennial challenges that lead to the sector operating below potential and as such, lagging behind other global competing producers like Australia and South Africa (HDC, 2005; Mbaka et al., 2006). Insufficient knowledge on good agricultural practices as well as pest and disease management, in addition to the inaccessibility of pathogen-free planting materials are some of the major challenges that face passion fruit production.(Mbaka et al., 2006; EU, 2009; Kleemann et al., 2010; Wangungu et al., 2010). Changing climate patterns are also contributing to the decline in passion fruit production. This is by favoring population densities and emergence of new species of the pests as has been the case with other crops (Emden and Service, 2004; EU, 2009).

<table>
<thead>
<tr>
<th>Year</th>
<th>Production area (ha)</th>
<th>Output ('000' Kg)</th>
<th>Yield (kg/ha)</th>
<th>Value ('000' Kshs)</th>
</tr>
</thead>
<tbody>
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<td>3958</td>
<td>53396</td>
<td>13490.65</td>
<td>1601850</td>
</tr>
<tr>
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<td>49662</td>
<td>8860.30</td>
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<tr>
<td>2010</td>
<td>6745</td>
<td>55094</td>
<td>8168.12</td>
<td>1873925</td>
</tr>
</tbody>
</table>

2.2 Morpho-agronomic descriptors for characterization of passion fruit

A description of variety should help in resolution of identity conflicts arising in registration and protection of cultivars (Castro et al., 2012). Morpho-agronomic characterization of germplasm as well as new varieties using descriptors is a key consideration in breeding programs. The term descriptor is used to refer to a character or attribute that is used to discriminate between varieties, with redundant descriptors being seen during evaluation of many traits and thus many descriptors are classified accordingly as unnecessary due to their low contribution to variability (Dahe et al., 1997; Castro et al., 2012; Oliveira et al., 2012).

Elimination of redundant descriptors is an important strategy in that it ensures reduction of the work required to collect data without causing significant losses in genotype discrimination (Oliveira et al., 2006; Oliveira et al., 2012). Some of the techniques used to determine the descriptors with high information content include regression discriminant analysis (Beale et al., 1967; Mardia et al., 1979) as well as principle components (Cruz et al., 2004). The distribution of phenotypic variation is associated with the nature and number of characters that are used in the analysis and is concentrated in the first components only when few agronomically important traits are evaluated (Pereira et al., 1992).
2.3 Molecular markers and genetic variability

A molecular marker is a DNA sequence or a gene, which is situated on a chromosome (Collard et al., 2005, Schulmann, 2007). They are also referred to as DNA markers. These markers enable detection of differences between individuals by showing polymorphism, thus enabling the detection of genetic differences between individual organisms or species (Collard et al., 2005).

Molecular markers find numerous applications in different areas of science. These include: genetic mapping, finding mutant genes of hereditary diseases, paternal tests, individual identification, epidemiology, food safety, population history and population studies (Hartl and Jones, 2005). Since the introduction of DNA makers in the 1980s, agricultural practices have become simplified and broadened for both scientific and commercial uses to reveal new and crucial information about crops, such as disease resistance (Collard et al., 2005). The DNA markers are used to study genetic diversity and construct linkage maps, as well as tracing desirable traits for crops by plant researchers (Schulmann, 2007).

Molecular markers have many advantages compared to morphological and biochemical markers. For instance, compared to morphological markers, the environmental influence can be disregarded when applying DNA markers. They are also superior in that they do not depend on the plant development stage and are ideal to detect inter and intraspecific molecular polymorphism (Costa et al., 2012). But, still comparing the two marker groups, the
morphological markers have an advantage over DNA markers in that they do not require a lot of expensive and time consuming laboratory work, which is necessary when working with DNA markers (de Vicente and Fulton, 2003).

For efficient utilization of genetic variability in plant breeding, it requires proper assessment and quantification of the germplasm. The germplasm must therefore be characterized according to several morphological traits (Crochemore et al., 2003; Plotze et al., 2005; Viana et al., 2010), agronomic behavior (Abreu et al., 2009) and molecular diversity (Cerqueira-Silva et al., 2008; Bellon et al., 2009; Cerqueira-Silva et al., 2009, 2010; Viana et al., 2010) to enable advances in description of genetic divergence among passion fruit accessions.

2.4.1 Types of DNA markers

Molecular markers have been used in plant breeding programs as well as in activities related to the conservation of genetic resources. The most common DNA markers are the random amplified polymorphic DNA (RAPD) (Welsh and McClelland 1990; Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Vos et al., 1995), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), diversity array technology (DART) (Wenzl et al., 2004), sequence-tagged sites (STS) (Bradshaw et al., 1994), single nucleotide polymorphism (SNP) (Wang et al., 1998), single simple repeat (SSR) (Tautz 1989; Weber and May, 1989), and inter-simple
single repeat (ISSR) (Zietkiewicz et al., 1994). Different types of DNA markers are available and new methods are continually being developed. There is however no ideal for all applications, and as such researchers are required to weigh both pros and cons of the methods when starting a new project (Harlt and Jones, 2005).

The DNA markers are grouped into two main groups based on their different abilities to show dominant marker (homozygosity) and co-dominant marker (heterozygosity) (Hartl, 1988; Hartl and Jones 2005). Dominant DNA markers for genetic diversity in plants include: RAPD, DNA, DAF, AP-PCR, ISSR and AFLP. On the other hand, the most commonly used co-dominant markers are: RFLP, SSR, CAPS, EST and SNP (de Vicente and Fulton, 2003). Both dominant and co-dominant markers can be used in detection of DNA polymorphism, which is further used to assess the level of genetic variation in diverse populations, and can also indicate population history, patterns of migration, and breeding structure (Hartl and Jones, 2005).

Despite the prevalence of molecular biology techniques in recent decades, leading to a reduction in the cost of most molecular markers, factors such as the availability of resources and background information are still determining factors in the choice of evaluation methods and markers to be used (Cerqueira-Silva et al., 2014a). There has been limited application of molecular marker techniques to a few *Passiflora* species. This is despite the availability of approximately 4000 fragments of both DNA and RNA sequences from
Passiflora data from the NCBI nucleotide database. (Cerqueira-Silva et al., 2014a).

Despite the limitations that are inherent to the use of dominant markers in passion fruit studies, genetic diversity estimates based on wild and commercial accessions have greatly contributed to the understanding of the genetic variability. These DNA markers include RAPD and AFLP with SSR markers being among the least exploited (Ganga et al., 2004; Bellon et al., 2009). The DNA markers have also been used to explain lack of correlation between the estimated genetic distances and geographical origins of the accessions being evaluated (Ganga et al., 2004). Nevertheless, estimates of variability using RAPD markers achieved with commercial accessions of *Passiflora edulis* indicated low genetic variability (Viana et al., 2003; Cerqueira-Silva et al., 2010). These studies have suggested that the contradiction between the low genetic diversity seen with molecular markers and the large variability observed in commercial plantations is mainly due to the major environmental influence on morphological characteristics. There was observed to be greater genetic distance among existing accessions of purple and yellow fruits (Viana et al., 2003; Bellon et al., 2007).

The accessions of both purple and yellow fruits are considered to be genetically distinct despite belonging to the same species (Bellon et al., 2007). The RAPD markers have also contributed to estimation of diversity in wild species of passion fruit, such as *P. cincinnata, P. nitida*, and *P. setacea*.
(Junqueira et al., 2007; Cerqueira-Silva et al., 2010; Cerqueira-Silva et al., 2012). In all these studies, great genetic variability was observed, with percentages of polymorphic loci ranging from 64% in *P. nitida* (Junqueira et al., 2007) to 93% in *P. setacea* (Cerqueira-Silva et al., 2012).

Inter-simple sequence repeats are regions found between microsatellite repeats (Zietkiewicz et al., 1994; Godwin et al., 1997; Bornet and Branchard, 2001; de Vincente and Fulton, 2003). These regions are semi-arbitrary markers that are amplified by PCR in the presence of a primer that is complementary to a target SSR. Inter Simple Sequence Repeats are useful in detecting genetic polymorphism, and have been used to fingerprint closely related individuals (Zietkiewicz et al., 1994) and for genetic diversity and variability studies (Tsumura et al., 1996).

Inter-simple sequence repeats have been in use since 1994 for a wide range of organisms (Zietkiewicz et al., 1994). Some of the applications include DNA fingerprinting, diversity analysis as well as genome mapping (Godwin et al., 1997; Bornet and Branchard, 2001; de Vincente et al., 2004). Some of the advantages of using ISSR include that it is quick, easy to apply, highly reproducible and polymorphous. Its greatest advantage is that no prior information about genomic sequence is required (Bornet and Branchard, 2001). The main disadvantage of the ISSR technique is its lack of co-
dominance and the consequent resolution of effectively bi-allelic loci which is the band presence versus band absence. (Tsumura et al., 1996).

Amplified Fragment Length Polymorphism (AFLP) is a PCR technique that uses restriction fragment analysis. Amplified Fragment Length Polymorphism markers are DNA fragments detected after PCR amplification, which provide a number of attractive features in genome fingerprinting of different complexities (Vos et al., 1995). The advantages of the AFLP technique include production of high polymorphisms, sequence information is not required and results are reproducible (Robinson and Harris, 1999). Restrictions of AFLPs are associated with the large number of steps involved as well as the fact that it is mainly a dominant marker (Wolfe and Liston, 1998).

2.4.2 Microsatellite markers

Microsatellites can be described as short nucleotide tandem repeats of a motif, usually one to six bases long. They are often referred to as simple sequence repeats (SSR) and are present in genomes of living organisms such as bacterial, fungal, plant, animal and human beings. Microsatellites are highly polymorphic, specific, easy to amplify, highly abundant and evenly distributed throughout genome (Bornet and Branchard, 2001; Weising et al., 2005b).

Studies have revealed that the most abundant repeated motifs in plants are (A)n, (AT)n, (GA)n, and (GAA)n with both homozygotes and heterozygotes
being detectable since microsatellites are co-dominant markers (Weising et al., 2005a; Weising et al., 2005b). The microsatellites are nowadays one of the most important genetic markers for population studies, due to their ability to show extensive variation between individuals as well as within populations (Coetes and Byrne, 2005).

Due to the broad spectrum application of SSR, it has found preference in plant breeding, conservation biology, population genetics as forensics, paternity analysis and gene mapping (Coates and Byrne, 2005). Microsatellite based techniques require little amount of DNA, which does not necessarily have to be of high quality. Another advantage is the simple interpretation of results, with them being co-dominant, multi-allelic, polymorphic, and reproducible thus, highly informative content can be generated (de Vicente and Fulton 2003; Schlötterer, 2004).

The main disadvantage of SSR is the requirement of a known sequence to be amplified. Development of new microsatellites is also time consuming and notably expensive (Coates and Byrne, 2005; Weising et al., 2005b). Another disadvantage is the phenomena of null-alleles, which are non-amplifying alleles and appear frequently. When having homozygosity, a null-allele leaves no bands but when having heterozygosity it leaves one visual band. This interferes and complicates reading of data, since it will have to be registered as a homozygote individual when actually being a heterozygote. To reduce this error due to null alleles, population studies should contain as many and diverse
SSR primers as possible so that different multiple microsatellite loci are investigated (Weising et al., 2005b). Another shortcoming facing the SSR technique is the initial cost of finding and sequencing the loci. They are also known to have high mutation rates and as such have limited use for phylogenetic analysis (Chambers and Avoy, 2000).

Comparative genetic variability studies have been successfully conducted on Passiflora using Randomly Amplified Polymorphic (RAPD) molecular markers (Viana et al., 2003; Perez et al., 2007; Viana et al., 2010) and inter-simple sequence repeat (ISSR) (Santos et al., 2011). There are however, only a few studies using microsatellite markers for the characterization of Passiflora involving several species (Paiva et al., 2014).

2.5 Application of genetic variability in plant breeding

Due to the economic and social importance of Passiflora, many studies have been conducted to develop varieties adapted to different cropping systems and climatic conditions (Santos et al., 2010). Activities related to the collection, conservation, characterization, and usage of Passiflora germplasm have been implemented to ensure that genetic variability is available for exploitation in breeding programs. Genetic diversity refers to variation of genes within species, that is, the heritable variation found within and between populations of organisms. Consequently, all variation resides in the sequence of the four base pairs that make up the DNA molecule and, as such, constitute the genetic code (de Vincente and Fulton, 2003).
Genetic diversity can also be identified at all levels of organization in the nucleus. These include the amount of DNA per cell, chromosome number and DNA structure. New genetic variation is continuously generated in individuals through mutations of chromosomes and genes, which in organisms with sexual reproduction, are propagated by recombination (de Vincente and Fulton, 2003). The genetic diversity of a population within a species is also affected by a number of evolutionary factors, such as the mating system, seed dispersal, gene flow, natural selection, geographic range, and the diversity center (Hamrick and Godt, 1989).

Although broad genetic diversity is expected in *Passiflora* because of the geographic distribution of the genus (Lopes, 1991), the divergence among collected genotypes at production areas is limited. The consequences of these phenomena are changes in gene and allele frequencies that account for the evolution of populations. Similar situations can occur through artificial selection such as breeding (de Vincente and Fulton, 2003). Selection of genotypes with greater phenotypic uniformity will always lead to narrowing of the genetic base regardless of the goals and methods used in improvement of the variety. This will also affect the characteristic self-incompatibility of yellow passion fruit that leads to allogamy and consequently genetic variability. Depending on the origin of genetic material and the degree of selection, loss of alleles can negatively affect the maintenance of the productive potential of improved genotypes (Santos *et al*., 2010).
Genetic variability is expected even among plants of the same accession because passion fruit is allogamous, due to its self-incompatibility. This is still the case even if they are the progeny of controlled pollination between related plants (Bruckner et al., 2005; Cerqueira-Silva, 2014b). Similar results were previously observed in accessions of commercial species (*P. edulis* and *P. alata*) (Santos et al., 2010). The maintenance of genetic variability in breeding populations requires monitoring at various stages of improvement, in order to ensure a successful and uninterrupted development of new varieties with increased yield, fruit quality as well as disease resistance (Reis et al., 2011).

The genotype by environment interaction is a constant challenge facing breeders. This is due to the complications brought about by the selection of accessions evaluated in different environments. In plant breeding, the greater the genetic diversity among genotypes and environments, the greater the importance of the interaction. This is especially the case in environments with considerable difference in temperature and precipitation variations (Borém, 1997).

Plant genetic resources include the existing genetic variation that has potential uses for the future of mankind. These genetic resources include traditional varieties, landraces, commercial cultivars, hybrids, and other plant materials developed through breeding. It also includes wild relatives of crop species; and others that could be used in the future for agricultural and environmental
benefits. Hence, plant genetic resources should be conserved, with the ultimate reason being to ultimately use these resources as a source of potentially useful genetic variation (de Vincente and Fulton, 2003). The ability to resolve genetic variation may in most scenarios is more directly related to the number of polymorphisms identified through the marker system (Sivaprakash et al., 2004). The use of molecular markers in early screening of the most promising plants for recombination therefore has the advantage of optimizing the use of time and resources spent on breeding (Reis et al., 2011).

2.6 Application of alleles in genetic diversity studies

There is a relationship between the number of alleles detected at a locus and the types of simple repeats existing within the targeted microsatellite DNA. As such, the larger the variation in the repeat motif in the microsatellite, the larger the number of alleles detected (Islam et al., 2012). It is known that genetic divergence is useful in expressing the difference in the allele frequency of a population, and that the effect of the recombination of different alleles and types of gene action allows higher genetic gains. This is by either exploring the additive effects to develop synthetic varieties or even by exploiting the allelic interactions of dominance and over-dominance for the development of hybrids (Reis et al., 2011). Despite the numerous advantages realized in breeding and selection, a great number of alleles have been lost through breeding and selection (Allard, 1996; Hoisington et al., 1999). With regard to the estimates observed for SSR loci from passion fruit, it is important to note
that there is low allelic diversity and tendency toward the fixation of alleles. This is due to the low values of observed heterozygosity, in comparison with the values of expected heterozygosity (Oliveira et al., 2008; Penha et al., 2013).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of plant materials

Fully ripe fruits and fully expanded leaves were collected for phenotypic characterization whereas young leaves were collected from healthy, vigorously growing vines for molecular studies. These materials were collected from passion fruit genotypes belonging to the varieties; KPF 4, KPF 11, KPF 12, Brazil, and purple, from the KALRO breeding orchards at the centre for horticultural crops- Kandara, Murang’a. All samples were assigned to populations based on the variety, with each population being composed of four (4) plants. Samples were also collected from different geographic locations in Embu County; Kenya and Kenyatta University School of Agriculture Farm. These samples had undetermined genotypes and were assigned one population. For the phenotypic study, replication was done five times per plant and three biological replicates.

Variety and category information of all the genotypes in this study was recorded (Appendix 1). The leaves for molecular studies were dried in silica gel granules and transported to Kenyatta University Plant Transformation Laboratory. In the laboratory, the leaf samples were shock frozen in liquid nitrogen and kept at -80°C awaiting DNA extraction.
3.2 Phenotypic characterization

Morpho-agronomic traits developed by CIRAD, IPGRI (now Bioversity International) were targeted on all the plants from which leaf samples for molecular studies had been obtained. The morpho-agronomic traits under study included; leaf length, leaf width, fruit length, fruit diameter and seed length whose data was recorded in centimeters. Fruit mass was taken using an analytical balance and recorded in grams. The recorded data was entered in Excel worksheet and used for phenotypic analysis using the MINITAB software version 16.

3.3 Molecular characterization

3.3.1 Deoxy ribonucleic Acids (DNA) extraction

About 2cm$^2$ of each leaf sample was placed in 2ml eppendorf tubes with 2 metallic beads, closed and immediately dipped into liquid nitrogen. The tube was then vortexed at high speed and returned to liquid nitrogen when the tissue was about to thaw. This was done until it was ground to a fine powder, after which the metallic bearings were removed. Using a pipette, 500μl of CTAB buffer containing 0.2% β – Mercapto-ethanol, were added to each tube and mixed. The tubes were then incubated at 65$^o$ C for 30 minutes, with mixing after every 10 minutes by inverting them three times. This was followed by addition of 500μl chloroform- isoamyl alcohol (24:1) with gentle mixing until the components were completely mixed, after which centrifugation at 13,000 rpm for 10 minutes was done. A pipette was used to
aspirate the aqueous layer into clean tubes followed by addition of two thirds volumes of isopropanol. The components were mixed and incubated overnight at -20°C. The tubes were then centrifuged at 13,000 rpm for 10 minutes and the supernatant discarded. This was followed by addition of 500μl of 70% ethanol and the tubes tapped and centrifuged at 13,000 rpm for 10 minutes, after which the supernatant was poured. This washing step was repeated, and the open tubes were placed upside down on clean paper towel in order to dry the pellet. The pellet was finally dissolved in 40μl DNAse free water.

3.3.1.1 Confirmation for presence and quality of DNA

A 1% agarose gel was used to estimate genomic DNA concentrations and quality by comparing the fluorescent signal from DNA stained with Sybr green dye. This was done on TBE electrophoresis by running 5μl of genomic DNA at 80 V for one hour. Gels were viewed under UV illuminator and photographed using a digital camera. Formation of distinct and bright bands was used as an indication of a quality DNA.

3.3.2 Primer selection

Twelve sets of SSR primers of the PE series (Passiflora edulis Sims), developed by Oliveira et al. (2005) were used (Table 3.1).
Table 31: SSR loci, primer sequence, motif, expected allele size (bp) of the primers

<table>
<thead>
<tr>
<th>LOCUS</th>
<th>FORWARD PRIMER (5'-3')</th>
<th>REVERSE PRIMER (3'-5')</th>
<th>REPEAT MOTIF</th>
<th>ESTIMATED bp</th>
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<tbody>
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<td>178</td>
</tr>
</tbody>
</table>

3.3.3 Polymerase chain reaction (PCR)

Each reaction contained 22 mM KCl; 20 mM Tris-HCl (pH 8.9 at 25°C); 1.8 mM MgCl₂; 22 mM NH₄Cl; 0.05% Tween® 20; 5% glycerol; 0.06% IGEPAL® CA 360; 0.2 mM of each dNTPs; 0.5 μM of each primer; 2 μg (1μg/μl) DNA template and 25 units/ml of One Taq DNA Polymerase to make a final volume of 25 μl. A negative control that contained the PCR master mix without primers and genomic DNA was run for every batch. Amplification was conducted in a thermocycler; Applied Biosystems (E2720 thermo-cyclcer). It involved 35 cycles each with one denaturation step at 94 ºC for 4 minutes; an initialization step at 94 ºC for 30 seconds; an average of the annealing temperatures of both primers of each marker (ranging from 45 to 48 ºC) for 30 seconds; followed by an extension step at 68 ºC for 60 seconds; and final extension at 68 ºC for 7 minutes.
3.3.4 Agarose gel electrophoresis analysis of the PCR products

The PCR products from the different markers were separated on 3% agarose gels to enable visualization of polymorphism. The PCR products were prepared by adding 5 μl loading buffer (0.05% Bromophenol blue, 1 mM EDTA, 10% Ficoll, 20% sucrose and 5 M urea) and 5 μl of Sybr® green dye to 5 μl of each sample. Each well on the gel was loaded with all the 15 μl of the loading mixture. One well was left on one side of the gel and was loaded with 5 μl of 100 bp DNA ladder (Invitrogen®). The gels were then run at 80 V for one and a half hours when the blue dye reached the anode wick. Visualization of genomic DNA was done under a UV Trans-illuminator and gel photos taken using a digital camera.

3.5 Data management and statistical analysis

Phenotypic analysis was done for the difference in means through one-way analysis of variance followed by Tukey’s post hoc test to separate means. Using the 7 morpho-agronomic traits for the 54 genotypes, a dendrogram was also constructed from their mean values. Eucledian distance tool was employed before forming complete linkage distance among the different genotypes. Cluster analysis yielded a dendrogram that was used to examine the phenotypic relatedness among the 54 Passiflora edulis genotypes. Principal component analysis (PCA) was also conducted in order to determine the source of morpho-agronomic variation based on the 7 phenotypic traits.
among the 54 *Passiflora edulis* genotypes. This was done using Minitab® version 16 software.

For molecular characterization, each DNA fragment was treated independently and scored as a discrete variable, with presence being reported as 1 while absence was reported as 0. Variability of the polymorphic markers was analyzed by simple matching coefficient of similarity as described by Cruz, (2008) using Powermarker® version 3.25 software. A dendrogram was then constructed using neighbor joining tool of DARWIN® version 6 with bootstrapping at 1000 permutations. Viewing of the phylogenetic tree was done using Treeview® software. Polymorphism information content (PIC) value for the SSR markers was also calculated using Power marker® version 3.25 software. On the other hand, Analysis of molecular variance (AMOVA) using GenAlex® version 6.502 software was carried out to determine variation within and among the different populations (Peakall and Smouse, 2012).
CHAPTER FOUR

RESULTS

4.1 Phenotypic characterization

The measurement of the leaf, fruit and seed traits for the 54 passion fruit genotypes and their mean values are shown in Appendix 2. From the tabulated results, KR4-1 which belongs to the KPF-4 variety, collected from KALRO orchards had the highest value of leaf length (14.82 cm) while KRC-2 which was a coastal yellow passion fruit (Brazil variety) collected from KALRO orchard had a value of 8.60 cm. In relation to the leaf width, there was no significant difference among all the genotypes although the data range indicated wide variation. KR4-4 had the highest mean value of leaf width (13.36 cm) while genotype SGE-ID1 had the lowest mean value (9.62 cm) (Appendix 2).

Longitudinal fruit length had wide variations ranging from 5.10 (PKS-TD1) to 10.58 (KR12-1 and KR12-3). Two genotypes averaged 0.00 cm since they did not bear fruits during data collection and no fruiting history and could therefore not be used to describe fruit length variation. On the other hand, there was no significant difference among mean fruit length values of some of the individuals; KID-ID1, JUS-ID1, PKS-ID1, MMD-ID1, KNG-ID1, SGE-ID1 and SGE-ID2 (Appendix 2). The tabulated results of the mean fruit longitudinal length also showed no significant differences for most genotypes. For example, there was no significant difference in the mean longitudinal lengths of genotypes; KMD-TD1, BMM-TD1, PKS-TD1, KUA-TD1, SNV-TD1, BMM-TD2, KMD-TD2 and SGE-TD1.
The mean values for fruit length among genotypes of the purple variety population which is one of the parent genotypes for the hybrid varieties (KPF-4, KPF-11 and KPF-12) had no significant difference. Genotypes, KRC-1, KRC-2, KRC-3 and KRC-4 which belong to the coastal variety were also found to have no significant difference among their mean values for longitudinal fruit length.

On the other hand, equatorial fruit diameter ranged from 3.74 cm (KMD-TD1) to 10.18 cm (KR12-1). The two non-fruiting genotypes were not used in description of fruit diameter due to their lack of fruits during data collection. There was no significant difference among the means of fruit diameter for most genotypes. As was the case with fruit length, individuals belonging to the purple passion fruit variety did not have significant difference. In relation to seed length, there was variation among the genotypes but no significant variation among genotypes in a particular assigned population. The values ranged from 0.32 cm (KR12-4) 0.78 cm (PKS-TD1) (Appendix 2).

Rind thickness ranged from 0.52 cm (SGD-TD1) to 0.86 cm (KR12-2). There was no significant difference between the mean rind thickness of several genotypes such as JUS-N1, KR12-4, PKS-N3, KR11-1, KRP-3, KRP-4, KUA-N1, KRP-2, KNG-FP1, SNV-N1, KNG-FP2, KR12-1, SGE-N2, KNG-N2, KR4-1, KR11-4 and KRC-4 (Appendix 2). Mean values for two genotypes; MMDNF1 and MMDNF2 were not considered since they were not fruiting and as such their fruit based parameters were assigned a value 0.
There was also wide variation in fruit mass among the populations. The mean fruit mass ranged from 28.62 g (BMM-TD2) to 121.82 g (KR11-1) (Appendix 2). There was no significant variation among several genotypes including JUS-N1, KR12-4, PKS-N3, KR11-1, KR4-1, SGE-N4, KR4-2, SGE-N1, KR12-3, KUA-N1KNG-FP2, KR11-4 and KRC-4 (Appendix 2). The mean values for fruit mass of MMDNF1 and MMDNF2 were not considered in the description variation in fruit mass of the genotypes under study since the plants were not fruiting and therefore were assigned values 0. On the other hand, mean values for seed length paired genotypes PKS-N3, KMD-TD1, PKS-ID1, PKS-TD1, BMM-TD2, MMD-ID1, KNG-ID1, SGE-ID2, SGE-ID1, KRC-1, KRC-3 and KRC-4 indicating that there was no significance difference between their mean seed length. There was also no significant difference between genotypes JUS-N1, PKS-N3, KID-ID1, BMM-TD1, JUS-ID1, KRP-3, KR11-2, KRC-2, KRC-3, KR11-4 and KRC-4 (Appendix 2).

4.2 Euclidean distance based dendrogram of mean values of the 7 morpho-agronomic traits.

The genotypes were discriminated into two main clusters; I and II with cluster I comprising two sub-clusters as shown in Figure 4.1. Each of the sub-clusters in cluster I was further divided into several clades carrying various genotypes. From the dendrogram, most of the known genotypes were clustered close to each other. For example, KRP-1, KRP-2, KRP-3 and KRP-4, all purple genotypes were clustered together. KR11-1, KR11-2, KR11-3 and KR11-4
were also clustered together at a similarity value close to 100%. However some of the known genotypes were outliers in clustering. For example, despite KRC-3, KRC-2 and KRC-4 being clustered together in the tree, KRC-1 which belonged to the same variety with them was placed distantly from them. Other populations whose genotypes did not have homogeneity were, KR12 (KPF-12) and KR4 (KPF-4). Genotypes belonging to the undetermined population were clustered together with those of known genotype. For example, PKS-N3 which was an undetermined genotype, clustered with the coastal genotypes (KRCs). The clustering of undetermined genotypes in the same cluster with the known ones also included genotype SGE-N2 which clustered together with KR4-4 and KR4-1 at a similarity value close to 100%, an indication that they may be genetically related; based on the evaluated descriptors.

![Figure 4.1: Euclidean distance based dendrogram developed from mean values of the 7 morpho-agronomic traits.](image-url)
4.3 Principal component analysis (PCA)

The results for principal component analysis for all the 7 morpho-agronomic traits for the 54 genotypes as indicated in table 4.1. For the seven traits, all the 3 principal components exhibited more than one Eigen values, showing about 88.7% variability among the 7 traits under study. The Eigen values were 3.5880, 1.6130 and 1.0062 for the principal component one (PC1), principal component two (PC2) and principal component three (PC3) respectively. The PC1 accounted for 51.3%, while PC2 showed 23.0% and PC3 exhibited 14.4% variability among the genotypes for the 7 morpho-agronomic traits under study. There was highly positive correlation between the Eigen values for PC1 and all the 7 morpho-agronomic traits under study as shown in table 4.1. On the other hand, the Eigen value for PC2 was negatively correlated to leaf length and leaf width and positively correlated to all the remaining morpho-agronomic traits. The Eigen value for PC3 was negatively correlated to leaf length, leaf width rind thickness as well as seed length. This value was however, positively correlated to the remaining morpho-agronomic traits. The traits were retained for morphological discrimination of the genotypes since much of variation (51.3%) was brought about by PC1 whose values were all positive. On the other hand the scatter plot of the 54 genotypes based on the 7 morpho-agronomic traits indicated that some of the genotypes were pairing very closely on the scatter plot, complementing the findings of the dendrogram that some of the genotypes had similarity value close or equal to 100%. An example of such a combination is that between MMD-NF1 and MMD-NF2. The two genotypes were also placed distantly from the other genotypes, a similar finding to the dendrogram where they were clustered in one main cluster.
Table 4.1: Eigen vectors, Eigen values, total variance and cumulative variance for 54 genotypes based on 7 morpho-agronomic traits.

<table>
<thead>
<tr>
<th>Traits</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf Length (cm)</td>
<td>0.119</td>
<td>-0.691</td>
<td>-0.015</td>
</tr>
<tr>
<td>Leaf Width (cm)</td>
<td>0.070</td>
<td>-0.597</td>
<td>-0.524</td>
</tr>
<tr>
<td>Fruit Length (cm)</td>
<td>0.511</td>
<td>0.036</td>
<td>0.095</td>
</tr>
<tr>
<td>Fruit Diameter (cm)</td>
<td>0.512</td>
<td>0.055</td>
<td>0.110</td>
</tr>
<tr>
<td>Rind Thickness (cm)</td>
<td>0.082</td>
<td>0.391</td>
<td>-0.824</td>
</tr>
<tr>
<td>Fruit Mass (g)</td>
<td>0.497</td>
<td>0.005</td>
<td>0.110</td>
</tr>
<tr>
<td>Seed Length (cm)</td>
<td>0.451</td>
<td>0.096</td>
<td>-0.118</td>
</tr>
</tbody>
</table>

Figure 4.2: Scatter plot of the genotypes developed from mean values of the 7 morpho-agronomic traits.
4.4 Determination of molecular diversity

4.4.1 Quality of extracted DNA

The quality of DNA is a crucial requirement that determines the success of SSR-PCR amplification. Gel photos of genomic DNA on agarose gel were taken (figure 4.3).

![Gel images showing DNA of some of the samples of the 54 genotypes extracted using CTAB method](image)

**Figure 4.3:** Gel images showing DNA of some of the samples of the 54 genotypes extracted using CTAB method

A: Negative control; B: KRC-2; C: PKS-N1; D: JUS-ID1; E: KNG-FP2; F: MMD-ID1; G: SGE-N4; H: KID-ID1; I: JUS-ID1; J: SGE-N3; K: KR11-2; L: KMD-TD2; M: KNG-ID1.

Visual comparison of bands on gel photos with the results above showed that all DNA samples extracted from the leaf samples of all genotypes were of good quality and as such could be used for PCR.

4.5 Determination of polymorphism using SSR markers

Of the 12 SSR markers, 1 marker (PE 20) was found to be monomorphic and showed only one allele (uniform band size of 300 bp) among all the genotypes and as such could not be used for the variability studies (figure 4.4). Eleven
microsatellite markers were polymorphic and showed consistent banding patterns and amplification of each variety and were ultimately chosen for the study of genetic variability among the selected genotypes (figures 4.5 and 4.6).

**Figure 4.4:** Gel image showing PE20 PCR products separation of some of the samples of the 54 genotypes separated on 3% agarose.

A- -ve control; B-Jus-N1; C- KRP-2; D- KNG-FP1; E- JSE-N3; F- SGE-N4; G- KR11-1; H- PKS-N3; I- SGE-N3; J- KR11-3; K- KUA-N1; L- KNG-FP2; N- SGE-ID2; M: Molecular Ladder (100bp)

**Figure 4.5:** Gel image showing amplification products of some of the samples of the 54 genotypes separated on 3% agarose gel.

A- -ve control ; B-Jus-N1; C- KRP-2; D- KNG-FP1; E- JSE-N3; F- SGE-N4; G- KR11-1; H- PKS-N3; I- SGE-N3; J- KR11-3; K- KUA-N1; L- KNG-FP2; N- SGE-ID2; M: Molecular ladder (100 bp).
A total of 60 alleles were detected at the loci of the 11 microsatellite markers across the 54 passion fruit genotypes. The number of alleles per locus ranged from 2 to 8, with an average of 5.4545 alleles. The highest number of polymorphic alleles was seen in markers PE 04, PE 74 and PE 42 while the least number of polymorphic alleles was seen in marker PE 38 as shown in table 4.2.

The results of gene diversity, computed using the PowerMarker® V3.25 software are shown in table 4.2. The average gene diversity among all the selected 54 Passiflora edulis genotypes was 0.4834. On the other hand, observed heterozygosity for all markers was 0.0000. The marker per marker gene diversity values ranged from 0.3695 (PE 04) to 0.6783 (PE 90) as shown in table 4.2. Polymorphic information content (PIC) value is a measure of polymorphism among varieties for a marker locus used in linkage analysis.
Table 4.2: List of the 11 polymorphic SSR markers used in this research study, the respective allele number, gene diversity and PIC values.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Major Allele Frequency</th>
<th>Allele No</th>
<th>Gene Diversity</th>
<th>Observed Heterozygosity</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE75</td>
<td>0.5185</td>
<td>4</td>
<td>0.4993</td>
<td>0.4993</td>
<td>0.3747</td>
</tr>
<tr>
<td>PE90</td>
<td>0.4259</td>
<td>4</td>
<td></td>
<td>0.6783</td>
<td>0.6179</td>
</tr>
<tr>
<td>PE38</td>
<td>0.537</td>
<td>2</td>
<td>0.4973</td>
<td>0.4973</td>
<td>0.3736</td>
</tr>
<tr>
<td>PE24</td>
<td>0.6019</td>
<td>4</td>
<td>0.417</td>
<td>0.417</td>
<td>0.3301</td>
</tr>
<tr>
<td>PE18</td>
<td>0.5463</td>
<td>4</td>
<td>0.4914</td>
<td>0.4914</td>
<td>0.3706</td>
</tr>
<tr>
<td>PE58</td>
<td>0.6204</td>
<td>5</td>
<td>0.512</td>
<td>0.512</td>
<td>0.4306</td>
</tr>
<tr>
<td>PE04</td>
<td>0.7176</td>
<td>8</td>
<td></td>
<td>0.3695</td>
<td>0.2929</td>
</tr>
<tr>
<td>PE74</td>
<td>0.6435</td>
<td>8</td>
<td>0.4412</td>
<td>0.4412</td>
<td>0.3424</td>
</tr>
<tr>
<td>PE42</td>
<td>0.6667</td>
<td>8</td>
<td>0.4036</td>
<td>0.4036</td>
<td>0.3129</td>
</tr>
<tr>
<td>PE66</td>
<td>0.5463</td>
<td>6</td>
<td>0.5466</td>
<td>0.5466</td>
<td>0.4848</td>
</tr>
<tr>
<td>PE11</td>
<td>0.6481</td>
<td>7</td>
<td>0.4607</td>
<td>0.4607</td>
<td>0.3689</td>
</tr>
<tr>
<td>Mean</td>
<td>0.5884</td>
<td>5.4545</td>
<td>0.4834</td>
<td>0.4834</td>
<td>0.3909</td>
</tr>
</tbody>
</table>

4.6 Pairwise genetic dissimilarity analysis

A dissimilarity matrix based on the “C.S Cord 1967” targeting shared microsatellite alleles was used to determine the relatedness among the *Passiflora edulis* genotypes. The pairwise genetic dissimilarity values ranged from 0.7669 to 0.0000 (Appendix 3). Genotypes KRC-2 and KRP-2; KRC-2 and KRP-4 had the highest level of dissimilarity; 76.69 %. On the other hand, KMD-TD1 and KMD-TD2 genotypes which were collected from the same orchard had a dissimilarity matrix of 0.000%. Genotypes KR12-1 and KR12-3, both hybrids from KALRO-Kandara also had a dissimilarity of 0.00%.
4.7 Clustering of *Passiflora edulis* genotypes using Neighbor joining

The phylogenetic tree revealed that there was genetic relatedness among the genotypes within the different populations based on the 11 polymorphic SSR markers. The genotypes were clustered into three major clusters; I, II and III as shown in figure 4.7. The three major clusters were also broken further into several sub-clusters. It was also observed that all known genotypes (KRP, KR11, KR12, KR4 and KRC) were all distributed into various clusters with each population of the known genotype being in the same phylogenetic cluster. Furthermore it was also noted that the cluster bearing the KRPs (KARI-purple genotypes) was the farthest from the cluster carrying the KRCs (KARI-coastal genotypes). The two populations are the parent genotypes from where the hybrid varieties (KPF-4, KPF-11 and KPF-12) were developed. On the phylogenetic tree, these hybrid varieties are clustered at intermediate distances between the two parent varieties (KRCs and KRPs).
Figure 4.7: A Neighbor Joining tree showing the genetic relationships among the 54 selected *Passiflora edulis* genotypes based on the 11 SSR markers under study.
4.8 Analysis of molecular variance (AMOVA)

The level of genetic variation was determined using Analysis of Molecular Variance (AMOVA). Nine percent (9%) (P<0.001) of genetic variation partitioned among populations and 91% (P<0.001) within individuals as shown in table 4.8.

**Table 4.8: Summary of analysis of molecular variance (AMOVA). Degrees of freedom (df), sum of squares (SS), mean of square deviation (MSD), % variation and P-values.**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Df</th>
<th>SS</th>
<th>MSD</th>
<th>% variation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>6</td>
<td>32.872</td>
<td>3.652</td>
<td>9%</td>
<td></td>
</tr>
<tr>
<td>Within Populations</td>
<td>53</td>
<td>106.036</td>
<td>2.410</td>
<td>91%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>138.907</td>
<td>6.062</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

4.9 Principal Coordinate Analysis

Principle coordinate analysis (PCoA) based on Nei’s genetic distance was used to visualize the genetic relationship among the genotypes as shown in figure 4.8. The first two principle axes accounted for 50.64% and 14.67% variation respectively. The known genotypes were located on the same axis while those from the undetermined population (FN) were distributed across the two principal axes.
Figure 4.8: Principle coordinate analysis of the P. edulis genotypes based on 11 SSR markers.
5.1 DISCUSSION

The yellow passion fruit (*Passiflora edulis* f. *flavicarpa* (Deg)) is a fruit species of great importance, which has, nonetheless, rarely been evaluated in studies of genetic improvement. For proper establishment of such studies, it is important to investigate the genetic structure of the cultivated populations to identify promising parents that can generate sufficiently heterotic hybrids. These hybrids in turn result to superior segregating genotypes, allowing a more effective genetic improvement program for the crop (Viana *et al.*, 2004). KPF-4, KPF-11 and KPF-12 are such hybrids that were developed by KALRO to combine the superior traits of purple passion fruit variety and those of the coastal yellow variety (Brazil). The genetic variation which is an important resource in plant breeding has not been determined for these relatively new hybrids. Therefore, the measurement of genetic variability of these species is fundamental for both plant breeding and conservation programs of many species (Paiva *et al.*, 2014).

From the tabulated results (appendix 2), the difference in leaf lengths of KR4-1 which belongs to the KPF-4 variety (14.820 cm) and KRC-3 which was a coastal yellow passion fruit variety (8.600 cm) can be explained by genotypic variation that is known to exist between hybrids and parent genotypes (Santos *et al.*, 2014). Both genotypes KR4-1 and KRC-3 were located on the same orchard and thus shared agronomic and environmental conditions. The relatively low leaf length of KRC-3 could be due to the known adaptation of the variety to
relatively drier and saline environmental conditions. Plants adapted to these conditions are known to have reduced leaf area in order to reduce water loss due to evaporation. The relatively higher leaf length of KR4-1 could be due to the fact that it is hybrid. This is comparable to the results found by Santos et al. (2014), who found a relatively higher mean leaf length value of a hybrid variety (14.91 cm) as compared to non-hybrid parents (11.80 cm and 11.85 cm). The two mean leaf length values of KR4-1 and KRC-3 were significantly different from each other and as such could be used to discriminate the two genotypes. The mean leaf width had no significant difference, which could be explained by the lack of agronomic and environmental influence on this across all the genotypes under study.

The wide variation of seed length; 0.3200 cm (KR12-4) to 0.7800 cm (PKS-TD1) with an overall mean of 0.669 cm means a variation in seed fitness. The size of the seed affects fitness of the plant growing from it. This variation in seed length could be explained by difference in position on the inflorescence or the fruit (Giles, 1990). The mean value was slightly higher than that obtained for *P. setacea* (0.429 cm) and *P. edulis* (0.506 cm) by Santos et al. (2014). The lack of significant similarity of seed length among genotypes in the same population is an indication of wide variation in seed length.

The averaged mean values for rind thickness; 0.482593cm (4.82593mm) was lower than that obtained for interspecific hybrids of *P. setacea* and *P. edulis* by Santos et al. (2014) (5.40mm). The value was however slightly higher than that
of *P. setacea* (2.78 mm) and slightly lower than that of *P. edulis* (6.25 mm) obtained by the same group. The value was also slightly higher than that found by Santos *et al.* 2009 (3.25 to 3.54 mm). The value for rind thickness was also lower than obtained by Silva *et al.* (2015) (between 5.66 mm to 5.88 mm) and Cavalcante *et al.* (2007) (6.0 to 7.11 mm). Breeding programs select genotypes with considerably reduced rind thickness, which may be used to indicate greater amount of pulp for the populations studied (Santos *et al.*, 2014). Negreiros *et al.* (2008) reported that both the fresh fruit market and the juice industry highly regard skin thickness as a relevant factor in fruit ranking because it is inversely proportional to juice yield. Therefore, from the data presented in Appendix 1, genotypes SGE-ID1 and SGE ID2 were favorable in terms of pulp and juice yield. These genotypes can therefore be adopted for crosses targeting higher juice and pulp yield.

The mean fruit diameter (7.72 cm), was equal to that obtained by Santos *et al.* (2014) for *P. edulis*, and closer to that obtained by Reis *et al.* (2011) (7.5 cm). The influence of environment and agronomic practice could possibly be the cause of wide variation in fruit diameter among the studied genotypes. However this can be ruled out from the fact that from the data presented (Appendix 2), there was no similarity limited to the different geographic locations. This assertion was confirmed by the similarity of different genotypes found in different geographic locations, despite the morpho-agronomic dissimilarity between genotypes from one locality. For example, individual PKS-TD1 and KMD-TD1 had fruit diameter values of 3.860 cm despite being in different
orchards and thus experiencing a possible difference in agronomic practice. This similarity in fruit diameter could therefore be attributed to genetic factors considering that these genotypes were apparently producing fruits with similar forms (they produced fruits with total deformity).

The mean fruit length (8.06 cm) of the 54 genotypes was close to that obtained for *Passiflora edulis* (8.150 cm) by Santos *et al.* (2014). This value was also comparatively close to that found by Reis *et al.* (2011) who obtained a value of 8.2 cm (820mm). This may be interpreted to imply that there is no significant influence of environment on fruit length considering that this study was not performed under controlled or uniform agronomic conditions but obtained similar results to those by Santos *et al.* (2014) and Reis *et al.* (2011), who examined the variation under controlled conditions. It is however notable that there is great variation among fruit length of 54 genotypes. The genotype with the least fruit diameter was PKS-TD1 (5.10 cm) while that with the highest was KR4-1 (10.08 cm). This is certainly a wide variation confirmed by the Tukey's post-hoc pairing (Appendix 2). This wide variation may be attributed to genetic variation among the 54 genotypes. The values for two genotypes (MMDNF1 and MMDNF2) were not considered in description of variation in fruit length since they were non-fruiting and as such would bring biasness in description of the range of fruit based descriptors.

Fruit length and width are important agronomic traits in passion fruit breeding. Fruits of the yellow passion fruit normally grow more in length than in width,
which is the preferred fruit form for the consumer market (Oliveira et al., 2008). The fruit form index is an important aspect that is useful in classification and standardization of passion fruit in the fruit market where it influences the acceptance and judgment of the product in some markets (Purquerio and Cecilio-Filho, 2005). Yellow passion fruit for fresh consumption, is classified by size (Meletti, 2001) and external aspect (Hafle et al., 2009), given that consumers prefer oval fruits (Oliveira et al., 2008). This external aspect of the fruit has been faced by various inconsistencies in passion fruit despite most of the other agronomic traits having little variation. For example, despite the plants under study being at different geographic locations hence a difference in environmental conditions, traits such as leaf width did not have significant difference among all the 54 genotypes being studied. Relatively little significant variation was also observed on the leaf length among the 54 genotypes under study. However great variation was observed in the fruit based.

The respective Eigen values indicated that the first principal component had a higher contribution to total variation compared to the second and third principal components. The principal components technique is useful in phenotypic variability studies in that it allows the evaluation of importance of each trait/character of the accessions being studied over total variation, hence allowing elimination of less discriminating characters (Daher et al., 1997). The contribution of the first principal component (51.3%) to total variance was lower than that obtained by Castro et al. (2012) for the first two components (57.13%). The cumulative value of the first two components in this study (74.30%) was
close to the cumulative value for the first 4 principal components by Castro et al. (2012) (74.29%). Martel et al. (2003) also found a figure close to this (57.13%) for the first two components on their work on Amazonian peach palm. Oliveira et al. (2012) found a figure higher than the previous two and much closer to that of this work at 52.09% on their work on Carica papaya.

Conversely some studies have obtained a much lower value. For example, in their study for selection of suitable descriptors for Euterpe palm, Oliveira et al. (2006) recorded a value of 35.8%. This was considerably small as compared to that found in this work since it was less than half of the cumulative value for the first two components (74.29%). The high cumulative variance may be explained by the fact that distribution of variation is associated with the nature and number of characters used in the study. This variation is concentrated in the first principal component especially when evaluating few agronomically important traits or certain groups such as flowers and fruits (Perreira et al., 1992). Quantitative descriptors should be discarded when they have high correlation with principal components of the lowest variance. However none of the descriptors had a high correlation with the lowest variance (PC3) and as such could be used for this study (Table 4.1).

The first Eigen value (3.5880) was close to that found by Mariguele and Silva (2010) who got a value of 3.37 on phenotypic traits of custard apple trees evaluated in the first year. This value was also close to 3.5434, obtained by Ghanavati et al. (2011) and 3.147 obtained by Asudi et al. (2010). On the other hand the second and third eigen values (1.6130 and 1.0062) were higher than
those obtained by Mariguele and Silva (2010) in the first year (0.76 and 0.55 respectively). The distribution of the genotypes on the scatter plot indicated the existence of wide variation among genotypes distantly located in the plot as well as closeness of those placed closer to each other. Those genotypes appearing very closely on the scatter plot could be interpreted to be biological replicates of each other. Closeness of genotypes from the known populations indicates their relatedness. For example, the graphical representation of genotypes, MMD-NF1 and MMD-NF2 was distant from the other genotypes, an indication that the two genotypes were phenotypically distant from the other genotypes, based on the 7 morpho-agronomic traits evaluated. The graphical closeness of the two genotypes on the principal axis is an indication of their biological relatedness, which could be interpreted to indicate that they are biological replicates. The findings of the scatter plot corroborate the dendrogram clustering discussed below.

On the other hand, clustering of the genotypes based on the 7 morpho-agronomic traits into two main clusters is an indication of existing variation in the genotypes of the two clusters. Further clustering in cluster I of is an indication of the great phylogenetic divergence among genotypes in the cluster. The similarity value between the two genotypes (MMDNF1 and MMDNF2) in cluster II indicates little variation (100% similarity) between them. Discrimination of the two genotypes in this cluster compared to the remaining genotypes in cluster I can be explained by the lack of fruit based traits, thus bringing wide variation between them and the rest of the genotypes. Their
clustering at 100% similarity is an indication that the two genotypes had the similar ancestry with a probability of being full siblings. However, this clustering may have been only as an effect of lack of fruit based parameters which were 5 of the 7 traits evaluated. These findings agree with those of the scatter plot which plotted the two genotypes on the same graphical location.

The similarity between the two main clusters, I and II, (0%) (Figure 4.1) confirms the wide variation between the two clusters. The great phenotypic distance between genotypes MMDNF2 and MMDNF1 with other 52 genotypes makes them candidate genotypes for crosses with genotypes in the cluster I. From the dendrogram; sharing of a cluster among the genotypes of known varieties confirms that they had morphological similarity, and as such share a common ancestry compared to other genotypes. Clustering of the undetermined genotype, PKS-N3, with Coastal genotypes at a similarity value close to 100% could also be interpreted to mean that it belonged to the coastal variety. The similarity of about 33% in sub-clusters of the main cluster I and further clustering at about 55% is an indication of the successive divergence in the clusters.

The clustering and hence similarity of some genotypes despite experiencing a possible difference in agronomic and environmental conditions credits the use of quantitative traits despite the varying influence of environment and agronomic practice. For example individuals, JSE-N3 which was obtained from an orchard in Embu County and KUA-N1 from Kenyatta University
School of Agriculture farms were clustered together (about 90% similarity). This is despite the two individuals being from different geographic locations and possibly different agronomic practices. This observation is vindictive of the earlier observation of little environmental influence on the morpho-agronomic descriptors.

In relation to the molecular objective, the considerably low number of alleles for all loci, ranging from 2 (PE38) to 8 (PE04, PE42 and PE74), and mean of 5.4545 agrees with findings of other related studies. Locus PE 18 had a total of 4 alleles similar to the number reported by Paiva et al. (2014). Both were dinucleotide microsatellites with nine replicates each. There was similarity in allelic variation with those found by Caze et al. (2012) and Paiva et al. (2014) who had a range of 2-5 and 2-9 alleles per locus respectively. The locus PE66 had a total of 6 alleles slightly higher than the number found by (Paiva et al., 2014). The respective means were 3.42 and 5, values close to the mean of this work at 5.4545. Other studies on the Passiflora genus have found a relatively higher number of alleles on a specific locus. For instance, Oliveira et al. (2005) obtained up to 20 alleles at one locus. However, the average number of alleles per locus (5.3) in the sample of 43 individuals was close to other studies by Caze et al. (2012) and Paiva et al. (2014) as well as this study. The low number of alleles per locus has been characteristic of the genus Passiflora thus suggesting that these loci are concentrated in preserved regions, with low mutation rate (Cerqueira-Silva et al., 2012; Paiva et al., 2014). The
relationship between allele number detected at a locus and the types of repeats is that the larger the variation in the repeat motif, the larger the number of alleles detected (Islam et al., 2012).

The polymorphic information content (PIC) is useful in the calculation and estimation of how much each primer is informative among the genotypes studied. The mean PIC value (0.390) for the 11 polymorphic markers was slightly above that found by Santos et al. (2011) who obtained a value of 0.28. The difference between the two means indicated that there was a difference in the levels of informativeness of the markers used in this study with that by dos Santos et al. (2011). The PIC ranged from 0.2929 to 0.6179. According to a classification of markers by their PIC value, Botstein et al. (1980) considered a marker highly informative if it had PIC value above 0.5, while those with values ranging between 0.25 and 0.50 were classified as reasonably informative and when values were below 0.24, the markers were described as mildly informative. Based on this classification, PE90 (0.6179) was highly informative while the rest of the markers were reasonably informative. The PIC value for PE66 (0.4848) was lower than that obtained by Paiva et al. 2014 and closer to the value of 0.5 obtained by Reis et al. (2011). This considerably small difference was responsible for the difference in classification where in this study the marker was classified as reasonably informative while in the other studies it was highly informative.
On the other hand, the PIC value for marker PE38 was 0.3736 a value close to that obtained by Paiva et al. (2014); (0.31). Reis et al. (2011) obtained a relatively lower value (0.24) a value describing the marker as mildly informative. Observation of lower PIC values can be attributed to the concentration of gene frequencies, which leads to deviation from the condition of maximum information content of a locus. This phenomenon occurs when all alleles have similar frequencies (Paiva et al., 2014).

The allele frequencies ranging from, 42.59% (PE90) to 71.76% (PE04) have a relationship with the PIC values and hence the informativeness of the markers. These values are used to indicate how much allelic frequency was concentrated in only one allele. The higher the allele frequency, the lower the PIC value and consequently, the lower the informativeness of the specific marker. The high frequency (71.76%) in PE 04 explains the lower PIC values and indicates that, in the present work, this locus generated the lowest content of information on the genotypes under analysis.

Gene Diversity ranges of 0.4036 (PE42) to 0.6783 (PE90), with an average value of 0.4834 (Table 4.2) was comparable to the findings of other studies. The mean gene diversity value was closer to those by Boussaid et al. (2010); 0.449 in *Coffea canephora*, and 0.3834 in *Stipa tenacissima*. The low values for observed heterozygosity can be explained to be as a result of inbreeding. It is imperative to note that the low values of observed heterozygosity (0.000 for all
markers), compared with the values of expected heterozygosity, suggest low allelic diversity and tendency toward the fixation of alleles, at least for the evaluated germplasm (Cerqueira-Silva et al., 2014b). Related studies have obtained similarly low values for observed heterozygosity. For example, Kylin, (2010) obtained values ranging from, 0.000 to 0.262. In their work on development of SSR markers for *Passiflora edulis*, Oliveira et al. (2005) reported observed heterozygosity values ranging from 0.00 to 1.00 with most of the markers registering a value of 0.000. Conversely, other studies on *Passiflora* have reported most microsatellite markers having heterozygosity values above 0.000. For example, Paiva et al. (2014) reported values between 0.000 to 0.9815, with only one out of the twelve markers having a value of 0.0000.

The pairwise genetic dissimilarity values that ranged from 0.7669 to 0.0000 (Appendix 3) can be used to indicate the wide variation among some genotypes while the low dissimilarity ones show common ancestry between those genotypes that have them. The highest level of dissimilarity (76.69 %) between genotypes KRC-2 vs. KRP-2 and KRC-2 vs. KRP-4 can be interpreted to mean that these combinations were the most distantly related compared to other genotypes. The fact that KRC-2 was distantly related to the purple genotypes can be explained by the underlying molecular variation between the purple variety and the coastal yellow varieties despite them belonging to the same species (Bernacci et al., 2008). On the other hand, the
dissimilarity matrix of 0.000% between KMD-TD1 and KMD-TD2 can be interpreted to mean they are full siblings.

Genotypes; KR12-1 and KR12-3, both hybrid genotypes from KALRO-Thika also had a dissimilarity of 0.00%. KR12-2 and KR12-4 also replicates collected to represent the KPF-12 hybrid cultivar also had a dissimilarity of 0.00%. These clusters can be interpreted to mean that these genotypes had the lowest genetic distance and possibly share a common ancestry and as such are replicates. The genetic distance of (0.000) was similar to that found in wheat genotypes; Balaka vs. Aghrani and Triticale vs BAW-1036 by Islam et al. (2012). Other studies have reported higher values than those obtained in this study. For example, Costa et al. 2012, obtained values ranging from 0.049 for the accessions GP09-07 vs GP09-04 to 0.407 for accessions BGM322 vs BGM140. The low dissimilarity of 0.000% may be attributed to common ancestry or the possibility of introgression of similar traits during the time of genetic improvement.

The Neighbor joining tree presented in Figure 4.7 revealed that there was genetic relatedness among the genotypes in the different populations based on the 11 polymorphic SSR markers. The clustering of the genotypes into three major clusters; I, II and III is an indication of genetic divergence of the genotypes under study. The grouping provided clear distinction between the different populations of known varieties, an indication of their uniqueness, compared to other genotypes. The furthest distance of genotypes KRP-1 and
KRC-2 on the phylogenetic tree may be due to the fact that they may be having diverse ancestral origins. Other possible explanations for this distance high gene flow caused by cross pollination among the varieties and chromosomal mutations (Santos et al., 2010). KRP-1 belongs to the purple variety while KRC-2 belongs to the coastal variety. This phylogenetic distance may be explained by the known genetic distance that exists between the varieties which the two genotypes belong to the Purple variety and coastal yellow variety respectively. The accessions of purple and yellow fruits are considered to be genetically distinct despite belonging to the same species (Bellon et al., 2007).

The homogeneity of distribution of genotypes from KALRO breeding program to various clusters with non-homogeneous distribution of the accessions from orchards managed by farmers can be interpreted to mean that the known genotypes had genetic uniformity compared to the undetermined genotypes which were not uniform, genetically. All the hybrids from KALRO and parent cultivars (purple and coastal varieties) were clustered into groups carrying genotypes from each cultivar, indicating that these accessions and cultivars might have a similar genetic background.

From the dendrogram, distance of the populations bearing genotypes from the KARI- Purple variety (KRP-1, KRP-2, KRP-3 and KRP-4) and the coastal variety (KRC-1, KRC-2, KRC-3 and KRC-4) indicates genetic basis of the difference between the two varieties. This is confirmed by the known ancestry distance between the varieties of the two populations (Bernacci et al., 2008).
Another genotype PKS-N3 placed in the undetermined population was clustered together with the coastal varieties from KALRO, an indication that it belonged to the coastal variety. This is an indication of the molecular relatedness of the genotypes within each population (Santos et al., 2010).

These results that genotypes belonging to the purple and coastal yellow varieties were the most distant are in agreement with a study regarding diversity studies conducted in *P. edulis* by Bellon et al. (2007). They observed greater genetic distance between accessions of fruits with yellow and purple skins. Therefore, despite these genotypes being recognized as the same species (Bernacci et al., 2008), the findings of this study reinforce the need to distinguish accessions of *P. edulis* by the color of their fruit (Bellon et al., 2007). This variation may be understood as a phenotypic marker of more robust molecular variability (Cerqueira-silva et al., 2014b). This may be the case considering that much of the phenotypic variability was based on the fruit and seed parameters, but this assertion may only hold with an assumption that the morpho-agronomic descriptors captured in this study had relationship with the microsatellite markers used. This calls for further molecular studies to determine the linkage between the microsatellite markers and the various quantitative traits.

Furthermore, the clustering of genotypes from field together with those of known varieties from KALRO orchards is an indication of genetic relatedness. One may assume that the genotypes are from the same place or that there may have been an exchange of germplasm between the two geographic and breeding
locations. In assessing the diversity of some genotypes of *Theobroma cacao* using microsatellite markers, Irish *et al.* (2010) found redundant accessions because of the presence of hybrids from the same cross (sibling seeds). The groupings made by neighbor joining confirmed that the polymorphism observed by amplification of SSR loci in accessions of *P. edulis* was sufficient to distribute the accessions of this species. Other studies have achieved phylogenetic discrimination with much fewer markers. For example, in *Vaccinium* species (blueberry), there was sufficient formation of groupings with only three pairs of microsatellite primers (Silva *et al.*, 2008).

From the results of this study, and the number of markers used, there was consistence with the reality of the collections under this study. This is especially the case with the populations of known genotypes where there was consistence of results with the perceived genetic relatedness. Furthermore, the results obtained in this study suggest that the different accessions can be useful for cross-breeding purposes because they are genetically distant in relation to the microsatellite markers analyzed. In particular, these results justify the work by KALRO that developed the hybrid varieties (KPF-4, KPF-11 and KPF-12) from both the purple skinned varieties (KARI-P) and the yellow skinned coastal varieties. The two varieties were the most distantly related on the phylogenetic tree.

From the AMOVA table, the variation that arose from the differentiation among population (9%) was close to that obtained by Cerqueira-Silva *et al.* (2014b)
who reported 7% variation among populations. This variation is an indication that the *Passiflora* genus has a relatively low variation among populations. On the other hand the intra-population variation (91%) was equal to that obtained by Ansari *et al.* (2012). Jatoi *et al.* (2010) also reported a value of 91% in their study of ginger (*Curcuma amada*). The low variation among populations can be indirectly confirmed by the poor bootstrapping values for some of the cluster nodes (Ansari *et al.*, 2012).

The PCoA results support the findings obtained from neighbor joining clustering. This is evident from the location of the known genotypes on the same principal axis. The results of graphical analysis of the two main coordinates revealed existence of dissimilarity between genotypes belonging to the known populations such as the KARI-purple and KARI-coastal. The location of some of the undetermined genotypes closer to the known ones confirms the findings of the Neighbor joining tree and thus the undetermined genotypes can be confirmed to have similar ancestry to the known populations. The value for principal coordinate 1 (50.64) was higher than the findings of Paiva *et al.* (2014) who obtained a value of 41%. On the other hand, the value for principal coordinate 2 was lower than that obtained by Paiva *et al.* (2014). The graphical closeness of some of the genotypes on the principal coordinates indicates their closeness, revealing a possibility of being biological replicates.
The wide genetic variability seen in the genotypes being studied is known to exist within *P. edulis*. This can be exploited in both natural and breeding populations such as the KALRO breeding orchards. The variability is also of interest for the localization of genomic regions that might be responsible for variation for important crop traits such as fruit length and width. In order to map genes and DNA sequences, good genetic maps are required, which will enable the identification of QTLs. Quantitative trait loci (QTL) mapping has contributed to the understanding of genetic composition of complex traits, such as yield and fruit quality and disease resistance, that are a result of the cumulative effects of several genes (Fischer *et al.*, 2004; Fanizza *et al.*, 2005).

Despite the wide molecular variation (across all the 11 polymorphic markers), much of the phenotypic variation was based on the fruit and seed parameters. This therefore is an indication of a possible lack of association between the markers and those agronomic traits with little significant differences. According to Souza and Sorrells (1991), the association between morpho-agronomic traits variability with that of molecular markers may be weak, due to inadequate representation of the genome by molecular markers. They also indicated that this association may be also weak due to the absence of association between the loci that control the various agronomic traits and SSR markers under study. Reis *et al.* (2011), noted that this lack of association may be due to the non-adaptiveness of molecular markers unlike the phenotypic characteristics, which are influenced by natural and artificial selection as well as environmental interaction.
5.2: CONCLUSIONS

i. Phenotypic characterization proved useful in the determination of genetic variation in *Passiflora edulis* genotypes under study. The 7 morpho-agronomic traits sufficiently discriminated the selected genotypes into various clusters.

ii. Molecular characterization sufficiently discriminated the selected genotypes based on the 11 polymorphic markers. For example, the NJ joining tree clustered all the known Genotypes (those collected from KALRO) into homogenous clusters indicating their genetic relatedness as well as variation with genotypes from other populations.

iii. The indication high similarity between some genotypes indicates that there is high inbreeding in the different populations. This is also confirmed by the low observed heterozygosity.

5.3 RECOMMENDATIONS

Based on this study, it is recommended that breeders source for breeding material from distant geographic locations or breeding localities in order to bring about diverse alleles; mitigating for the observed inbreeding.
5.4 SUGGESTIONS FOR FURTHER STUDIES

i. Recruitment of more descriptors such as color of branches, leaf shape, division of the leaf blade, pulp color and prevailing skin color as used by Santos et al. (2014).

ii. Studies should be conducted to determine if there is significant phenotypic variation in *Passiflora edulis* along different growth intervals as well as further investigation on the influence of environment to different morpho-agronomic descriptors.

iii. Study to determine the actual relationship between SSR markers and the traits that are represented by the different loci as is the case with crops like rice whose comprehensive gene mapping has previously been conducted and QTLs identified as well as their close SSR markers. This will enable further exploration of the correlation between phenotypic and molecular characteristics of the genotypes because as it is, there is no known relationship between the two characteristics. The lack of relationship between phenotypic traits and SSR markers was reported by Reis et al. (2011) who used the same sets of markers. They indicated that this lack of association could be due to the fact that most of the variation that is detected by molecular markers is not adaptive, unlike the phenotypic characteristics, which are affected by natural, artificial selection and interaction with the environment (Vieira et al., 2007).
REFERENCES


### APPENDICES

**Appendix 1:** Population, genotype, and category of the respective plants used in this study.

<table>
<thead>
<tr>
<th>POPULATION</th>
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<th>CATEGORY</th>
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**KEY:**
KARI-P - purple genotype collected from KALRO
KARI-12 - KPF-12 genotype collected from KALRO
KARI-C - coastal genotype collected from KALRO
FD - genotype collected from farmers orchards
KARI-4 - KPF-4 genotype collected from KALRO
KARI-11 - KPF-11 genotype collected from KALRO
Appendix 2: Analysis of variance of the 7 morpho-agronomic traits for the 54 *Passiflora edulis* genotypes

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<th>Leaf Width (cm)</th>
<th>Fruit Length (cm)</th>
<th>Fruit Diameter (cm)</th>
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<th>Fruit Mass (g)</th>
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</table>

**MEAN**

|          | 12.49 | 11.54 | 8.06 | 7.72 | 0.48 | 77.27 | 0.67 |

Values are expressed as Mean ± SEM. Values followed by the same super script are not significantly different (P>0.05).
Appendix 3: CS. Cord coefficients dissimilarity matrix among pairs of 54 *Passiflora edulis* genotypes

|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |    