GENETIC CHARACTERIZATION AND BIOCHEMICAL ASSAY OF PASSION FRUIT (*Passiflora edulis Sims*) VARIETIES GROWN IN SELECTED REGIONS OF KENYA FOR DROUGHT TOLERANCE

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A research thesis submitted in partial fulfillment for the award of Masters of Science Degree in Molecular Biology and Bioinformatics of Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

This thesis is my original work and has not been submitted to any university for the award of any degree.

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DEDICATION

I dedicate this work to my late mum (Mrs. Mary Adhiambo) for her endless support and inspiration throughout my life.

ACKNOWLEDGEMENT

I incurred many debts of gratitude as I researched and wrote this thesis.

Special thanks and glory to Allah for His faithfulness that has seen me this far.

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Thank you all.

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ABBREVIATIONS AND ACRONYMS

µ	Micro
AA	Ascorbic acid
AFLP	Amplified fragment length polymorphism
AMOVA	Analysis of molecular variance
APX	Ascorbate peroxidase
CAT	Catalase
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic
g	Grams
GR	Glutathione reductase
HCDA	Horticulture crops development authority
ISSR	Inter simple sequence repeats
m	Milli
M	Molar
MDA	Malondialdehyde
n	Nano
ORF	Open reading frame

PCoA	Principal coordinate analysis
PCR	Polymerized chain reaction
POD	Peroxidases
PPO	Poly phenol oxidase
RAPD	Random amplified polymorphic DNA
ROS	Reactive oxygen species
rpm	Revolutions per minute
SOD	Super oxide dismutase
SRAP	Sequence related amplified polymorphism
SSR	Simple sequence repeats
USD	United State dollar
UV	Ultra violet

ABSTRACT

Passion fruit (*Passiflora edulis* [Sims]) is currently ranked third among fruit exports from Kenya and has great potential since demand for both fresh fruit and processed juice is on a continuous increase. Although assessment of genetic variability and biochemical characterization of germplasm for drought tolerance is indispensable for improvement and development of superior cultivars, little information is currently available on the genetic diversity and drought tolerance of passion fruit cultivated in Kenya. The objective of this study was to determine the genetic diversity of passion fruit varieties from passion fruit growing regions in Kenya using sequence-related amplified polymorphism (SRAP) markers and to characterize the varieties for drought tolerance. Twenty two passion fruit varieties were analyzed with 24 pairs of SRAP primers. Seven out of 24 pairs of SRAP primers displayed polymorphism and stable amplification profiles. A total of 931 clear bands were amplified with an average of 133 bands per primer pair, of which 610 (65.5%) bands were polymorphic. The similarity coefficients among the 22 passion fruit germplasms ranged from 0.51 to 1.0 with an average of 0.755. The 22 passion fruit varieties were classified into two groups by cluster analysis using unweighted pair- group method with arithmetic mean (UPGMA) with 12% similarity. Shannon's diversity index (SDI) was 0.0934 and Nei's gene diversity index (NGDI) was 0.1370 in the present study. The study findings demonstrate the existence of genetic variability among passion fruit varieties grown in different regions of Kenya. This suggests the potential application of these varieties in breeding programs by exploiting the use of molecular markers for selection of specific traits.

To characterize varieties for drought tolerance, the purple and yellow passion fruit varieties were subjected to drought stress by limiting water supply and the changes in the level of chlorophyll, reactive oxygen species, malondialdehyde, antioxidant enzymes evaluated. Prolonged drought

stress showed significant effect on the yellow varieties as characterized by chlorosis (low chlorophyll) associated with effects of elevated levels in hydrogen peroxide and malondialdehyde and their inability to employ antioxidant mechanism to mitigate the challenge as the varieties recorded low level of antioxidant enzymes. Purple varieties experienced minimal effect as indicated by relative stable chlorophyll content, low level of hydrogen peroxide and a stable malondialdehyde content; this can be attributed to the increased level of the antioxidant enzymes. Our study postulated that the ability of the purple passion fruit varieties to tolerate drought stress in comparison to the yellow varieties could be linked to their ability to enhance production of antioxidant defense mechanisms.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Passion fruit is generally associated with species of the *Passifloraceae* family, particularly those belonging to the genus *Passiflora* (Do Nascimento *et al.*, 2016). The genus *Passiflora* is highly diverse in terms of number of species within the family, with approximately 520 species distributed in the tropical regions of America, Asia, and Africa (Feuillet and MacDougal, 2004). Most passiflora are diploid with the passion fruit containing 18 chromosomes (2n = 18 [9 pairs]); that favors breeding to obtain interspecies hybrid (Yotoko *et al*., 2011). The interest in Passiflora is focused primarily on edible fruit species *Passiflora edulis* Sims, an important fruit crop grown worldwide for both export and domestic markets (FAO, 2011). This species is popularly known as yellow or purple passion fruit.

Passion fruit has great commercial potential in Kenya supporting the livelihood of both small and large scale famers (HCDA, 2013). In 2013, commercialization passion fruit produce was ranked third in the horticulture sector generating USD 22.5 million (HCDA, 2013). The fruits of passion fruits contain high nutritive and medicinal value and are sources of minerals and Vitamins (Amata *et al*., 2009). Despite the economic importance and different potential uses of passion fruit, there has been a limited understanding on genetic diversity of the germplasm and the tolerance of different varieties cultivated in Kenya for drought stress. Drought is a major abiotic constraint in plant growth and development (Fahad *et al*., 2017). Drought stress has a negative impact in plant metabolism, inducing a significant number of changes at the morphological, physiological, biochemical and metabolic levels in plant system (Tas and Tas, 2007). Consequently plants have developed a number of mechanisms to adapt to drought stress

conditions. Based on the strategy employed, plants can escape drought situation for a period of time or tolerate drought stress and as such can either be classified broadly as tolerant or non-tolerant.

Characterization of genetic and biochemical variation among passion fruit genotypes for drought tolerance is imperative for its effective conservation, management of germplasm and efficient utilization in breeding schemes. The genetic background of the plant is a crucial factor for plant breeders when selecting the parental material for breeding (Viana *et al*., 2003; Segura *et al*., 2002). Traditionally, diversity in passion fruit is estimated by measuring variations in morphological or qualitative traits such as flower color, growth habit (Vicente *et al*., 2005) or quantitative agronomic traits such as yield potential, fruit weight, fruit size or biotic (fungi, bacteria, nematodes and viruses) and abiotic (heat, drought and soil fertility) stress tolerance (Oliveira *et al*., 2013; Junqueira *et al*., 2003) that do not correctly reflect genetic relatedness between accessions (Pereira *et al*., 2015). Furthermore, morphological and agronomic traits are affected by environmental changes and are developmental stage dependent. This limits their use for development of hybrids with specific ecological adaptations (Moraes *et al*., 2005). In contrast to these defects, DNA molecular markers represent a potential tool for evaluation of genetic diversity not only for crop improvement efforts, but also for efficient management and conservation of plant genetic resources (Cerqueira-Silva *et al*., 2015; Kumar *et al*., 2009).

Genetic diversity of passion fruit has been studied with a number of molecular marker techniques such as Random Amplified Polymorphic DNA, RAPD (Cerqueira-Silva *et al*., 2010; Bellon *et al*., 2009; Crochemore *et al*., 2003; Aukar *et al*., 2002; Fajardo *et al*., 1998), Amplified Fragment Length Polymorphism, AFLP (Segura *et al*., 2002), Simple Sequence Repeats, SSRs (Cerqueira-Silva *et al*., 2015; Cerqueira-Silva *et al*., 2014) and Inter Simple Sequence Repeats, ISSRs

(Ferraz and Jorge, 2011). The mentioned markers (RAPD, AFLP, SSR and ISSR) have a number of disadvantages limiting their application in genetic diversity studies leading to the application of Sequence-Related Amplified Polymorphism (SRAP) markers in genetic diversity studies (Li and Quiros, 2001). The marker (SRAP) are more powerful than SSR, ISSR, or RAPD markers in revealing genetic diversity among closely related cultivars (Budak *et al*., 2004) and are easier to assay than AFLPs (Li and Quiros, 2001).

Sequence-Related Amplified Polymorphism is a molecular marker technique based on two-primer amplification that preferentially amplifies open reading frames (ORFs) of genes (Li and Quiros, 2001). The forward primers are designed to preferentially anneal exonic regions, and the reverse primers preferentially anneal intronic regions and regions with promoters (Li and Quiros, 2001). The observed polymorphism originates from the variations in the length of these exons, introns, promoters, and spacers, both among individuals and between species (Li and Quiros, 2001). Due to their unique primer design, SRAP markers are more reproducible, more stable, and highly simple in terms of operation in comparison to other molecular marker techniques (Li and Quiros, 2001). SRAP not only amplifies the interval between genes and their non-coding flanking regions, but is also linked to actual genes, hence allowing the generation a fingerprint of the coding sequences (Yu *et al*., 2008).

The objective of the study was to evaluate the genetic diversity and relationships of passion fruit varieties grown in Kiambu, Trans Nzoia, Siaya, Nairobi and Meru counties in Kenya using SRAP markers and to utilize biochemical assays to establish drought tolerance of passion fruit varieties.

1.2 Statement of the problem

Low production level of 8 tons per hectare is still witnessed in passion fruit farming despite the potential of 24 tons per hectare. The major cause of low production in passion fruit farming is drought stress. It is postulated that by 2050 more than 50% of the arable land will be experiencing drought (Vinocur & Altman, 2005). Drought stress accounts to 60% of yield losses in Kenya. Kenya falls in the category of "economic water stress" thus experiencing periodic drought conditions. No previous studies have been done to identify drought tolerant genotypes of passion fruit grown in Kenya.

Despite the significance of genetic diversity study (Ambaw and Dessalegn, 2017), assessment of the genetic variability of passion fruit varieties grown in different regions in Kenya has not been carried out. The limited information on the genetic variability of passion fruit grown in Kenyan has made it impossible to improve the production level passion fruit as well as the quality of the produce. The consequence has been low production level and produce of low quality.

1.3 Justification

Passion fruit farming in Kenya is currently a lucrative agribusiness supporting both large scale and small scale farmers. In 2013, commercialization of passion fruit generated USD 22.5M (HCDA, 2013) despite its significance Kenya still does not meet its production potential of 24 tons per hectare significantly due to drought. Studies geared to identifying passion fruit varieties capable of tolerating drought stress are required in order to mitigate the challenges associated with the condition. In addition to overcoming challenges associated with drought stress, drought tolerant varieties yield more fruits in a few months drought period in comparison to the susceptible varieties (Adee *et al*., 2016).

Molecular markers are important tools in germplasm characterization as they facilitate different steps in pre-breeding and breeding programs thus reducing the time required for the release of cultivars. The study of genetic diversity of passion fruit is essential as it will provide information geared towards the improvement of passion fruit production that has been a major course of concern over the years through breeding programs to assist in the generation of superior cultivars with desirable qualities and also providing a frame work for bio-conservation activities. Therefore, evaluation of genetic variability and determination of passion fruit genotype tolerant to drought stress is imperative to assist in mitigating low production that has been witnessed for some time.

1.4 Hypotheses

1. There is no genetic variability in different varieties of passion fruit grown in selected regions in Kenya.
2. There is no passion fruit variety tolerant to drought stress.

1.5 Research objectives

1.5.1 Overall objective

To assess the genetic diversity and biochemically evaluate passion fruits varieties grown in selected regions in Kenya for drought tolerance.

1.5.2 Specific objectives

1. To assess the genetic diversity of passion fruit varieties using sequence related amplified polymorphism markers.
2. To evaluate the response of passion fruit varieties to drought stress based on biochemical assays.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botany and taxonomy of passion fruit

Passion fruit is a strong, climbing vine that uses tendrils to grip other plants or the support material. It can grow 20 to 30 feet high once established and has a life span of 5 to 7 years (Thokchom and Mandal, 2017). It has herbaceous and shallow roots with its alternate leaves having entire or toothed margins. Flowers are colorful, bisexual and possess a complex corolla and superior ovary on gynophores. The flowers are sterile thus requires insects for pollination to occur (Mbora *et al*., 2008). Fruit development in purple passion fruit takes place after about seven to nine months whereas that of yellow passion takes place after one to three years after pollination (Chayut *et al*., 2014).

Passion fruit (*Passiflora edulis*) belongs to the *Passifloraceae* family, the exact number of species in the *Passifloraceae* family is not known as new species are being identified. The current literature points the number of the families to be approximately 700 species with the genus *Passiflora* proving to be highly diverse with approximately 520 species (Feuillet and MacDougal., 2004). Cytogenetic information on passion fruit illustrates four different chromosome number distributed among the four sub-genera of *Passioflora*. Most *passioflora* are diploid with the passion fruit containing (2n = 18) genetic composition which favors genetic breeding to obtain interspecies hybrids (Yotoko *et al*., 2011). The interest in *Passiflora* is focused primarily on edible fruit species *Passiflora edulis* Sims, which is an important fruit crop grown worldwide for both export and domestic markets (FAO, 2011). This species is popularly known as yellow or purple passion fruit (Figure 2.1).

Figure 2.1: Passion fruit crop (*Passiflora edulis*): (A) Yellow passion fruit – growing vines (left) and fruits (right); (B) Purple passion fruit – growing vines (left) and fruits (right).

2.2 Origin, distribution and economic importance of passion fruit

Passion fruit originated from Southern Brazil through Paraguay to Northern Argentina (Thokchom and Mandal, 2017). Initially the plant was mainly grown in the tropics where the temperature was relatively favorable, however, this has changed over the years, and the plant is now cultivated in both tropic and sub-tropic regions of the globe. In Kenya, the plant was introduced in 1920 with major commercialization activities taking place in the mid-20th century. Passion fruit is mainly grown in Rift Valley (Elgeyo Marakwet and Uasin Gishu counties), Eastern (Meru and Embu counties), Central (Murang'a and Kiambu counties), Western (Bungoma county) and Nyanza (Kisii) where the climatic conditions are favorable (Amata *et al*., 2009). The yellow passion fruit variety is a tropical plant whereas the purple variety is a sub-

tropical. The varieties require an optimum temperature of 20^0 to 30^0 C, an altitude of 1000 to 2000 m and annual rainfall of 1000 to 2500 mm. The vines require a well-drained soil (pH 6-7) with high organic matter (Thokchom and Mandal, 2017).

Passion fruit has great commercial potential in Kenya since demand for both fresh fruit and processed juice is on the increase (HCDA, 2013). In 2013, passion fruit contributed USD 22.5 million from a production of 62,207 Metric Tons under an area of 4,377.2 hectares (HCDA, 2013). Passion fruit is a rich source of minerals and Vitamins A, C, and D (Amata *et al*., 2009) as well as a source of alkaloids, flavonoids, and carotenoids that are beneficial to human health (Dhawan *et al*., 2004). The seeds are sources of essential fatty acids (55 – 66% linoleic acid, 18 – 20% oleic acid, and 10 – 14% palmitic acid), which may be used in the food and cosmetic industries (Malacrida and Jorge, 2012). Compounds in passion fruit plants with anxiolytic, antihypertensive, sedative, and analgesic properties are well documented (Ngan and Conduit, 2011; Konta *et al*., 2014).

2.3 Constrain to passion fruits production

There has been a continuous fluctuation in the annual production of passion fruits across the globe. Kenya for instance, has continued to record low average yields of 8-9 tons per hectare over the years despite its potential of 19-24 tons per hectare (Mbaka *et al*., 2006). Such fluctuations have had negative economic impact on farmers who rely on the passion fruit for their livelihood.

Drought stress is a major contributor to low production accounting to over 60% of yield losses (Fahad *et al*., 2017). Kenyan experiences erratic weather conditions in addition to falling under the category of 'economic water stress' (Figure 2.2). Other factors contributing to low production include pest and diseases (Amata *et al*., 2009).

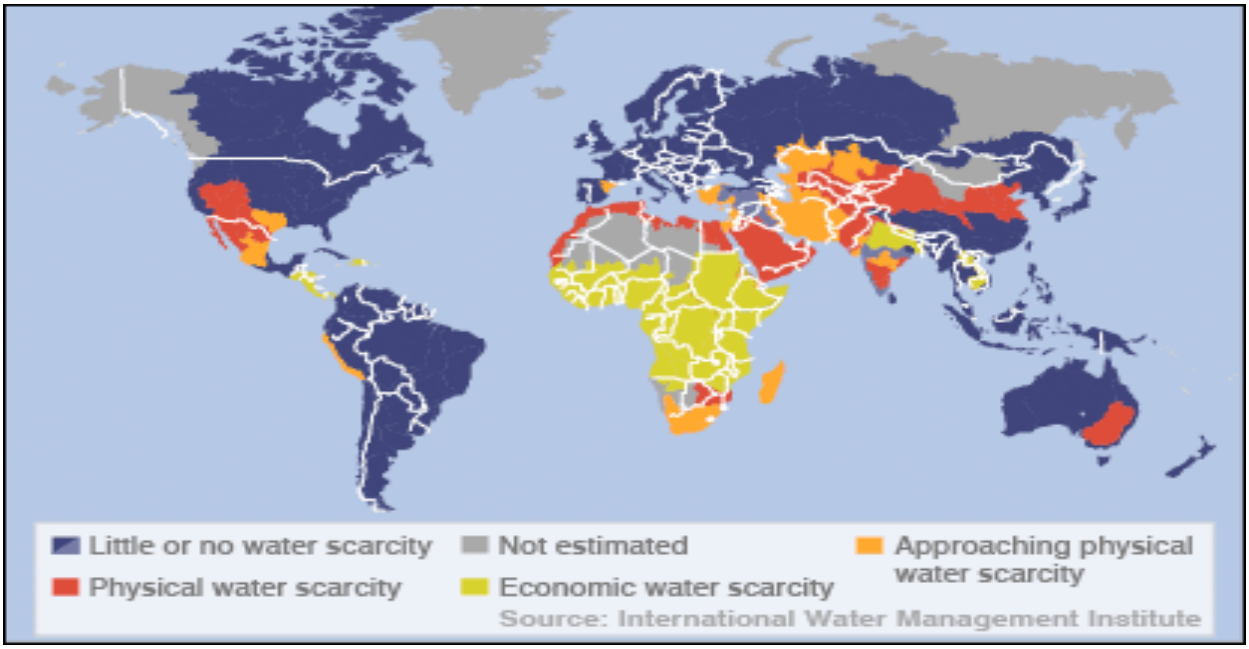

Figure 2.2: Global distribution of water scarcity (Areas of physical and economic water scarcity)

2.3.1 Manifestation and plant response to drought stress

Drought is an important manifestation of abiotic constrain in plant growth and development (Fahad *et al*., 2017). Drought stress has a negative impact in plant metabolism, inducing a significant number of changes at the morphological, physiological, biochemical and metabolic levels in plant system (Tas and Tas, 2007). Consequently, plants have developed a number of mechanisms to adapt to drought stress conditions. Based on the strategy employed, plants can be able to escape the situation for a period of time or they can tolerate drought stress. Thus based on the response plant can either be classified as either tolerant or non-tolerant (Craine *et al*., 2012).

The first cause of action employed in drought stress involves release of abscisic acid (ABA) as it stimulates stomatal closure, hence reducing water loss, carbon dioxide uptake and NADP+ regeneration by the Calvin Cycle (Sharma *et al*., 2012).

Reactive oxygen species (ROS), which can be free radicals or non-radical molecules (Sharma *et al*., 2012), are important components of the signaling pathways' network, as they act as regulators in cellular responses and cell physiology of plant to environmental factors (Sharma *et al*., 2012). Hydrogen peroxide is the most essential non-radical ROS (Sharma *et al*., 2012). Increased production of hydrogen peroxide has been reported to occur during biotic and/or abiotic stress, such as pathogen attack, wounding, UV radiation, exposure to intense light, drought, salinity, and/or chilling (Sharma *et al*., 2012; Ślesak *et al*.,2007). Under normal physiological conditions, ROS, including hydrogen peroxide, are byproducts of plant metabolic pathways and, thus, are being continuously produced in different cellular compartments (Tripathy and Oelmüller, 2012). ROS under normal or stress conditions are normally eliminated by an antioxidative defense system, whose components are located in given cellular compartments (Tripathy and Oelmüller, 2012).

Accumulation of ROS causes an oxidative stress, by oxidizing the photosynthetic pigment, proteins, DNA, and lipids (Gill and Tuteja, 2010) hence an equilibrium between ROS production and scavenging is of significance (Apel and Hirt, 2004). The levels of hydrogen peroxide in addition to other ROS act as a reliable marker in determining the level of oxidative stress (Shulaev and Oliver, 2006).

Plants have employed non-enzymatic as well as enzymatic antioxidant systems to keep the levels of ROS low to protect cells from oxidative damages (Mittler, 2002). Non-enzymatic mechanism involve application of β-carotenes, ascorbic acid (AA), α-tocopherol (α-toc), reduced glutathione whereas enzymatic mechanism includes: superoxide dismutase, guaiacol peroxidase, ascorbate peroxidase, catalase, polyphenol oxidase and glutathione reductase (Xu *et al*., 2008). Superoxide dismutases (SODs) are considered to be the first enzymes employed in the scavenging of ROS

and are responsible for the dismutation of radical oxygen to hydrogen and oxygen (Gratao *et al*., 2005). Catalase, ascorbate peroxidase and peroxidase enzymes are involved in detoxification of hydrogen perioxide to water and oxygen (Gratao *et al*., 2005). The ability of plants to scavenge ROS and reduce their damaging effects may correlate with the plant's tolerance to drought (Cruz de Carvalho, 2008).

2.4 Characterization of plant genetic diversity

Characterization of genetic variation among passion fruit genotypes is imperative for its effective conservation, management of germplasm and efficient utilization in breeding schemes (Ambaw and Dessalegn, 2017). The genetic background of the plant is a crucial factor for plant breeders when selecting the parental material for breeding (Viana *et al*., 2003; Segura *et al*., 2002). Traditionally, diversity in passion fruit is estimated by measuring variations in morphological or qualitative traits such as flower colour, growth habit (Vinente *et al*., 2005) or quantitative agronomic traits such as yield potential, fruit weight, fruit size, biotic (fungi, bacteria, nematodes and viruses) and abiotic (heat, drought and soil fertility) stress tolerance (Oliveira *et al*., 2013; Junqueira *et al*., 2003) that do not correctly reflect genetic relatedness between accessions (Pereira *et al*., 2015). Furthermore, morphological and agronomic traits are not enough in numbers to cover the whole genome, they are affected by environmental changes and are developmental stage dependent; therefore, limiting the knowledge of germplasm structure for development of hybrids with specific ecological adaptations (Arif *et al*., 2010). To overcome these challenges, DNA molecular markers represents a potential tool for effective characterization of genetic diversity for crop improvement efforts as well as in efficient management and conservation of plant genetic resources (Cerqueira-Silva *et al*., 2015; Kumar *et al*., 2009).

2.4.1 Molecular markers used in characterization of passion fruit

Genetic diversity of passion fruit has been studied with a number of molecular marker techniques such as Random Amplified Polymorphism, RAPD (Cerqueira-Silva *et al*., 2010; Bellon *et a*l., 2009; Crochemore *et al*., 2003; Aukar *et al*., 2002; Farjado *et al*., 1998), Amplified Fragment Length Polymorphism, AFLP (Segura *et al*., 2002), Simple Sequence Repeats, SSRs (Cerqueira-Silva *et al*., 2015; Cerqueira-Silva *et al*., 2014) and Inter Simple Sequence Repeats, ISSRs (Ferraz and Jorge, 2011). RAPD technique is simple, convenient, and inexpensive, but low stability and poor reproducibility limits its utilization (Roodt *et al*., 2002). On the other hand, AFLP technology presents good reproducibility and high polymorphism, but is complex, requires multiple steps and shows pseudo-polymorphism when methylation-sensitive restriction enzymes are used (Budak *et al*., 2004). Further, SSRs are stable, abundant, co-dominant, highly polymorphic and reproducible, but they require sequencing for primer development for each species, which makes the method time-consuming and expensive (Li and Quiros, 2001). ISSR though widely distributed throughout the genome and hence highly polymorphic has the disadvantage of being a dominant marker and thus unable to differentiate heterozygosity at a given locus (Budak *et al*., 2004).

2.4.1.1 Sequence related amplified polymorphism (SRAP) markers

Sequence-Related Amplified Polymorphism (SRAP) is a novel molecular marker technique based on two-primer amplification that preferentially amplifies open reading frames (ORFs) of genes (Li and Quiros, 2001). The forward primers are designed to preferentially amplify exonic regions, and the reverse primers preferentially amplify intronic regions and regions with promoters (Li and Quiros, 2001). The observed polymorphism originates from the variations in the length of these exons, introns, promoters, and spacers, both among individuals and between

species (Li and Quiros, 2001). Due to their unique primer design, SRAP markers are more reproducible, more stable, and high simplicity in terms of operation in comparison to other molecular marker techniques (Li and Quiros, 2001). SRAP not only amplifies the interval between genes and their non-coding flanking regions, but is also linked to actual genes, hence allowing the generation of fingerprints of the coding sequences (Yu *et al*., 2009). In addition, SRAP markers are more powerful than SSR, ISSR, or RAPD markers in revealing genetic diversity among closely related cultivars (Budak *et al*., 2004) and are easier to assay than AFLPs (Li and Quiros, 2001).

The marker (SRAP) has been successfully applied in cultivar identification, genetic map construction, genealogical classification, gene tagging and cloning, marker-assisted selection, germplasm resource evaluation and for prediction of heterosis (Aneja *et al*., 2012; Robarts and Wolfe, 2014). Further, SRAP markers were employed in studying population structure, genetic diversity and genetic linkage map of plants such as; cucumber (Zhang *et al*., 2010), sugarcane (Alwala *et al*., 2008), eggplant (Mutlu *et al*., 2008), and castor (Zheng *et al*., 2010), as well as in grasses including elephant grass (Xie *et al*., 2009), buffalo grass (Budak *et al*., 2004), alfalfa (Castonguay *et al*., 2010), and *Vicia faba* (Alghamdi *et al*., 2012). SRAP has also been used to assess germplasm resources and parental selection in plants such as citrus (Gulsen *et al*., 2010), sour orange (Polat *et al*., 2012), and lotus (Deng *et al*., 2013) as well as in studying parasites of human and animal health significance, for instance *Fasciola* (Li and Quiros *et al*., 2001) and *Schistosoma japanicum* (Song *et al*., 2011). This wide applicability of SRAP technique demonstrates that these markers are effective and reliable for investigating the degree of genetic polymorphism in different genomes.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

Twenty-two (22) varieties of passion fruit were obtained from different passion fruit growing regions (Siaya, Trans-Nzoia, Meru, Kiambu, and Nairobi) in Kenya (Table 3.1; Figure 3.1).

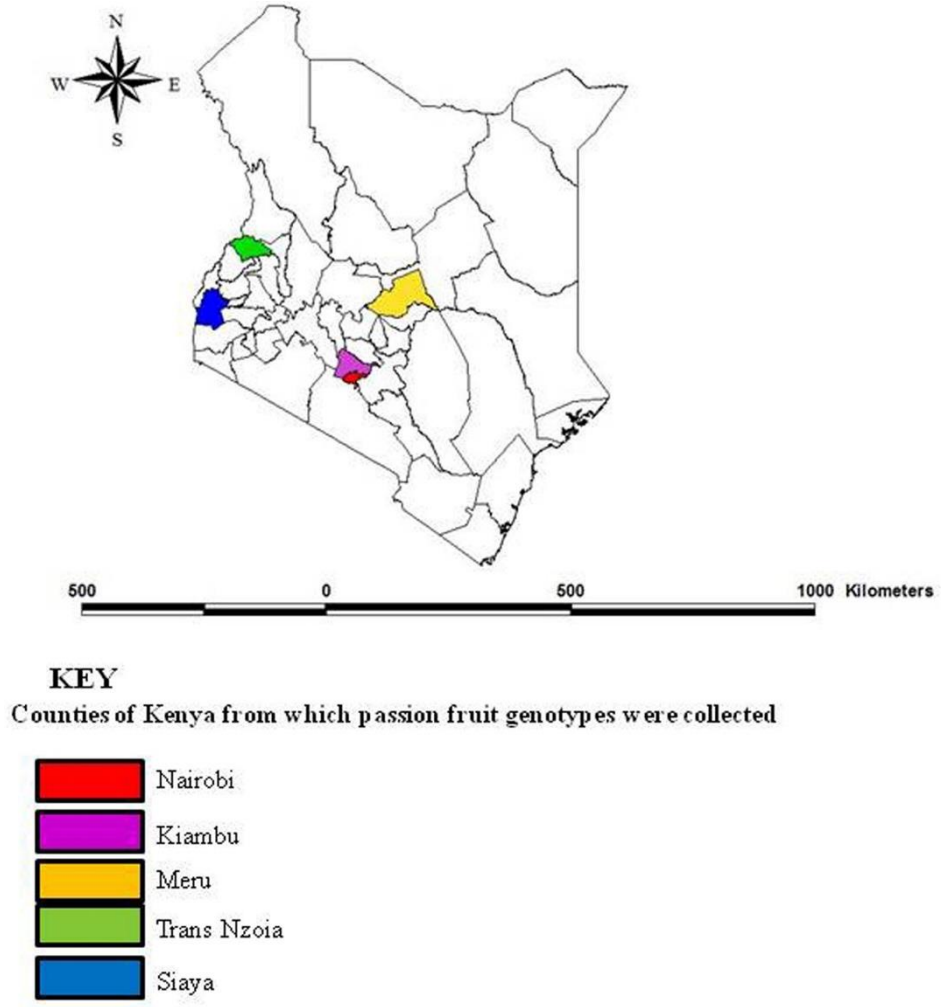

Figure 3.1: Map of Kenya showing Counties from which passion fruit varieties used in this study were collected.

The passion fruit varieties were collected from the fields as fruits and brought to the Department of Biochemistry, University of Nairobi (Kenya) where seeds were extracted and dried to about

10% moisture content under the sun. Five seeds from each variety were sown into pots containing a mixture of peat and sand in a glasshouse. Young leaves from three-week-old established plants in the glasshouse were used for DNA extraction and molecular analysis.

Two varieties of passion fruits; purple and yellow from Jomo Kenyatta University of Agriculture and Technology (JKUAT) and Kenya Agricultural and Livestock Research Organization (KALRO) were used in the biochemical characterization of passion fruit for drought tolerance.

Table 3.1: Identification code and place of collection of twenty-two (22) passion fruit varieties used in the studies of genetic diversity.

S/No.	Code	Entry number	Type of passion fruit	Place of collection	Region/County
1	PF001	NRB01	Purple	Embakasi	
2	PF002	NRB02	Purple	Westlands	
3	PF003	NRB03	Purple	Dagoretti	Nairobi
4	PF004	NRB04	Purple	Starehe	
5	PF005	NRB05	Yellow	Chiromo campus, UoN	
6	PF006	KTL01	Purple	Kwanza	
7	PF007	KTL02	Purple	Kiminini	
8	PF008	KTL03	Purple	Cherengani	Trans-Nzoia
9	PF009	KTL04	Purple	Central Kitale	
10	PF010	KTL05	Purple	Saboti	
11	PF011	MER01	Yellow	Tigania	
12	PF012	MER02	Yellow	Tigania	Meru
13	PF013	MER03	Yellow	Buri	
14	PF014	JUJ01	Yellow	JKUAT, Juja	
15	PF015	JUJ02	Purple	JKUAT, Juja	Kiambu
16	PF016	TKA01	Yellow	KALRO, Thika	
17	PF017	TKA02	Yellow	KALRO, Thika	
18	PF018	SYA01	Landrace	Boro	
19	PF019	SYA02	Landrace	Aduwa	
20	PF020	SYA03	Landrace	Awelo	Siaya
21	PF021	SYA04	Yellow	Boro	
22	PF022	SYA05	Landrace	Siaya Town	

UoN = University of Nairobi; JKUAT = Jomo Kenyatta University of Agriculture and Technology; KALRO = Kenya Agricultural and Livestock Research Organization.

3.2 DNA extraction and quantification

Genomic DNA was isolated from young and healthy leaf tissues of 3-week old plants using cetyl trimethyl ammonium bromide (CTAB) method (Osena *et al*., 2017). Approximately 200 mg of the leaf tissue sample was weighed and crushed into a homogenous paste in 700 µl of CTAB buffer [(2% CTAB, 1.4 M sodium chloride, 0.2 M ethylene diamine tetra acetic acid (EDTA), 1 M Tris-HCl and 4% polyvinyl pyrrolidone (PVP), with a final pH of 7.5] and 150 µl of 20% sodium dodecyl sulphate (SDS). The homogenate was transferred into a clean 1.5 ml Eppendorf tube and incubated in a water-bath at 55 °C for 20 minutes. The tubes were inverted after every five minutes during the incubation period. The sample was then centrifuged in a micro-centrifuge at 13,800 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new sterile Eppendorf tube followed by addition of equal volume of chloroform: isoamyl alcohol (24:1). The tube content was vortexed and then centrifuged at 13,800 rpm for 7 minutes at 4 °C. The chloroform: isoamyl alcohol extraction step was repeated once and the top layer was transferred to a new Eppendorf tube followed by addition of 50 µl of 7.4 M ammonium acetate and 2 volumes of ice-cold absolute ethanol. The sample was incubated at -20 °C for 20 minutes to precipitate out DNA from the solution. The tube was then centrifuged for at 13,800 rpm for 10 minutes at room temperature (25 °C) in order to pellet DNA. The supernatant was discarded and 500 µl of a wash solution (75% ethanol and 15 mM ammonium acetate) was added to clean the DNA pellet by centrifugation at 12,000 rpm for 5 minutes at room temperature (25 °C). This wash step was repeated twice and the supernatant discarded after each centrifugation step. The pellet was then air dried for 10 minutes and dissolved in 70 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA). DNA samples were treated with RNase through addition of 0.6 mg/ml

Ribonuclease A followed by incubation of the sample in a water bath at 37 °C for 30 minutes. The DNA was stored at -20 °C until use.

The quality and quantity of the extracted DNA samples were estimated using UV spectrophotometer and by resolving the DNA samples on 0.8% agarose gel in 1× Tris–acetate–EDTA (TAE) buffer. The isolated DNA was prepared and stored at 4 °C until use.

3.3 Primer selection and SRAP-PCR amplification

The SRAP analysis was carried out according to published protocols previously established by Li and Qurios (2001), with minor modifications. Twenty-four SRAP primer combinations were tested for their ability to prime to DNA of three (3) randomly selected passion fruit varieties. Primer combinations were excluded from the study if their banding patterns were difficult to score or if they failed to amplify consistently in all 3 varieties. Seven SRAP primer combinations (Table 3.2) that produced consistent amplification and clear banding patterns were selected for analysis of genetic diversity of 22 passion fruit varieties.

Table 3.2: List of SRAP primers sequences for the amplification of gene fragments used in assessment of genetic diversity of passion fruit varieties.

Locus	Forward primer (5' to 3')	Reverse primers (5' to 3')
ME1-EM7	TGAGTCCAAACCGGATA	GACTGCGTACGAATTCAA
ME1-EM9	TGAGTCCAAACCGGATA	GACTGCGTACGAATTCGA
ME1-EM12	TGAGTCCAAACCGGATA	GACTGCGTACGAATTATG
ME2-EM10	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCAG
ME2-EM11	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTCCA
ME2-EM12	TGAGTCCAAACCGGAGA	GACTGCGTACGAATTATG
ME5-EM7	TGAGTCCAAACCGGAAG	GACTGCGTACGAATTCAA

PCR reactions were performed in 20 µl volume using 1× GoTaq Green Master Mix (Promega Corporation, Madison, USA), 10 µM of each primer (i.e. both forward and reverse), 50 ng of

template DNA and the reaction mix was topped up to 20 µl with nuclease-free water. PCR amplification were performed in a MJ Mini^{TM} programmable Thermal Cycler (Bio-Rad, Singapore) using thermocycling conditions as follows; initial denaturation at 94 °C for 5 minutes and 5 cycles of denaturation at 94 °C for 1 minute, annealing at 35 °C for 1 minute and extension at 72 °C for 1 minute, followed by 30 cycles of denaturation at 94 °C for 1 minute, primer annealing at 50 °C for 1 minute, and extension at 72 °C for 1 minute. The amplification process was completed with a 7-minute final extension at 72 °C and the PCR products were maintained at 10 °C. The PCR reaction for each SRAP primer was performed at least twice, only clear and completely reproducible bands were used in data evaluation.

PCR products were resolved by electrophoresis on a 2.5 % ethidium bromide-stained agarose gel in 1× TAE buffer. Electrophoresis was carried out at 60 V for 60 minutes and PCR products were visualized using E.A.S.Y® Doc plus Gel Documentation system (Herolab GmbH Laborgeräte, Germany). The molecular weights of the PCR products were estimated using a 100 base pairs DNA marker (Bioneer, South Korea) loaded alongside the samples.

3.4 Growth conditions and drought stress treatments

Three-weeks-old potted passion fruit seedlings of both varieties were collected from KALRO and JKUAT and transferred to the glasshouse at the College of Biological and Physical Sciences, University of Nairobi. The seedlings were transplanted and grown in 2-L plastic pots (10 cm in diameter and 12 cm in height) containing a mixture of 1/3 peat and 2/3 sand with a drainage layer at the bottom of the pot. Each pot was irrigated every three days with tap water to field capacity. One month after transplanting, the plants were divided into two sets (control (well irrigated) and water deficit) with each set having three replicates. Water deficit was applied by withholding irrigation for 7 and 14 days followed by 7 days of recovery period for each

treatment. Leaf samples were harvested from plants in the different treatments and stored in polythene bags at -80° C for subsequent physiological and biochemical analyses.

3.5 Measurement of chlorophyll content

Photosynthetic pigments were determined using spectrophotometer according to Upadhyaya *et al*. (2010). Fresh leaf samples (100 mg) were weighed and ground in 1 ml of 100% (v/v) ethanol, with a pestle and mortar, and incubated in the dark for 24 hours. The extracts were then centrifuged at 3,500 × *g* for 5 minutes. The supernant was transferred in a clean cuvette and its absorbance read at wavelengths of 647 nm and 664 nm. Chlorophyll contents were expressed in μg g^{-1} FW. Chlorophyll a (Chl a), chlorophyll b (Chl b) and total chlorophyll (Chl T) contents were calculated as described by Upadhyaya *et al*. (2010) using the formula: Chl a = $11.94A_{664} – 1.93A_{647}$; Chl b = $20.36A_{647} – 5.50A_{664}$; Chl T = Chl a + Chl b.

3.6 Estimation of lipid peroxidation

Malondialdehyde content was estimated following a protocol described by Hodges *et al*. (1999). Exactly 0.2 g of leaf sample was homogenized in 4 ml of 0.1% (w/v) ice cold trichloroacetic acid (TCA) solution using a mortar and pestle. An additional 1 ml of TCA was added to the suspension and the homogenate was centrifuged at 3,500 × *g* for 5 minutes. Zero point five milliliters of the supernatant was dispensed in a clean tube and 1ml of 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added and the mixture incubated in a water bath (95 °C) for 30 minutes. The mixture was cooled after incubation, centrifuged at 3,500 × *g* for 10 minutes and its absorbance was read at 532 nm and 600 nm. The level of lipid peroxidation was expressed as nmol malondialdehyde g^{-1} fresh weight.

3.7 Estimation of hydrogen peroxide

Hydrogen peroxide content was estimated spectrophotometrically following a procedure described by Velikova *et al*. (2000). Exactly 0.5 g of the leaf samples were ground to a suspension in a 2 ml ice cold 0.1% (w/v) TCA and the resulting homogenate centrifuged at 3,500 × *g* for 30 min at 4 °C. Finally, 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide were added to 0.5 ml of the supernant and the absorbance read at 390 nm. Hydrogen peroxide content was calculated using the extinction coefficient 0.28/mM/cm and expressed as μmol g^{-1} FW.

3.8 Estimation of antioxidant enzymes activities

Peroxidase, ascorbate peroxidase and catalase were assayed as described by Venisse *et al*. (2001). Exactly 0.5 g of leaf sample was homogenized in an ice cold 2 ml of homogenate buffer (100 mM potassium phosphate buffer pH 6.8, 0.2 mM EDTA and 1% w/v polyvinylpyrolydone (PVP)) and the homogenates centrifuged at 3,500 × *g* for 20 minutes at 4 °C. The supernatants were used to assay for enzyme activities.

Peroxidase activity (EC 1.1.11.1.7) was estimated by measuring an increase in absorbance at 470 nm due to the formation of tetra guaiacol (extinction coefficient of 26.6 mM^{-1} cm^{-1}) as elucidated by Chance and Maehly (1995). Fifty microliters of the homogenate was added to 2 ml of the reaction mix containing 50 mM sodium acetate buffer (pH 7), 25 mM guaiacol and 25 mM H_2O_2. The absorbance of the mixture was read at 470 nm.

Ascorbate peroxidase activity (EC 1.11.1.11) was assayed spectrophotometrically by monitoring oxidation of ascorbic acid and measuring the change in absorbance at 290 nm. Exactly 10 µl of the prepared homogenate was added to 1 ml of the reaction mixture containing 0.2 mM

Tris/HCL buffer (pH 7.8), 0.25 mM ascorbic acid and 0.5 mM H_2O_2. Enzymatic activity was determined from ascorbate's extinction coefficient 2.8 mM^{-1} cm^{-1} (Nakano and Asada, 1981).

Catalase activity (EC 1.11.1.6) was determined by measuring a decrease in absorbance in pursuant to the decomposition of H_2O_2 at 240 nm for 1 min as elucidated by Cakmak *et al*. (1993). A 3 ml reaction mixture consisted of 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 µl of crude enzyme extract at 25 °C. The enzyme activity was calculated from the extinction coefficient (40 mM^{-1} cm^{-1}) of H_2O_2.

3.8 Data collection and analysis

Each SRAP primer pair was considered to be one genetic marker. Only distinct, reproducible and clearly distinguished DNA fragments in all genotypes were used in the data analysis. SRAP profile data were scored manually for each fragment in every genotype for each primer pair and recorded as (1) representing presence of a fragment and (0) representing the absence of fragment. The observed number of alleles, genetic similarity, Shannon information index and genetic distance estimated based on Nei's coefficient between pairs were analyzed using Popgene software, version 3.5 (www.ualberta.ca/~fyeh/popgene.pdf). Polymorphism information content per locus was calculated according to Anderson *et al*. (1993). Principal coordinate analysis and analysis of molecular variance were performed using GENALEX 6.41 (Peakall and Smouse, 2006). The distance matrices were generated based on Jaccard's similarity coefficient (Jaccard, 1908). Similarity matrices were subjected to cluster analysis of unweighted pair group method with arithmetic mean and dendrograms constructed using FigTree software (Version 1.4.2).

Biochemical data were subjected to Analysis of variance at 95% significance level ($P < 0.05$) using GenStat statistical software version 15.1.8035 ICRISTAT.

CHAPTER FOUR

4.0 RESULTS

4.1 Polymorphism revealed by SRAP markers

A total of 24 primer combinations were tested for their ability to amplify three randomly selected genotypes by PCR. Only, seven SRAP primer combinations (Table 3.2) that showed reproducible polymorphic patterns were selected to analyze all 22 passion fruit genotypes. The seven SRAP primer combinations generated a total of 931 bands with an average of 133 alleles per primer pair accounting for the 65.5% of polymorphic bands. The number of amplicons produced by each primer set ranged from 5 (ME2-EM12) to 10 (ME1-EM7) and (ME5-EM7), with an average of 8.29 amplicons per primer pair. The number of polymorphic amplicons ranged from 3 (ME1-EM9) and (ME2-EM10) to 9 (ME5-EM7), with an average of 5.43 amplicons per primer pair (Table 4.1). The Polymorphic Information Content (PIC) values measured from all the primers ranged from 0.1460 (ME1-EM9) to 0.4058 (ME5-EM7), with an average of 0.2302 (Table 4.1). The gene diversity varied from 0.1460 (ME1-EM9) to 0.3595 (ME5-EM7), with an average value of 0.2330. Shannon's information index at the ISSR level ranged from 0.2098 to 0.5216, with an average value of 0.3441. Figure 4.1 represents an example of the electrophoresis pattern obtained with markers ME1-EM9 and ME2-EM10 in six genotypes of passion fruit.

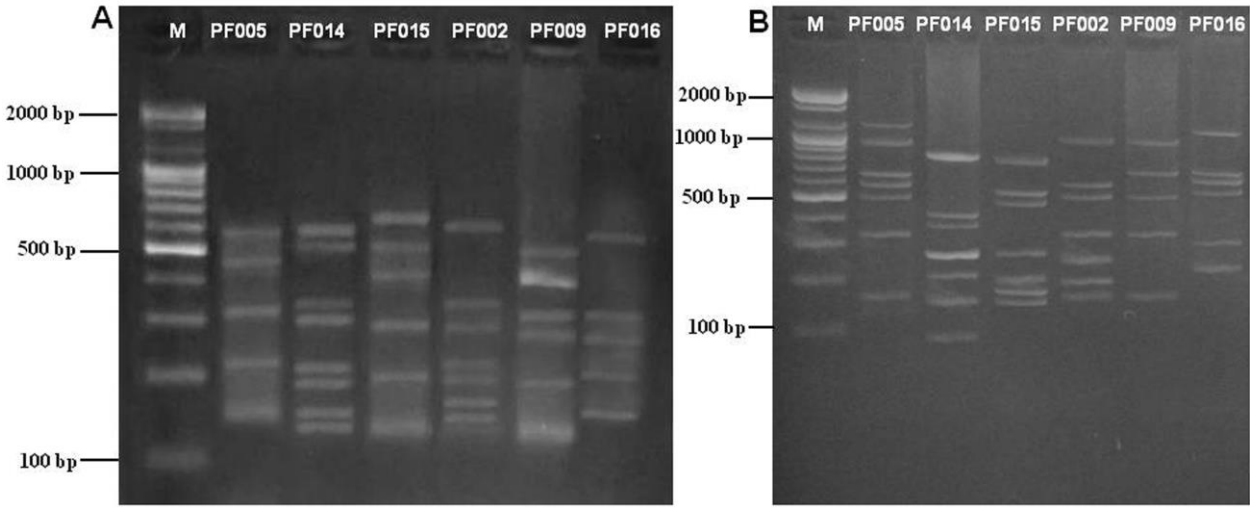

Figure 4.1: SRAP amplification profile of primer combination (A) ME1-EM9 and (B) ME2-EM10 in six varieties of passion fruit. Lane M represent 100 bp molecular weight marker (Bioneer, South Africa) and Lanes PF005, PF014, PF015, PF002, PF009 and PF016 are codes of respective passion fruit varieties.

Table 4.1: The number of polymorphic fragments, gene diversity and Shannon information index per SRAP primer combinations used for analysis of 22 passion fruit varieties.

Primer pair	NAB	NPB	PPB	PIC	GD	I
ME1-EM7	10	4	40	0.1884	0.1884	0.2655
ME1-EM9	9	3	33	0.1460	0.1460	0.2098
ME1-EM12	8	7	88	0.1956	0.1663	0.2825
ME2-EM10	7	3	43	0.1777	0.2031	0.2857
ME2-EM11	9	7	78	0.2479	0.3035	0.4396
ME2-EM12	5	5	100	0.2479	0.2479	0.3959
ME5-EM7	10	9	90	0.4058	0.3595	0.5216
Total	**58**	**38**	**-**	**-**	**-**	**-**
Mean	**8.29**	**5.43**	**66**	**0.2302**	**0.2330**	**0.3441**

NAB = Number of amplified bands; NPB = number of polymorphic bands; PPB = percent of polymorphic bands; GD = gene diversity; PIC = polymorphic information content; I = Shannon information index.

4.2 Similarity coefficient among passion fruit varieties

Genetic similarity matrix among all studied passion fruit varieties was obtained from amplified fragments produced by the seven SRAP markers using Jaccard coefficients. The similarity coefficient among the 22 passion fruit germplasm ranged from 0.51 to 1.0 with an average of

0.755. The highest similarity coefficient was 1.00 between code PF011 and PF012 (Table 4.2). However, the lowest genetic similarity of 0.51 was recorded between varieties PF004 and PF005.

Table 4.2: Pairwise genetic similarity index among 22 passion fruit varieties based on SRAP data.

	PF001	PF002	PF003	PF004	PF005	PF006	PF007	PF008	PF009	PF010	PF011	PF012	PF013	PF014	PF015	PF016	PF017	PF018	PF019	PF020	PF021	PF022
PF001	1.00	0.97	0.86	0.86	0.56	0.86	0.83	0.84	0.86	0.90	0.62	0.62	0.54	0.56	0.57	0.60	0.60	0.66	0.74	0.67	0.68	0.69
PF002		1.00	0.88	0.88	0.54	0.88	0.85	0.86	0.88	0.93	0.60	0.60	0.52	0.54	0.56	0.58	0.58	0.64	0.71	0.65	0.66	0.67
PF003			1.00	0.86	0.57	0.95	0.93	0.93	0.95	0.95	0.63	0.63	0.60	0.62	0.64	0.64	0.67	0.63	0.71	0.67	0.72	0.69
PF004				1.00	0.51	0.86	0.83	0.84	0.86	0.91	0.63	0.63	0.55	0.56	0.58	0.58	0.64	0.67	0.67	0.61	0.65	0.66
PF005					1.00	0.54	0.54	0.56	0.58	0.57	0.74	0.74	0.71	0.69	0.68	0.72	0.64	0.64	0.62	0.62	0.70	0.63
PF006						1.00	0.93	0.98	0.95	0.95	0.63	0.63	0.57	0.59	0.61	0.61	0.64	0.63	0.71	0.64	0.69	0.66
PF007							1.00	0.90	0.88	0.88	0.57	0.57	0.58	0.60	0.62	0.61	0.64	0.64	0.68	0.65	0.70	0.67
PF008								1.00	0.98	0.93	0.65	0.65	0.59	0.61	0.63	0.63	0.65	0.65	0.73	0.66	0.71	0.68
PF009									1.00	0.95	0.67	0.67	0.61	0.63	0.65	0.65	0.67	0.67	0.75	0.68	0.73	0.70
PF010										1.00	0.66	0.66	0.57	0.59	0.61	0.61	0.64	0.63	0.71	0.64	0.69	0.66
PF011											1.00	1.00	0.90	0.88	0.86	0.73	0.76	0.76	0.79	0.73	0.71	0.75
PF012												1.00	0.90	0.88	0.86	0.73	0.76	0.76	0.79	0.73	0.71	0.75
PF013													1.00	0.98	0.96	0.81	0.84	0.74	0.74	0.77	0.76	0.76
PF014														1.00	0.98	0.83	0.86	0.72	0.76	0.79	0.78	0.75
PF015															1.00	0.85	0.84	0.71	0.77	0.81	0.80	0.77
PF016																1.00	0.78	0.75	0.75	0.78	0.77	0.70
PF017																	1.00	0.77	0.67	0.70	0.83	0.73
PF018																		1.00	0.81	0.71	0.80	0.77
PF019																			1.00	0.88	0.76	0.80
PF020																				1.00	0.76	0.80
PF021																					1.00	0.79
PF022																						1.00

4.3 Genetic relationship among passion fruit varieties

Relationships among the 22 varieties were evaluated using principal coordinate analysis. The results of principal coordinate analysis were performed to present a spatial representation of the relative genetic distance among individuals revealed three distinct groups (Figure 4.2). The plane of the first three PCoA axis accounted for 65.30% of the total variation (first axis = 45.40%, second = 10.13 % and third = 9.40%). All varieties from Nairobi and Trans Nzoia Counties (except PF005) clustered in the first group and were distributed on the right side of the plane (Figure 4.2). Cultivar PF005 together with varieties from Kiambu and Meru Counties were clustered in the left plane as the second group (Figure 4.2). All varieties from Siaya County in addition to one variety (PF016) from Kiambu were separated as the third group on the upper left quadrant (Figure 4.2).

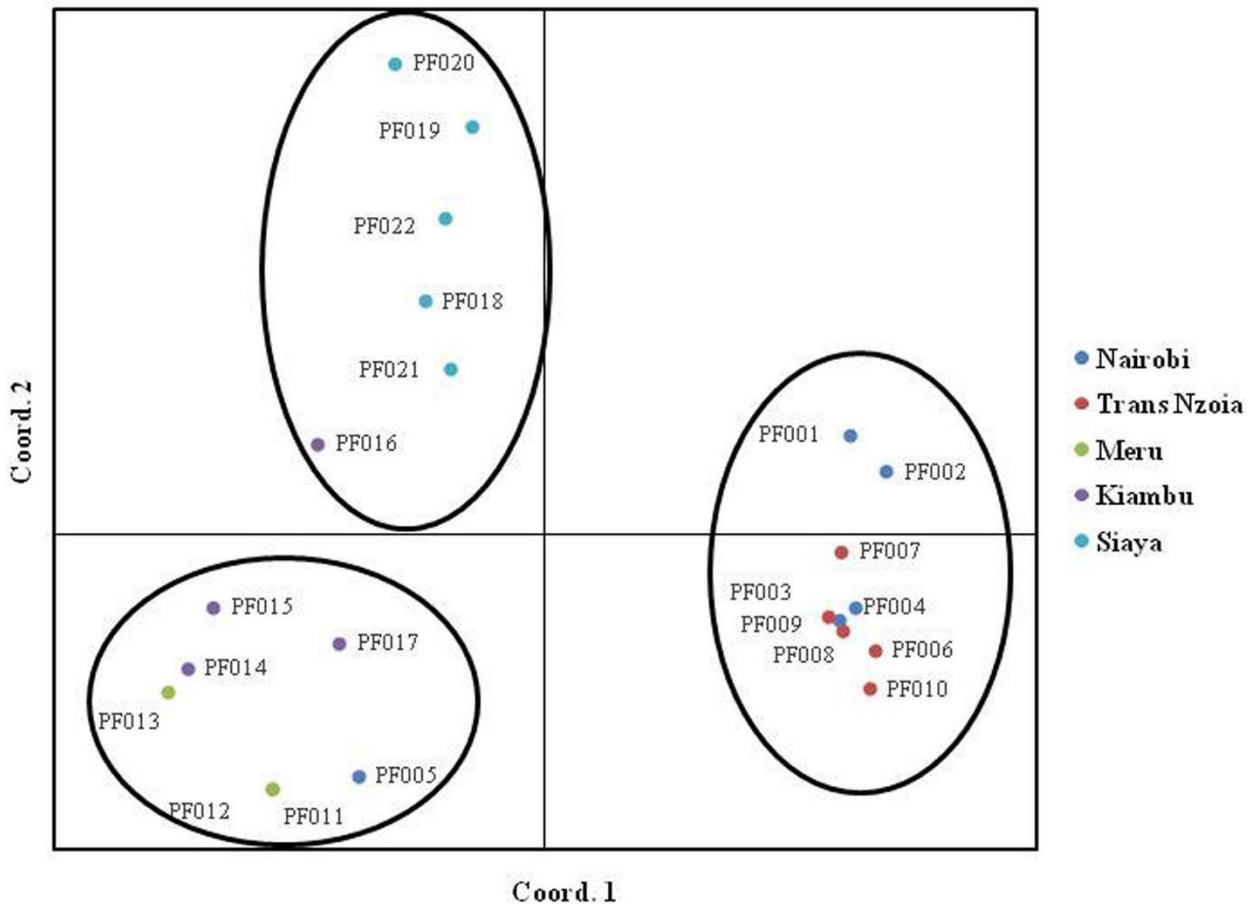

Figure 4.2: Principal coordinates analysis of 22 passion fruit varieties with 7 SRAP primers. Identification of 5 groups of varieties corresponded to samples listed in Table 1. Individuals are coded according to the County of origin.

To further describe the relationship between the above 22 varieties, based on the similarity coefficient, an UPGMA dendrogram of the passion fruit varieties was constructed (Figure 4.3). The tested varieties were divided into two major clusters at the lowest range of similarity (12%). The first cluster (A) included 13 varieties, while the second cluster (B) included 9 varieties. The first cluster included all the varieties from Meru, Kiambu and Siaya Counties, except PF005 that was obtained from Nairobi County. The first cluster (A) had two further sub-clusters; the first sub-cluster had 12 varieties whereas the other sub-cluster had only one variety (PF005) which failed to group with the rest. The second cluster was composed of purple varieties from Trans

Nzoia and Nairobi Counties. Only the yellow variety (PF005) that originated from Nairobi County was missing from the second cluster. In comparison to cluster A, cluster B also had two sub cluster with the first consisting of 8 varieties whereas the other one having a single variety (PF004). In summary, the above findings show that sub-clustering of passion fruit varieties followed the genetic background as well as the geographical location.

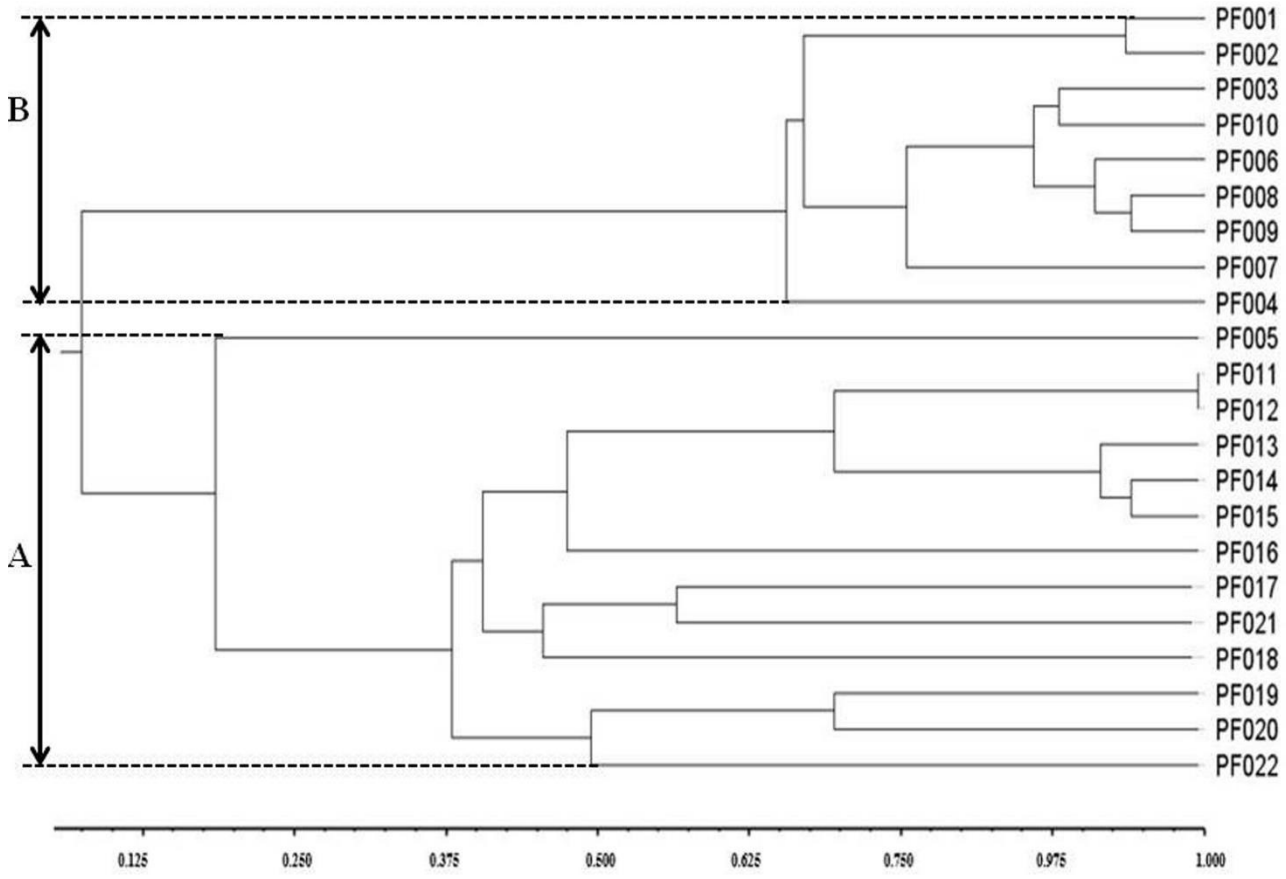

Figure 4.3: Clustering of 22 varieties of passion fruit obtained by unweighted pair group method with arithmetic average based on Jaccard's coeffient from SRAP data.

The dendrogram generated from the SRAP data according to the geographical origin of the varieties resulted in two main clusters (A and B) at 25% similarity level (Figure 4.3). The first cluster was further subdivided into two sub clusters. The first sub cluster included the varieties from Siaya County, while varieties from Meru and Kiambu Counties formed the second sub

cluster. The varieties from Trans Nzoia as well as Nairobi Counties formed the second cluster (Figure 4.4).

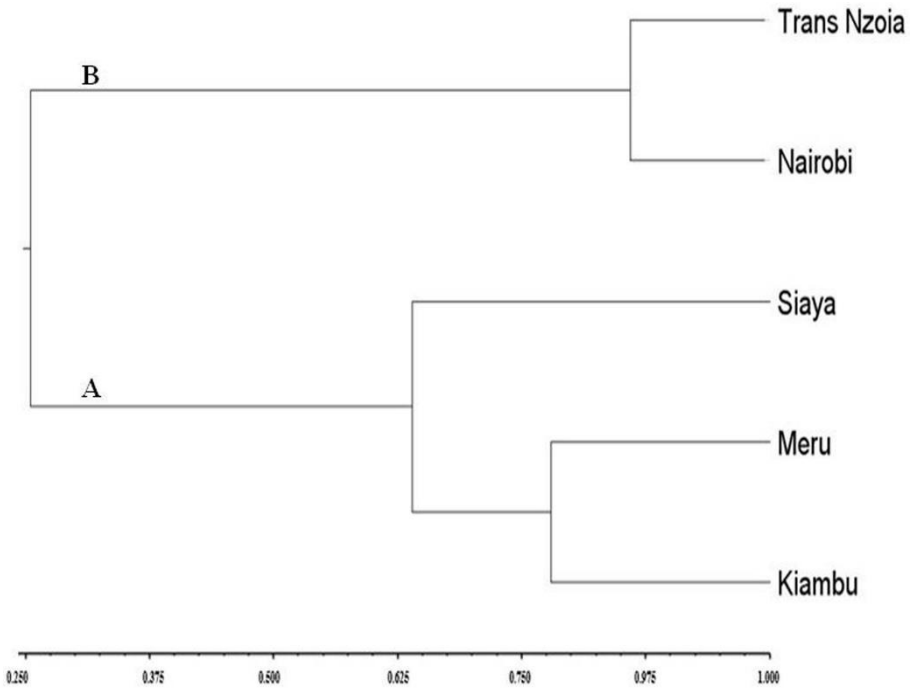

Figure 4.4: Dendrogram of passion fruit groups of the 5 regions/Counties based on genetic similarity

Nei's gene diversity index and Shannon diversity index were used to evaluate the germplasms' genetic diversity in the current study. We compared the genetic diversity in passion fruit genotypes from five Counties in Kenya. The estimates of the genetic diversity in each population are summarized in Table 3. Nei's gene diversity index of the five populations ranged from 0.0552 to 0.2446 whereas Shannon's diversity index was from 0.0388 to 0.1637. Among these five populations, those genotypes from Nairobi and Siaya Counties exhibited the highest level of variability (NGDI: 0.2446 and 0.1892, SDI: 0.1637 and 0.1291, respectively), whereas population from Meru County (NGDI: 0.0552 and SDI: 0.0388) exhibited the lowest level of variability as shown in Table 4.3.

Table 4.3: Shannon diversity index (SDI) and Nei's gene diversity index (NGDI) in the varieties of passion fruit from different regions/Counties

County	SDI	NGDI
Kiambu	0.0892	0.1301
Siaya	0.1291	0.1892
Nairobi	0.1637	0.2446
Trans Nzoia	0.0461	0.0658
Meru	0.0388	0.0552
All	**0.0934**	**0.1370**

4.4 Analysis of molecular variance

Analysis of molecular variance for the SRAP markers revealed that 52% of the total SRAP marker variation was due to among population variance, while 48% of the total SRAP marker variation was due to within-population variance (Table 4.4).

Table 4.4: Analysis of molecular variance for 22 passion fruit varieties in five populations based on SRAP markers.

Source of variance	Df	SSD	MSD	Variance component	Percentage of variation
Among populations	**4**	**84.848**	**21.212**	**4.001**	**52**
Within populations	**17**	**63.833**	**3.755**	**3.755**	**48**
Totals	**21**	**148.682**		**7.756**	**100**

Df, degrees of freedom; SSD, sum of squared deviation; MSD, mean squared deviation

4.5 Physiological and biochemical analyses

The physiological and biochemical analyses revealed that there were significant variations between the two passion fruit varieties in all the parameters analyzed at each point of water deficit treatments even under well-watered conditions.

4.5.1 Changes in chlorophyll content

Changes in chlorophyll (a, b, total) content in control, drought and recovered plants are presented in Figure 4.5, which showed that drought stress induced a gradual decline in photosynthetic pigment. There was no significant change in chlorophyll content in the well-watered control plants during the entire experimentation period in comparison to the decreased chlorophyll content observed in plants subjected to drought stress. Effect of drought stress on the chlorophyll content was severe on the yellow passion fruit varieties as there was a decline of chlorophyll content by 17%, whereas the decline in purple passion fruit varieties was by 14%.

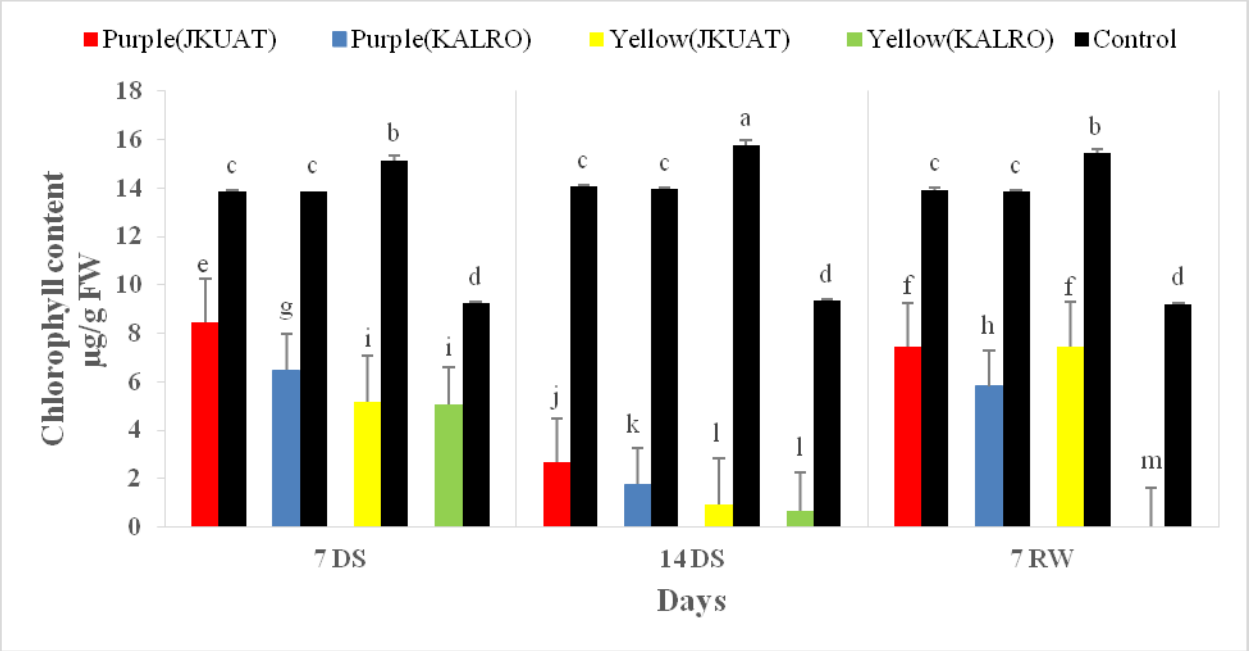

Figure 4.5: Effect of drought stress on chlorophyll content in different passion fruit varieties (purple and yellow). The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

4.5.2 ROS production and MDA in passion fruit varieties in response to drought stress

The levels of ROS accumulation and oxidative damage in all passion fruit varieties were increased under drought stress with no significant change being observed on plants under well-watered control conditions (Figure 4.6). When compared with well-watered control conditions, drought stress treatment increased the values of H_2O_2 and MDA in the range of 10 – 46% and 5 – 24%, respectively. The yellow and purple passion fruit varieties recorded a 44% and 32% increase in the level of H_2O_2, respectively. As indicated in Figure 4.7, the MDA content in passion fruit leaves was significantly higher in the varieties subjected to drought stress treatments. Significant differences ($p<0.005$) were observed in MDA values for 7 and 14-day drought stressed plants compared with well-watered control plants. Withholding water for 7 and 14-days increased MDA content to higher levels than well-watered control plants, respectively.

After re-watering, MDA content decreased in drought stress plants but remained higher for purple and yellow varieties than well-watered control plants. The drought stressed yellow variety from KALRO recorded the lower MDA values than those of the control.

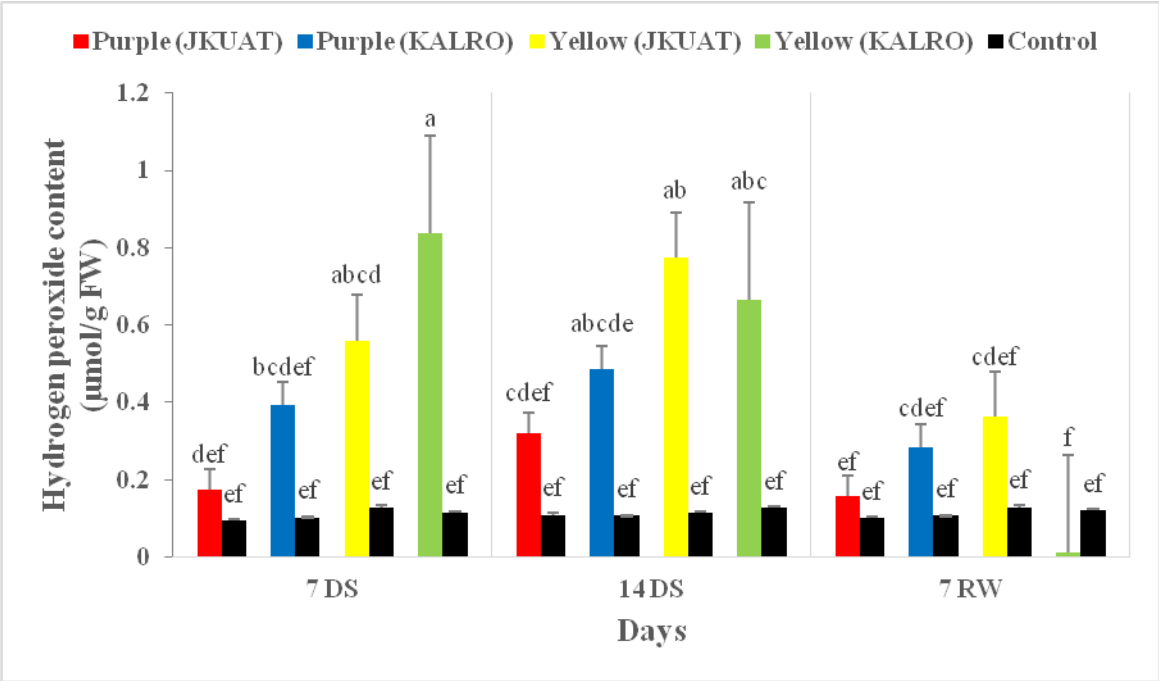

Figure 4.6: Effect of drought stress on the content of hydrogen peroxide in different passion fruit varieties (purple and yellow). The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

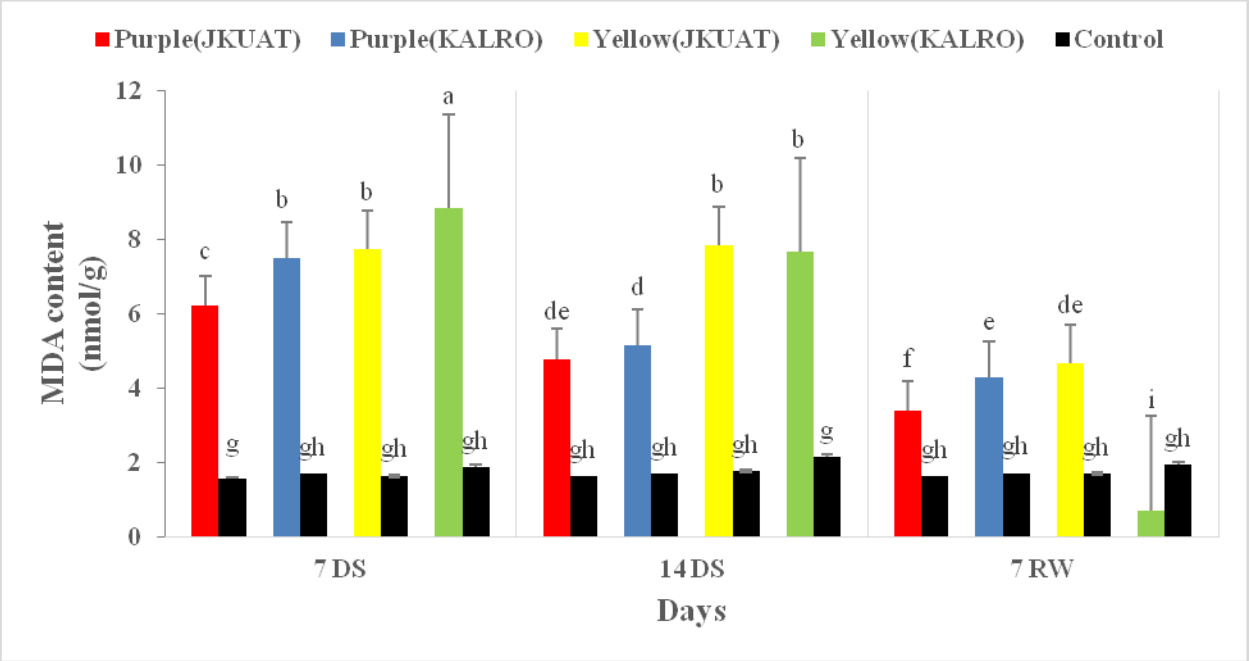

Figure 4.7: Effect of drought stress on MDA content in different passion fruit varieties (purple and yellow). The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

4.5.3 Changes in antioxidant enzymes activities

The effect of drought stress on the activities of antioxidant enzymes which plays significant role in scavenging of ROS was evaluated in response to drought stress. The plants under well-watered conditions (controls) showed no significant change in the levels of antioxidant enzyme activities. An increase in the activities of antioxidant enzymes was observed in drought stressed plants with the highest levels being recorded on the fourteenth day (Figure 4.8, 4.9 and 4.10). The purple passion fruit varieties recorded a significant increase ($P>0.05$) in the level antioxidant enzymes in comparison to the yellow varieties which showed no significant change. The levels of the antioxidant enzymes reduced significantly after re-watering.

Ascorbate peroxidase level increased by 41% in drought stressed purple passion fruit varieties in comparison to the control plants under well-watered conditions. Drought stressed yellow passion fruit varieties recorded no significant change in ascorbate peroxidase activity in relation to the

well-watered control plants. The purple varieties had a 31% increase in ascorbate peroxidase level as compared to the yellow varieties under drought stress (Figure 4.8).

Drought stress resulted in a significant increase in the activity of catalase in purple varieties at 25% in comparison to the yellow varieties at 17% (Figure 4.9). After re-watering, catalase activities decreased but remained higher in drought stressed plants as compared to well-watered plants after 7 days of re-watering.

The level of peroxidase in the drought stressed purple varieties was 30% higher than those of the well-watered control plants. No significant change was recorded between drought stressed and well-watered plants of the yellow passion fruit varieties. Purple varieties recorded a 16% increase in peroxidase activity in comparison to the yellow varieties following drought stress (Figure 4.10). After re-watering, peroxidase activities decreased but remained higher in drought stressed plants as compared to well-watered plants after 7 days of re-watering.

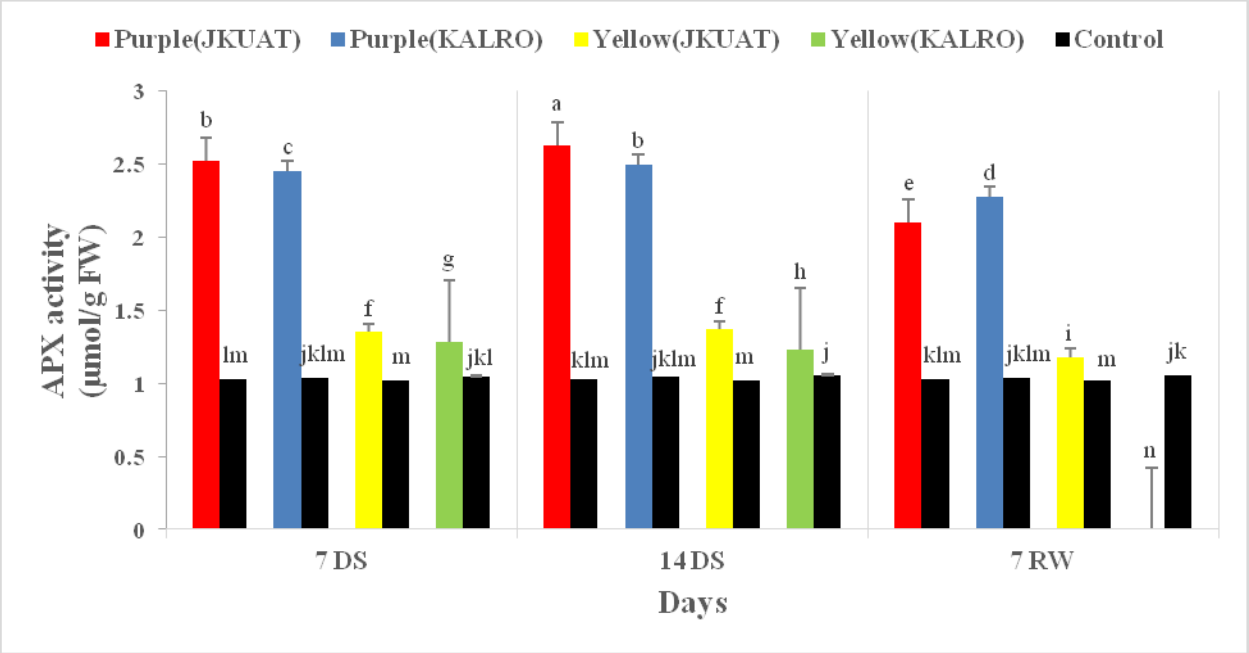

Figure 4.8: Effect of drought stress on ascorbate peroxidase activities in different passion varieties. The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

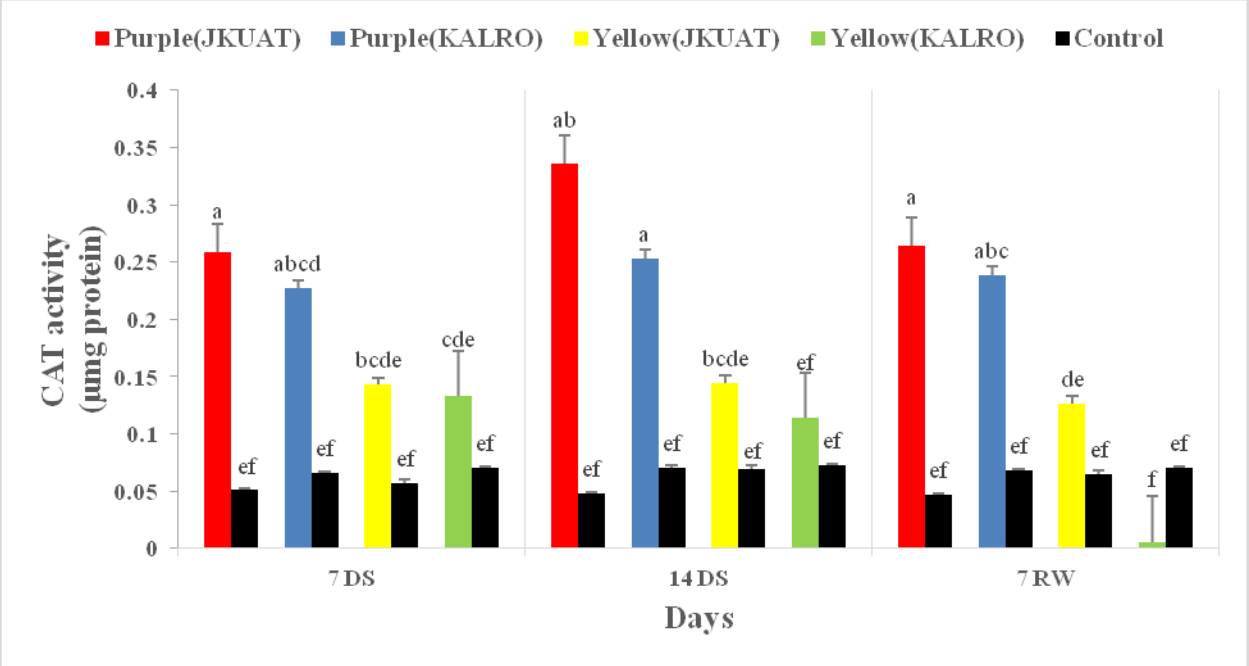

Figure 4. 9: Effect of drought stress on catalase activities in different passion fruit varieties (purple and yellow). The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

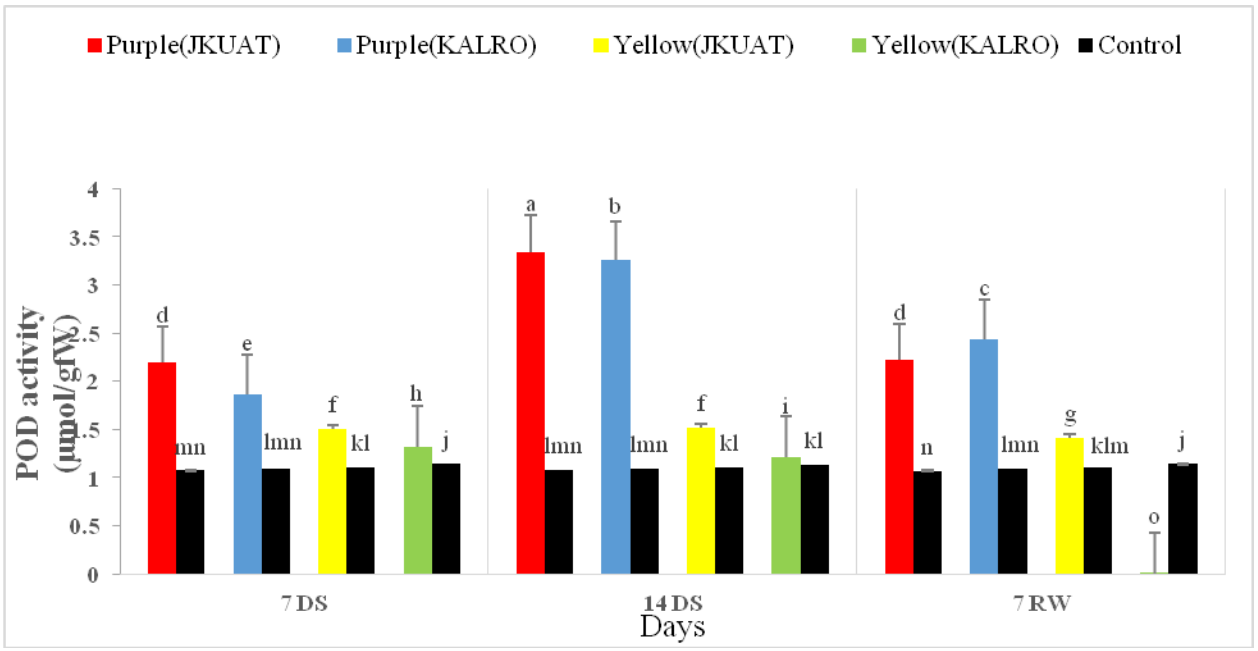

Figure 4.10: Effect of drought stress on peroxidase activities in different passion varieties (purple and yellow). The bar graph represents means of three replicates at a significance level of 0.05. DS and RW represent drought stress and re-watering, respectively.

CHAPTER FIVE

5.0 DISCUSSION

Knowledge of genetic variability within collections of genotypes provides crucial information for the effective conservation, germplasm resource management and utilization in breeding programs (Salem *et al*., 2008). Variability of genotypes is important to a breeder to develop high yielding varieties through selection, and therefore assessment of genetic variation is a major concern of plant breeders (Ambaw and Dessalegn, 2017). Genetic variation is essential in the production of new varieties with the objective of improving crop productivity and ability to withstand shocks from biotic and abiotic stresses (Salem *et al*., 2008). Genetic variability in passion fruit has been previously studied using different DNA molecular markers such as RAPD, AFLP, ISSR (Segura *et al*., 2002; Viana *et al*., 2003; Aukar *et al*., 2002; Santos *et al*., 2011). In this study we used SRAP markers to estimate genetic diversity of passion fruit genotypes cultivated in Kenya. Primer combinations with poor reproducibility in repetitive experiments were not scored. Fragments amplified with 7 primer combinations, selected from the screened 24 primer combinations, after repetitive experiments were scored. The amplified fragments were classified into strong, intermediate, or weak categories on the basis of their intensity; only the strong and intermediate fragments were scored, and the weak amplicons were rejected. This variation in amplification could be as a result of the copy number of the fragment in the genome and/or the degree of complementarity of the end sequences to those of the primers (Hu and Vick, 2003).

Although, the SRAP marker system was primarily developed for *Brassica* species (Li and Quiros, 2001), it has been shown to be a powerful technique for the assessment of genetic variability in many studies of other crop plants including cucumber, sugarcane, eggplant, and

castor, as well as in grasses including elephant grass, buffalo grass, alfalfa and *Vicia faba* (Alghamdi *et al*., 2012; Castonguay *et al*., 2010; Zhang *et al*., 2010; Zheng *et al*., 2010; Xie *et al*., 2009; Alwala *et al*., 2008; Mutlu *et al*., 2008; Ariss and Vandemark, 2007; Budak *et al*, 2004). Previous results showed that SRAP markers had many advantages including; simplicity, reliability, high degree of reproducibility and discriminatory power, as well as a high polymorphism rate (Li and Quiros, 2001). In this study, seven SRAP primer combinations amplified 58 fragments with an average of 8.29 fragments per primer. The molecular studies showed that 38 (66%) out of 58 PCR-amplified fragments were polymorphic and this level of achieved polymorphism was sufficient to distinguish all 22 passion fruit genotypes included in the study. Thus indicating high discriminatory ability of the SRAP technique in studying genetic variability. The mean number of bands per primer and polymorphism level of 66% observed in this study was when compared with data available for *P. edulis*, with a mean of 14.4 bands per primer and 73% polymorphism (Bellon *et al*., 2009) and also in other plant species including 100% polymorphism in faba bean (Alghamdi *et al*., 2012), and 93.4% in willow (Daneshvand *et al*., 2015). However, the polymorphic rate observed in this study was higher than that generated using SRAP markers in other plant species, including 56.0% in eggplant and related *Solanum* species (Li *et al*., 2010), 49.4% in mustard (Wu *et al*., 2009) and 43% in *Coffea arabica* (Mishra *et al*., 2011). The variation of polymorphism rate is a reflection to the extent of genetic divergence among and within the populations and/or genotypes studied and SRAP combinations used.

Polymorphic information content (PIC) values were used to measure the genetic diversity of passion fruit genotypes in our collection. The PIC value, which depends on the number of detectable alleles and their distribution, indicates a marker's utility for detecting polymorphism

within a population (Chesnokov and Artemyeva, 2015). The PIC values obtained from all the primers, included in this study, ranged from 0.1460 to 0.4058, with an average of 0.2302 demonstrating good discriminatory capacity of SRAP markers. The obtained Nei's genetic diversity average values of 0.2330 were consistent with the expectation for outcrossing species and with the values observed in a previous study for *P. edulis* (Oliveira *et al*., 2005). Additionally, Shannon's information index at the ISSR level ranged from 0.2098 to 0.5216, with an average value of 0.3441. These findings are similar to those reported in previous studies of other allogamous species, such as 0.383 in *Nelumbo nucifera* (Han *et al*., 2007) and 0.3834 in *Stipa tenacissima* (Boussaid *et al*., 2010). The genetic diversity revealed by SRAP in our study was relatively higher than those of passion fruit varieties revealed by ISSR markers (Ferraz and Jorge, 2011).

The genetic similarities of the 22 genotypes ranged from 0.51 to 1.0, with an average of 0.75, revealing high levels of genetic variation among the passion fruit genotypes studied. The highest similarity (of 1.0) was between code PF011 and PF012, indicating that they are related. The lowest genetic similarity coefficient (0.51) was recorded between PF004 and PF005, indicating that they are far much unlike, possibly because of differences in their genomes, as they are purple and yellow varieties, respectively.

The genetic diversity of a population in a species is affected by a number of evolutionary factors, including; the seed dispersal, gene flow, natural selection, geographic range, and the diversity center (Lázaro-Nogal *et al*., 2017). Studies of diversity among passion fruit accessions based on dominant markers were unable to demonstrate associations between the estimated genetic diversity and their geographic origins (Ganga *et al*., 2004). In the present study, the dendrogram constructed using UPGMA method suggested occurrence of two major clusters/groups. The

UPGMA cluster analysis of the genotypes based on the SRAP data illustrated considerable association between the molecular diversity and geographic origin of the passion fruit varieties. The first cluster (A) comprised of 13 varieties from Siaya, Kiambu and Meru Counties. The 'A' cluster consisted of 8 yellow and 1 purple passion fruit varieties, as well as 4 landraces scattered in three different sub-clusters. All genotypes from Trans Nzoia and Nairobi Counties, except PF005, were grouped in Cluster A. A common feature in these 9 varieties is that they are purple passion fruits. Although broad genetic diversity was expected in *Passiflora* because of the geographical distribution of this genus (Lopes, 1991), our findings on the divergence among collected genotypes at production areas suggests otherwise. This could be due to the germplasm exchange within Kenya and in most cases, the passion fruit producers grow their seedlings, either from seeds collected in their neighborhood or from fresh fruits purchased at the market. Several studies show the reduction of variability among *Passiflora* varieties found in a particular geographical region, compared to genotypes from other distant geographical regions (Viana *et al*., 2003; Bellon *et al*., 2009). Therefore, the relatedness observed in this study may suggest that the varieties may have similar origins, but were grown in different geographical localities. The SRAP markers in this study did not show complete separation of different forms of *P. edulis* (yellow and purple passion fruits), demonstrating high degree of genetic similarity. Genetic resources from different geographical areas can result in genetic diversity required for passion fruit breeding.

Genetic variability among accessions of *P. edulis* makes clear the broad genetic basis of species, and good prospects for its use in breeding programs. Therefore, the information generated from the study will be useful in future breeding programs aiming at domestication of this species as

well as the expansion of the genetic basis of resistance, especially in regard to woodiness virus disease, Fusarium wilt, and tolerance to drought.

Drought stress is one of the most significant factors limiting growth, development and productivity of crop plants in the field (Fahad *et al*., 2017). Chlorophyll is one of the major chloroplast components for photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate (Parry *et al*., 2014). Flexas and Medrano (2002) reported that water stress reduces green leaf color in C3 plants due to chlorophyll degradation and photo-oxidation. In the present study, chlorophyll content decreased in drought-stressed plants as compared to the well-watered control plants in both passion fruit varieties. The purple passion fruit varieties showed significantly higher chlorophyll content compared to the yellow varieties, suggesting that the yellow varieties were sensitive to drought stress. In addition to being an indicator of oxidative stress, chlorophyll content can be used to determine the plant level of tolerance or sensitivity to oxidative stress. A study by Pastori and Trippi (1992) on wheat and corn showed that resistant genotypes had higher chlorophyll content than the sensitive genotypes under the oxidative stress.

Malondialdehyde a byproduct of lipid peroxidation is an essential biomarker for oxidative stress in drought conditions. Mafakheri *et al*. (2010) described that due to decline in chlorophyll content, the capacity of light harvesting is lowered which ultimately increases the production of ROS. Higher ROS and MDA contents in the yellow variety as compared to purple variety might be associated with greater photosynthetic inhibition under drought stress and an increased potential for ROS production. ROS is capable of damaging the photosynthetic apparatus and cause oxidation of proteins, lipids, nucleic acids, and carbohydrates (Liu *et al*., 2015; Sabra *et al*., 2012). The findings from the showed that higher MDA concentration in drought-stressed

plants was associated with higher hydrogen peroxide content, especially in the severely drought-stressed plants. The lower MDA values in the drought stressed yellow varieties from KALRO is attributed to cell deaths as they were unable to overcome the effect of drought stress during the recovery period. The high levels of MDA in drought stressed plant is a direct correlation to the magnitude of drought condition as well as the sensitivity of plants to drought as observed in *Lonicera japonica* (Li *et al*., 2009).

Plants employ a number of strategies to mitigate challenges associated with drought stress. Antioxidant enzymes are considered the best strategies employed by plants to overcome challenges associated by drought stress (Ren *et al*., 2016). Their synthesis is subject to increase in substrate availability and hence the level of hydrogen peroxide plays a significant role in their synthesis (Ren *et al*., 2016). Peroxidases, catalases and ascorbate peroxidase are examples of some of the antioxidant enzymes produced in response to drought stress. Their level in plants under drought stress directly correlates to the plant's ability to overcome the negative effect drought stress (Sahitya *et al*., 2018; Ren *et al*., 2016). High levels of peroxidase, catalase and ascorbate peroxidase were recorded in drought stressed plants compared to the controls in the study. Similarly purple passion fruit cultivars recorded higher values of the mentioned enzymes in comparison to the yellow variety. The findings are consistent with studies on rice (Guo *et al*., 2006), maize (Jiang and Zhang, 2002), *Coffea canephora* (Pinheiro *et al*., 2004) and alfalfa (Rubio *et al*., 2002) that indicated an increased level the catalase and under drought stress.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

The findings of our study demonstrate the existence of a considerable amount of genetic variability among passion fruit genotypes grown in different regions of Kenya. This indicates the potential application of such genotypes in passion fruit breeding programs by exploiting the molecular markers for selection of specific traits.

It was also noted that the purple passion fruit variety were tolerant to drought stress in comparison to the yellow variety. This can be attributed to the significant rise in the activities of the antioxidant enzymes under the study (ascorbate peroxidase, peroxidase and catalase) in scavenging the reactive oxygen species.

6.2 Recommendations

Studies on passion fruit are needed in identification of molecular markers linked to drought tolerance. This will be of significance in generation of superior plants through marker assisted breeding.

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APPENDICES

Appendix 1: Publication

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Analysis of genetic diversity of passion fruit (*Passiflora edulis* Sims) genotypes grown in Kenya by sequence-related amplified polymorphism (SRAP) markers

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ABSTRACT

Passion fruit (*Passiflora edulis* [Sims]) is currently ranked third among fruit exports from Kenya and has great potential since demand for both fresh fruit and processed juice is on a continuous increase. Although assessment of genetic variability of germplasm is indispensable for improvement and development of superior cultivars, little information is available on the genetic diversity of passion fruit cultivated in Kenya. The objective of this study was to determine the genetic diversity of passion fruit genotypes from major growing regions in Kenya using sequence-related amplified polymorphism (SRAP) markers. Twenty four SRAP primer combinations were screened using three passion fruit genotypes and only seven that displayed polymorphic and stable amplification profiles were used to analyze 22 genotypes. The seven primer combinations amplified a total of 931 clear bands with an average of 133 bands per primer pair, of which 610 (65.5%) bands were polymorphic. The similarity coefficients among the 22 passion fruit germplasms ranged from 0.51 to 1.0 with an average of 0.755. The 22 passion fruit genotypes were classified into two groups by cluster analysis using unweighted pair-group method with arithmetic mean (UPGMA) with 12% similarity. Shannon's diversity index was 0.0934 and Nei's gene diversity index was 0.1370 in the present study. The study findings demonstrate the existence of a considerable amount of genetic variability among passion fruit genotypes grown in different regions of Kenya. This indicates the potential application of these genotypes in breeding programs by exploiting the use of molecular markers for selection of specific agronomic traits.