ASSESSING DIVERSITY OF *Solanum nigrum* L GROWN IN KENYA

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November, 2016
DECLARATION

This thesis is my original work and has not been presented for a degree in any other university for any other award.

Signature ........................................Date.........................

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DEDICATION

I dedicate this thesis to the Rwigi’s family.
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# ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AFLP</td>
<td>Amplified Fragment Length Polymorphism</td>
</tr>
<tr>
<td>CHO</td>
<td>Carbohydrates</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleotide phosphates</td>
</tr>
<tr>
<td>MAS</td>
<td>Marker Assisted Selection</td>
</tr>
<tr>
<td>PCBs</td>
<td>Polychlorinated biphenyls</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</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>SSR</td>
<td>Simple Sequence Repeats</td>
</tr>
<tr>
<td>STR</td>
<td>Simple Tandem Repeats</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic acid</td>
</tr>
<tr>
<td>VNTRs</td>
<td>Variable Number of Tandem Repeats</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-Strand Conformation Polymorphism</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>EST-SSR</td>
<td>Expressed Sequence Tag SSR</td>
</tr>
<tr>
<td>MEGA</td>
<td>Molecular Evolutionary Genetics Analysis</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris Borate EDTA</td>
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<tr>
<td>PCoA</td>
<td>Principle Coordinates Analysis</td>
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ABSTRACT

Solanum nigrum (commonly referred to as black nightshade) are world-wide weeds on arable land, gardens and rubbish pits. Nightshades have been used in the field of medicine in the manufacture of analgesics, ointments and vasodilators. The limited information available on this neglected and underutilized family hinders their development as well as sustainable conservation. Existing knowledge on the genetic potential of these plants is limited and the information regarding the genomic organization of Solanum nigrum complex species found in Kenya is scarce. The objective of this work was to evaluate diversity of S. nigrum populations grown in selected parts of Kenya using morphological and Simple Sequence Repeats (SSR) markers. A total of 120 samples from four populations were assessed. Four aerial characters were used in morphological characterization. Leaf surface (smooth and hairy), Leaf margin (entire and sinuate), Leaf shape (ovat, lanceolate and diamond), Fruit colour (Red, Purple small and Purple large). Tukeys HSD test revealed significant variation in the characteristics assessed (P≤0.05). Four SSR primers generated 63 polymorphic bands ranging between 14-17 bands per primer set. Polymorphic Information Content ranged from 0.1429 to 0.1855 with a mean of 0.1704. The average heterozygosity $H_e=0.1370$ for SSR markers used. Axis 1 and 2 of PCoA accounted for 44.65% of the variance in the population distribution. AMOVA revealed 14% variation among populations and 86% within populations. Variation among regions was not genetically evident. Nei’s genetic distance ranged from 0.010 (Kipkaren and Matanya) to 0.020 (Makuyu and Mauche). Dissimilarity analysis was performed using Unweighted Neighbor Joining with 1000 bootstraps in DARwin 6.0.5 software. The dendogram did not reflect morphological and genetic differences, a good indicator of missing genetic divergence between populations. Variation in the Agro Ecological Zone does not imply variation in the S. nigrum varieties grown. Comparison between morphological and molecular data revealed clustering of leaf surface and fruit colour with genetic data. However, there was no clustering between leaf shape and leaf margin with the same data. Observed morphological differences are mainly as a result of selection by human. Findings from this study show that S. nigrum complex exhibit low genetic diversity but high morphological variability. S. nigrum is a group of closely related plants as shown by low genetic distance and lack of clustering patterns. The output of this study will have far reaching applications in the development of markers linked to important agronomic traits that can be used in future breeding programs of the Solanum complex. The findings will further contribute to changing the taxonomic confusion within the Solanum nigrum.
CHAPTER ONE

INTRODUCTION

1.1 Background information

Indigenous leafy vegetables comprise a large group of species that are unrelated and whose leaves are used as food by many local communities in Africa. These vegetables play a crucial role in food security (Nandhini et al., 2014). Human consumption of their leaves and fruit as food is widespread, particularly in Africa and South East Asia (Jagatheeswari et al., 2013). *S. nigum* is popular vegetable in Kenya. Small holders who grow it in kitchen garden mainly for subsistence dominate the production of these vegetables. Yields have been declining because of low soil fertility and poor production practices by farmers (Juma, 1989).

Agricultural development and cultivation in developing countries are primarily based on subsistence and edible wild species. However, dietary utilization of non-domesticated plants has received little attention and a dramatic narrowing of the food base in many traditional societies has occurred (Tindall, 1977). Edible wild leafy vegetables play an important role in African agricultural and nutritional systems. Chweya (1997) listed several leafy vegetables used in Kenya. Some of these vegetables are treated as weeds in different parts of the country, and as indigenous vegetables in others. Such is the case of *Solanum* species, related to the black nightshades, the subject of this study.
Black nightshades are widely distributed in various habitats throughout the world, from tropical to temperate regions and from sea level to altitude exceeding 3500m. Their wide tolerance to habitat types, ability to flower while still young and prolific seed production all contribute to their success as weeds (Jennifer, 1977). *S. nigrum* is an important source of germplasm for crop improvement where it provides resistant genes against various diseases such as Early blight (Olet *et al*., 2011).

Unfortunately, there is a wide confusion over precise identification of the taxa involved, especially in areas where the species are most commonly used as food resources (Grieve, 2012). In Africa, this confusion is probably aggravated by the use of vernacular names whereby one name can apply to several species, or several names to the same species. Biosystematic studies of *S. nigrum* is needed to understand the connection between wild and cultivated plants (Chweya, 1997). About 200 indigenous plant species are used as leafy vegetables in Kenya, of these only four are fully domesticated and more than fifteen are semi domesticated while majority are wild. *S. nigrum* is among the most widely used leafy vegetable in East Africa being used by Nilotes and Bantus (Edward and Charles, 1990).

Black nightshades are cosmopolitan weeds reported in 61 countries affecting 37 crops (Zachary, 2010). Several studies have been conducted to estimate the genetic diversity using DNA markers in several crops including black nightshades (Anas and Yoshinda, 2004; Wang *et al*. 2009; Mutegi *et al*., 2011; Sayed *et al*., 2012). SSR markers were
used since they are highly polymorphic and abundant in the genome hence a powerful tool for revealing interspecific and intraspecific phylogenetic relationships (Barkley et al., 2005). In Kenya, studies on diversity of *S. nigrum* using SSR markers have not been exhaustively done and therefore, this study opens an opportunity for assessing the effectiveness of these markers in diversity studies. Results from the study shows that *S. nigrum* species is a group of closely related plants as postulated by previous work done outside Kenya.

### 1.2 Problem statement and Justification

Since the description of *S. nigrum* by Linnaeus more than 30 names have been published referring to the same specie (Péter and Hyvönen, 2011). The great number of junior synonyms has resulted in large number of conflicting and confusing species definitions. Despite the fact that the group has recently been studied extensively its taxonomy is still unsettled and debated (Péter and Hyvönen, 2011).

Taxonomic complexity associated with *Solanum nigrum* has long been accepted (Mwai, 2007). These species display varying amounts of phenotypic variation particularly in their vegetative features such as plant habit, leaf size, form and stem winging. However, it is not clear if the phenotype reflect genetic diversity, which is critical for future breeding of the species.
The limited information available on important and basic aspects of neglected and underutilized crops in this family, such as black nightshade, hinders the development of these species as well as their sustainable conservation (Angeline et al., 2010). There has been limited utilization of germplasm derived from species belonging to the *S. nigrum* in research, breeding studies and crop improvements (Chweya, 1997). Currently, the demand for edible *S. nigrum* complexes is increasing but production remains relatively low (Onyango, 1993). *Solanum nigrum* types grown display different characteristics with varied farmer preferences.

There is need to develop a clear distinction between black nightshade type grown in Kenya. This study was done to assess diversity of black nightshades cultivated in Kenya to determine future breeding strategies. Knowledge obtained in this study could be employed in the development of markers linked to important agronomic traits that can be used in future breeding of consumer preferred types. The findings will further contribute to changing the taxonomic confusion within the *S. nigrum*.

**1.3 Hypotheses**

i. SSR markers cannot discriminate types of *S. nigrum* grown in Kenya.

ii. There are no observable morphological variations in *S. nigrum* grown in Kenya.
iii. Morphological characteristics do not correlate to genetic diversity in *S. nigrum*.

1.4 Objectives’

1.4.1 General objective

To assess the diversity of *S. nigrum* grown in Kenya using morphological and Simple Sequence Repeat (SSR) markers.

1.4.2 Specific objectives’

i. To determine genetic diversity of black nightshades (*S. nigrum* L) using SSR markers.

ii. To determine morphological variations existing within black nightshades.

iii. To deduce the correlation between morphological and genetic variations in black nightshades.
CHAPTER TWO
LITERATURE REVIEW

2.1 Solanum nigrum L

Long ago, societies exploited edible wild plants to provide an adequate level of nutrition (Angeline et al., 2010). Recent studies on agropastoral societies in Africa indicate that these plants play a significant role in nutrition, food security and income generation (Defelice and Michael, 2003). The nutritional composition of resources, although not well documented, could be comparable to superior introduced cultivators. It is, therefore, worth noting that incorporation or maintenance of edible wild and non-cultivated plant resources could be beneficial to nutritionally marginal population, or certain vulnerable groups within populations especially in Kenya (Nekesa and Meso, 1993). Majority of diagnostic characters which have been used by some authors’ to identify the species belonging to section Solanum, are extremely variable, with some species within the section being variable morphologically.

2.2 Taxonomy

Solanaceae, to which Solanum nigrum belongs, is a cosmopolitan family containing many essential vegetables and fruits such as potatoes, tomatoes, green and red peppers (Lim, 2013) as well as ornamental plants such as Petunia, Schizanthus and Lycium spp (Grieve, 2012). It is composed of approximately 90 genera and between 2000-3000 species. Mbagwu and Nwachukwu (2007) described 70 genera with 2000 known
species. The family Solanaceae has a worldwide distribution with centers of diversity occurring in Central, South America and Australia (Chweya, 1997). It’s made up of flowering plant in the genus Solanum (black nightshade), Capsicum (pepper), Physalis (chinese lantern), Lycium (kraal honey thorn), Nicotiana (tobacco) among others. Solanum is the largest genus with 1700-1750 species which include plants such as *S. nigrum*, *S. tuberosum*, *S. lycopersicon* and *S. dulcamara* (Terri and Lynn, 2007). *S.nigrum* is one of the largest and most variable species in the group. The species has developed several adaptations to environmental conditions, mainly through phenotypic plasticity and biomass production (Lana and Krystic, 2002). The taxonomy of black nightshades is unsettled because of inter and intra specific hybridization, phenotypic plasticity and polyploidy (Lana and Krystic, 2002).

2.3 Biology

There are conflicting reports on lack or presence of primary dormancy in freshly harvested seeds (Bithell et al., 2002). The function of seed dormancy is probably adapted to time of germination so that environmental risks associated with seed establishment are low. Seeds that emerge late in the season may die in autumn due to low temperatures before reproduction. Variation in seed dormancy is ecologically significant for natives resulting in contrasting ecotypes following many generations of selections (Allen and Meyer, 2002). Seed dispersal occurs in two forms whose evolution is closely linked - dispersal in space and time. Seasonal dormancy of seeds enables black
nightshades time germination to appropriate conditions of seedling establishment and reproduction. Soil moisture, nitrate level, light and desiccation are factors that do not influence dormancy.

Black nightshades are annual herbs growing to heights of up to 0.6 m. In temperate regions black nightshades are in flower from July to September and the seeds ripen in August to October (Taab, 2009). The flowers are hermaphrodite and pollinated by insects. Fruit is about 9mm in diameter, some variations occur in colour of fruits with 66% of nightshades having black fruits while 34% produce pomes that never turn black (Zachary, 2010). Seedlings have cotyledons that are covered with short hairs along the margins. Roots are fibrous and shallow. Nightshades have weak, unarmed, smooth, usually hairless stems that branch widely and freely (Jennifer and James, 1977). Large species stand 90 cm and span 20 cm. Leaves are alternate, dark green, soft rather thin and often riddled with big holes like those of amaranth, which they sometimes resemble. The young leaves may have a coppery or purplish sheen on the underside. Size of leaves is quite variable and shape varies from ovate to lanceolate to diamond shaped (Schmidt et al., 2004).

Petioles are 1.2-2.5 inches long usually with a faint wing on each side. The inconspicuous five petaled flowers are whitish and about half inch across (Poczai and Hyvonen, 2011). Fruit is almost perfectly spherical, about the size of a blueberry, green at first but turn purplish black when ripe. They are subtended by a persistent five parted
calyx that is slightly smaller in diameter than the fruit (Rudy, 1998). Skins are somewhat tough, like tomato skin and encapsulate a soft juicy interior with numerous seeds.

Some nightshades have serrated leaf margins while others have entire margins. A great difference is also observed in emergence patterns between hairy nightshades and eastern nightshades (Lexa et al., 2015). Hairy nightshades appear to be later emerging species than eastern black night shades. Eastern black nightshades require 4 to 5 weeks from flowering up to seed production (Assia et al., 2007). Seeds become viable 1 to 2 weeks before berries begin to turn black. Hairy nightshades are highly affected by frost which prevents seed production (Assia et al., 2007).

Species belonging to the section constitute polyploidy genes, with diploid (2n = 2 x = 24), tetraploid (2n = 4 x = 48) and hexaploid (2n = 6 x = 72) species occurring throughout most geographical range. Octaploid plants 2n = 8 x = 96 have also been reported (Jennifer and James, 1977).

Natural hybridization has also been observed. Species belonging to the section are naturally self-pollinating, however, out and cross-breeding can occur, and natural inter and intra specific hybridizations, especially among the smallest-flowered diploids have been reported at higher polyploidy levels (Kantety and Rota, 2002).
2.4 Distribution and origin

Black nightshade is a common weed found in all inhabited continents. It has long and well-established history as food source for numerous cultures around the globe. In fact, it is among most widely used and well documented wild foods in the world, rivaled in this respect by weeds such as Lamb’s quarters, amaranth and stinging nettle (Schmidt et al., 2004). Black nightshades (S. nigrum) are worldwide weeds in arable land, garden (Grieve, 1995), rubbish pits, soil rich in nitrogen, in moderately light and warm situation. It is extensively cultivated in Cameroon and exported to Nigeria and Gabon (Mkabwa et al., 2008).

*S. nigrum* is native to Eurasia and was introduced in America, Australia and Africa (Mohy-Ud-Dint and Ahmad, 2010). The plant prefers light sandy, medium loamy and heavy clay soils and requires well-drained soil. The plant prefers acid to neutral and alkaline soils. *S. nigrum* occurs in wide range of habitats from sea levels up to 3500m above sea level (Chweya, 1997; Olet, 2004). Tolerance to drier conditions and habitats has been noted. It is found in habitats such as roadsides, often on arable land especially edges of cultivated fields and plantations, riverbanks and gullies. They also occur in disturbed habitats such as hedges of cultivated fields, railway cuttings, quarry sides, under trees, on forest, grassland margins, shingle beaches (Allen and Meyer, 2002). Nightshades grow in fertile soils, especially those high in nitrogen and phosphorus. Most species are frost susceptible dying with the onset of first frost in temperate regions (Nzioka, 1994).
Some of the major species within Solanum complex are: *Solanum americanum* – Commonly found in warm humid areas, particularly coastlands. *Solanum eldorettii*– also known as *Solanum eldoretianum* is a thin species with course leaves. It is restricted to Kenya highlands and Northern Tanzania and frequently grown by farmers in Kisii District and highland of Rift Valley Province of Kenya. However, it not popular with farmers due to the less acceptable taste. *S. nigrum* is commonly found in the tropics (Chweya, 1997).

*Solanum retroflexum* – found in horn and Southern Africa. It’s the main species grown by farmers in Limpopo province of S. Africa (Jansen et al., 2007). *Solanum scabrum* – largest species growing to 1.5 m with large fruits and leaves. Has large black to purple fruits and large leaves. The species was introduced to East and Southern Africa from humid West Africa. *Solanum villosum* – orange berried nightshade. It is distinguished from the rest by its orange to yellow berries which are edible. Common in dry areas and best known in East Africa, where it is picked from wild and cultivated types (Werne and Petra, 2010).

### 2.5 Economic importance

Vegetables play an important role in diet of many households in Kenya both urban and rural areas. Indigenous vegetables are more preferred because they are readily available
in rural areas and have good taste (Nzioka, 1994). Some indigenous vegetables are harvested from wild and those that grow as weeds in farms.

Black nightshades are cultivated as food crop, both for its leaves and fruits. According to Werner and Petra, (2010), fruits are used in preserves, jams and pies. Fruits have a pleasant musky taste (Hammington, 1974), which improves lightly after a frost.

Unripe fruit contains toxin solanine (Timothy and Kokwaro 1991). Fruit contains 2.5% protein, 0.6% fat, 5.6% CHO, 1.2% ash, minerals (iron and phosphorous) and vitamins B. S. nigrum is nutritious and rich in β-carotene (Dhellot et al., 2006). Seeds are rich in minerals such as iron, calcium and proteins especially methionine, an essential amino acid (Onyango, 1993). It’s also rich in vitamin A and C. Young leaves and new shoots, raw or cooked as a potherb are added to soups (Reid, 1997). The level of ascorbic acid depends on the method of cooking used. Increase in the cooking time and volume of water used lowers the ascorbic acid content. Boiling for more than 20 minutes can lead to up to 75-89% loss. However leaves boiled with six times the volume of water for 15mins result in 70% loss (Jagatheeswari et al., 2013).

Oxalates indicate presence of oxalic acid in plant material. When ingested by humans the acid combines with calcium to form an insoluble salt which the body cannot absorb. This renders calcium unavailable to the body. Black nightshade berries contain steroidal alkaloid glycoside, solanine, alpha and beta-solanigrine, alpha and beta-solamargine
(Mohy-Ud-Dint and Ahmad, 2010). They also contain the steroidal sapogenins, dysgenic and tigogenin, solasodine - hence its therapeutic properties (Defelice and Michael, 2003). Highest concentrations of toxins are in immature fruits hence only ripe fruits should be eaten,

The whole plant is antiperiodic, diaphoretic, diuretic, emollient, febrifuge, narcotic, purgative and sedative (Anderson, 2006). Extracts of the plant are analgesic, antispasmodic (Mohy-Ud-Dint and Ahmad, 2010), anti-inflammatory and vasodilator (Duke and Ayensu, 1985). The plant has been used in manufacture of analgesic, ointments and juice from the fruit has been used as analgesic for toothaches, treatment of dropsy (Keterere and Dibungi, 2010), digestive disorders (Ignacimuthu and Sankara, 2006), such as flatulence, colitis and peptic ulcers (Chiej 2010). Extracts are also used in treatment of asthma, removing phlegm from the bronchial tubes in asthmatics. Extracts from leaves are used in treatment of tumors especially liver cancer (Son et al., 2003), lung cancer, bladder and gastric carcinoma (Mueller et al., 2005; Ashwani and sagwal, 2012). Methanol extracts of *S. nigrum* have shown significant antioxidant activity in various assays including DPPH radical scavenging activity (Ramya et al., 2011; Ashwani and Sagwal, 2012)

Among the British herbals nightshade is used for infirmities that need cooling and binding. It is good against ‘St Anthonies fire’ the shingles, panic head and heart burning. It’s referred to as ‘cold saturine plant’ used to cool hot inflammation either internal or
external. It is known to cool throat, eye inflammation, running ulcers, testicular swellings, gout and ear pain (Ramya et al., 2011).

In India, an infusion of juice is used as an enema for infants with abdominal upset. It’s also used for treatment of anthrax pustules when applied locally. An alcoholic preparation of leaves is active against *Staphylococcus eurens* and *Escherichia coli*. In china, plant extract are known to reduce blood pressure (Grieve, 2012). Seed are reportedly used to treat gonorrhea and dysuria. Immature fruits contain steroidal glycoside which has considerable cancer activity (Jagatheeswari et al., 2013). These fruits are applied for toothache and squeezed on babies gum to ease pain during teething.

Both leaves and berries are used as a source of dyes used to colour sisal baskets (Nzioka, 1994) Purple berries of both *S. scabrum* and *S. americanum* are used a source of ink. Saponins and glycoalkaloids have larvicidal properties (Silva et al., 2005; Chowdhury et al., 2008), hence effectively used as pesticides. Extracts from black nightshades have been used to control mollusks (Shoeb et al., 1990; Chowdhury et al., 2008).

The species has been found to be effective in removing polychlorinated biphenyls from the soil and detoxifying them (Duke and Ayensu, 1985), especially when infected with bacterial parasite *Agrobacterium tumefaciens* (Anderson, 2006).
Solanum species are found on sale as a vegetable in both rural and urban market, hence a source of income for rural farmers who are mostly women (Nzioka, 1994). They are found on sale in markets in Cameroon, Ghana, Kenya, Madagascar and Nigeria. Zulu women are known to take baskets full of berries to sell in nearby villages and towns (Sergel and Irina 2009, Jagatheeswari et al., 2013), hence generating income for family use.

When nightshades are harvested with beans, juice from ruptured berries stain the grains and cause soil particles to cling on the beans. This reduces quality and economic returns of the crop (Rogger et al., 1981). Berries are similar to soybeans, peas and others seeds making them difficult to separate by sieving. Seed staining and berry contamination has resulted in severe dockage of many crops and rejection by processors of beans (Vicia faba) and pea (Pisum sativa). Seed companies and foundations reject soybean seeds that have been strained by juice or berries of black nightshades (Bithell et al., 2002). Stems, leaves and berries also form a wet and sticky mass in harvesting equipments plugging rotors and screens and thereby slowing or stopping harvesting.

Juice increases moisture in the stored grains thereby increasing mould problem. Livestock producers are concerned about poisoning of nightshades in pastures and berry juice on sheep wool (Keterere and Dibungi, 2010). Species related to black nightshades are notorious weeds of agriculture in most parts of the world. They mainly affect barley, wheat, melon, onion, pea, bean, potato, sorghum, tea, tomato sugarcane and several
vegetable (Nzioka, 1994). They compete for moisture, light and nutrients with these crops hence lowering the quantity of yields, and in the long run reduce income.

2.6 Constraints to production

During rainy season in West Africa, a viral disease, yellow rain clearing may attack the plants (Onyango, 1993). Black nightshades seem to be susceptible to bacterial wilt and fungi caused by *Ralstonia solanacearum* and *Cladosporium oxysporum* respectively. In some areas, late blight *Phytophthora infestans* has been observed (Philips et al., 2001). Main pests are red spider mites (*Tetranychus evansii*), black thrips (*Aphis fabae*), flea beetles (*Epilachria hirta*), cutworms and nematodes. Farmers also lack certified seeds and they therefore, use seeds from old crops (Maundu et al., 2004). There has been very little utilization of germplasm derived from species belonging to the *Solanum* group in research, crop improvements and breeding studies. No collection expeditions have been organized yet, largely because the plants used as vegetables are regarded as weeds or volunteers crops and have not been fully domesticated (Chweya, 1997).

2.7 Genetic diversity of Solanum nigrum

There are two hexaploid spp. of *S. nigrum* section present in Africa- *S. scabrum* and *S. nigrum* (Mkabwa et al., 2008). Present studies apply use of AFLP to study genetic diversity in *S scabrum*. This species group is often referred to as the *S. nigrum* complex. There are a number of morphogenetically distinct taxa, which show their greatest
diversity and concentration in the new world tropics particularly in South America (Poczai and Hyvonen, 2011). Taxonomic complexity inherent on the species has been caused by:

(i) Historical aspects where species belonging to this section have been subjected to extensive economic study and species related to S. nigrum reclassified innumerable times. Boundaries between many of the species are still ill defined with many of the new taxa proving to be more than slight morphological variants of those already described (Jennifer and James, 1977).

(ii) Phenotypic plasticity- S. nigrum specie display varying amounts of phenotypic variation, in features such as plant habit, leaf size and form, and stem winging. In addition senescence is often accompanied by smaller and fewer flowers and fruits than usual, while gene for anthocyanin pigmentation in flowers seem to be independent on light intensity and temperature for its expression. In some species, it is therefore difficult to define limits within which such features are genetically fixed (Chweya, 1997).

Genetic diversity studies have been done on African hexaploid species S. scabrum Mill and S. nigrum L using AFLP (Manoko et al., 2008). Studies have also been carried out on S. nigrum collections in the Gatersleben Genebank by Stracke et al., (2008). RAPD were used by Poczai et al. (2010) to determine genetic diversity of 12 accessions
belonging to section Solanum. SSR markers have been used in the recent times for most of the genetic diversity studies in African leafy vegetables (Angeline et al., 2010).

2.8 Use of Markers to Determine Diversity

Diversity amongst individuals or populations can be determined using morphological, biochemical and molecular markers (Datema et al., 2008). Morphological markers also known as visual markers are often detectable by eyes (simple visual inspection). They are phenotypic characters such as presence or absence of awn, leaf sheath, colouration, height, grain colour, aroma, leaf shape, fruit colour etc. Morphological and seed traits have long been used for studying taxonomy and species of S. nigrum. Morphological markers are associated with several general deficits that reduce their usefulness (Semagn et al., 2006). They are influenced by environmental factors, the developmental stage of a plant, deleterious effects, rare polymorphism, pleiotrophic and dominance.

Morphological characterization has been used for various purposes including identification of duplicates, studies of genetic diversity patterns and correlation with characteristics of agronomic importance (Karuri et al., 2010). Biochemical markers include secondary metabolites in plants, micro molecules, viz proteins and DNA. Analysis of secondary metabolites is restricted to plants that produce suitable range of metabolites which can easily be analyzed (Jonah et al., 2011). Metabolites used as markers should be neutral to environmental effects or management practices (Guterreiz et al., 2014).
In contrast, molecular markers based on DNA sequence polymorphism, are independent of environmental conditions and show higher levels of polymorphism (Mueller et al., 2005). They exhibit simple inheritance, are dominant throughout the genome, easy and fast to detect, exhibit minimum pleiotrophic effects, and detection is not based on developmental stage of the organism (Guterreiz et al., 2014). The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics (Semagn et al., 2006). Since the markers and the genes they mark are close together on the same chromosome, they tend to stay together as each generation of plants is produced (Malik et al., 2009).

A genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species (Ramachandran et al., 1997). It can be described as a variation which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, surrounding a single base-pair change (SNP) or a long one like minisatellite (Furton, 2004). Molecular markers offer numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development or defense status of the cell, not confounded by the environment, pleiotrophic and epistatic effects. According to Doyle and Doyle (1990), an ideal molecular marker technique should have the following criteria: be polymorphic
and evenly distributed throughout the genome, provide adequate resolution of genetic differences, generate multiple independent and reliable markers that have linkage to distinct phenotype and requires small amounts of tissue DNA samples.

Molecular genetic markers are valuable regions of DNA that provides genetic tools in the genetic linkage mapping (Varshey et al., 2004), association studies, for estimation of several population genetic parameters such as diversity, genes flow and inbreeding (Morganite et al., 1993). They are classified as PCR based and Non PCR (Kumar et al., 2009). Molecular markers have great advantages over the conventional breeding techniques which takes long. Majority of molecular markers used nowadays are microsatellite-like markers (Simple Tandem Repeats), Amplified Fragment Length Polymorphism (AFLP), Variable Number Tandem Repeats (VNTRs), Random Amplified Polymorphic DNA (RAPD), Single Strand Conformation Polymorphism (SSCP) and Restriction Fragment Length Polymorphism (RFLP) (Mohan and Suresh, 1997). Several DNA markers have been used to study genetic variation in crops. These markers differ mainly in their principles and yield varying amounts of data.

### 2.8.1 Restriction Fragment Length Polymorphism (RFLP)

These markers are most widely used hybridization based molecular markers. They were first used in 1975 to identify DNA sequence polymorphism for genetic mapping of adeno-virus serotypes (Lian et al., 2006). The technique is based on restriction enzyme that reveal patterns of difference between DNA fragment size of individual organisms.
RFLP involves fragmenting a sample of DNA by restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs. They measure variation at the level of DNA sequence, however, it requires large amount of DNA. Several types of DNA markers, developed to study genetic diversity and crop evolution are now considered better for diversity studies (Homar et al., 2011).

2.8.2 Amplified Fragment Length Polymorphism (AFLP)

This is a polymerase chain reaction-based technique for DNA fingerprinting, that has been widely used for studying genetic relationships among cultivars of different species or genera (Lexa et al., 2015). The technique is based on detection of genomic restriction fragment by PCR amplification and can be used for DNA of any origin or complexity (Jacob et al., 2003). AFLP procedure involves three steps: restriction of the DNA using common cutting restriction enzyme, selection amplification of the restriction fragments using designed primers and gel analysis of amplified fragments (Jonah et al., 2011). AFLP have been used in diversity studies of S. nigrum L. complex by Dehmer and Hammer, (2004). The results led to four major clusters uncovering significant difference in the levels of genetic diversity within and between species studied.

2.8.3 Random Amplified Polymorphic DNA (RAPD)

RAPDs detect nucleotide sequence polymorphism in DNA by using a single primer of arbitrary nucleotide sequence (oligonucleotide primer) mostly ten base pairs (William et
In the reaction a single species of primers anneals to the genomic DNA at different sites on the complementary strand. Advantages of RAPD markers include: use of small amount of DNA, non involvement with radioactive assays, non involvement in blotting or hybridization and being reproducible in different cases if all condition are adhered to (Crouch and Ortiz, 2004). However, limitations associated with them include: suffering from problems of repeatability in many systems, primers are relatively short, a mismatch or even a single nucleotide can often prevent a primer from annealing, its polymorphism are inherited as dominant or recessive characters causing loss of information relating to markers which show co-dominance (Crouch and Ortiz, 2004).

2.8.4 Randomly Amplified Microsatellite Polymorphism (RAMP)

This is a micro satellite based marker that shows a high degree of allelic polymorphism but they are labour intensive (Agarwal et al., 2008). The technique involves radiolabelled primers consisting of a 5' anchor and a 3' repeats which is used to amplify genomic DNA in presence or absence of RAPD.

New marker type SNPs is now on scene and has gained high popularity (Kumar et al., 2009). They prove to be universal as well as the most abundant forms of genetic variation among individuals of the same species (Mammadow et al., 2012). Although SNPs are less polymorphic than SSR markers because of their biallelic nature, they easily compensate their drawbacks by being abundant, ubiquitous and amendable to high and ultra-high thought out automations. However, complex genomes pose serious
obstacles for researchers interested in developing SNPs. This occurs due to high repetitive nature of the plant genome (Martin and Roger, 2007). SNPs are identified through the comparative sequencing of individual lines (Rajeev et al., 2007). They can be identified in basically unlimited number of single copy DNA.

2.8.5 Simple Sequence Repeats (SSR) Markers

These are ideal genetic markers for detecting differences between and within the species of genus of all eukaryotes (Farooq and Azam, 2002). They are short stretches of tandem repeat simple DNA sequence consisting of 2-7 base pair units arranged in the repeats of mono-, di-, tri-, tetra- and penta-nucleotides (A,T,AT,G,A,AGG,AAAG) with different length of repeat motifs (Zhao, 1993). The variations in the number of tandem repeat units result in highly polymorphic banding patterns (Farooq and Azam, 2002; Kantety and Rota, 2002), which are detected by PCR using specific flanking regions primers where they are known. Microsatellites can be used efficiently by different researchers to produce consistent data (Joshi et al., 2011).

SSR markers will be used to amplify the DNA due to their numerous advantages (Wang et al., 2009). Microsatellites have potential to affect all aspects of genetic information, including gene regulation, development and evolution (Koshi and King, 2006; Lawson and Zhang, 2006). Molecular markers have contributed to a greater genetic knowledge
of many plants. In addition, these markers have been used in *Solanum* genus for analysis of biodiversity and polygenetic studies (Sue *et al*., 1997).

Microsatellite loci have been isolated from partially digested genomic libraries of small insert size and thousand clones need to be screened through hybridization using repetitive DNA probes (Panaud *et al*., 1995). High number of alleles per locus in SSR causes some bias in diversity estimates due to increased heterozygosity level. High mutation rates also mean that microsatellites suffer from homoplasy problems. Marker-Assisted selection has been used in molecular characterization of many crops using different molecular markers (Mohan and Suresh, 1997). SSR markers are expensive to generate and can be laborious, many researchers have attempted to use SSR primers developed from one species for studies on related species (Matsuoka *et al*., 2002; Wang *et al*., 2003), as in the case of this study.

Microsatellites have highly mutable loci which may be present at many sites of the genome, hence highly polymorphic (Jones *et al*., 1997). Simple sequence repeats (Gous *et al*., 2013), are an important tools for genetic identification of germplasm (Ma *et al*., 2011). They provide an efficient tool for efficient selection of desired agronomic traits (Benor *et al*., 2008), because they are based on plant genotype and thus independent of environmental variation. SSR markers have some merits such as quickness, simplicity, rich polymorphism and stability (Andrew *et al*., 2004; Abdul *et al*., 2012), thus being widely applied in genetic diversity analysis, molecular map construction and gene map
construction of fingerprints (Xiao et al., 2006), genetic purity test analysis of germplasm diversity (Jin et al., 2010). They are more informative and variable than RFLP, RADP and AFLP (Sheej et al., 2014).

2.8.6 SSR principles

Simple Sequence Repeats can be amplified for identification by PCR using unique sequence of flanking regions as primers. The primers are designed to bind to these flanking regions, providing alleles of different lengths that can be resolved in an electrophoresis gel (Selkoe and Toone, 2006). These primers are generally 18-24 bp long and target highly conserved region in the genome (Kim et al., 2008). SSR detect variation in the number of short repeat sequences, usually two or three base pairs. The number of such repeat units has been found to change at high frequency and allow the detection of multiple alleles (Farooq and Azam, 2002).

2.8.7 Application of SSR markers

Microsatellites have been used in genome studies for genetic mapping (Andrew et al., 2004) comparative mapping, physical mapping and association mapping. Genetic mapping with marker selection was first reported in tropical trees and then soybeans (Zane et al., 2002). So far there are over 80 genetic maps constructed involving the use of SSR markers from many plants. Comparative mapping is the alignment of chromosomes or chromosomal fragments of related species based on genetic mapping of
common DNA markers. Comparative mapping has been successfully conducted in Solanaceae family (Zane et al., 2002), legumes (Zhu et al., 2005) among others. EST-SSR have been used in comparative studies of wheat, barley, rice and rye (Yu et al., 2004). SSR have also been used for physical mapping as anchor markers for joining large species of overlapped DNA fragments such as Bacterial Artificial Clones (BACs). They are used to construct a whole genome physical map of model crop species. Physical maps are useful for helping assemble genome DNA sequence and for positional cloning. Association mapping is significant in mapping molecular markers with phenotypic traits (Gupta et al., 2003). SSR marker assisted selection can also greatly enhance the efficiency of plant breeding programs (Masneuf-Pomarede et al., 2007) by either flanking SSR markers or Target gene SSR markers. This improves cultivar identification through Marker Assisted Selection (MAS).

Moreover SSR markers are used to determine population structure within and among natural population and identify parental progenitors (Douglas and Pamela, 2003). Since SSR markers are highly polymorphic, abundant genome wide and easy to use they have become the markers of choice for population genetics and evolutionary studies (Zane et al., 2002). Biomedical diagnosis of some disease conditions have been done using SSR, due to their association with certain mutants in coding region of DNA that can cause a variety of medical disorders (Gupta et al., 2003).
DNA testing in forensic studies involves use of SSRs, match identification for microsatellite can be very high hence high probability of providing evidence from crime scenes (Stagel et al., 2008). SSR markers are a powerful tool for revealing interspecific and intraspecific phylogenetic relationships (Barkley et al., 2005). Recently SSR markers have been developed for eggplant (Solanum melongena) and genetic diversity of these plants assessed (Stagel et al., 2008).

Primers that have been designed for closely related species may be used. Slipped strand mis-pairing is considered the main source of variation in the number of repeat units of a microsatellite. This results in length polymorphism that can be detected by gel electrophoresis (Matsuoka et al., 2002).
CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection sites

Four sites within Kenya were selected for this study; Matanya in Nanyuki sub county, Mauche in Njoro sub county, Kipkaren in Kapsabet sub county and Makuyu in Muranga South sub county (Figure 3.1).

3.1.1 Site selection

The following considerations influenced selection of the sites; (i) Agro-ecology; these areas fall under different agro-ecological zones. Mauche and Kipkaren occur in Agro-ecological zone 3, restricted to elevations of 900-1800 meters above sea level with rainfall between 950 and 1500mm per annum. Makuyu and Matanya occur in Agro-ecological zone 4 with same elevation as zone 3 but the rainfall is lower about 500-1000mm per annum (www.infonet-biovision.org-AEZs- the Kenya system. Environmental health).(ii) Social-cultural; there are communities with unique cultures and use black nightshades as traditional vegetables (Onyango 1993).

Mauche is inhabited by the Ndorobo community who are mainly hunters and gatherers, who depended on wild fruits and meat for food. However, with clearing of large part of Mau forest this community shifted from hunting and gathering to subsistence farming. Kipkaren hosts the Nandi people who are successful animal keepers. Use of traditional vegetables such as black nightshade dates back to 16th century as an accompaniment to
Ugali and Mursik (fermented milk) which was their main food. Matanya is inhabited by mainly pastoralist (Samburu) and Farmers (Kikuyu). While pastoralists depend on livestock the farmers grow food crop which include black nightshades for subsistence and sale. Makuyu is predominantly a Kikuyu region who practice crop farming commercially and for home use (www.kenya information guide, 2015). (iii) Consumption of black nightshade has greatly increased over time and the crop is now grown commercially (Maundu et al., 2004).

### 3.1.2 Sampling criteria

Stratified random sampling was done based on prior information on species grown and sampling partnership with farmers. Thorough consultations were done with area Agricultural officers before collection of materials. Sampling was done in a 1km radius in each location. Five farms where black nightshades were growing were selected at random ensuring no repetition occurred. The selected farms were small with sizes ranging between 0.5ha and 2ha and production of black nightshades done at a small scale. Six plants were randomly selected in each farm and one mature stem collected, hence a total of six stems per farm. Each of the collected stems had mature leaves and fruits. A total of 120 mature plant samples were collected from the four selected sites (each 30 plants). These materials were labeled based on the area of collection Makuyu (M), Matanya (N), Mauche (R) and Kipkaren (K).
Figure 3.1: Map showing four counties in Kenya where the *S. nigrum* samples were collected. (Source [www.d-map.org](http://www.d-map.org))
3.2 Morphological characterization

Collected sample were taken to Plant Transformation Laboratory in Kenyatta University where basic morphological characteristics were visually assessed using criterion described by Jennifer and James, (1977) and Nandhini et al., (2014) as shown in Table 3.1. Each of the traits was assessed, tabulated and the means calculated.

Table 3.1: Assessed morphological characteristics in S. nigrum populations.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Nature of leaf surface</td>
<td>Hairy, smooth</td>
</tr>
<tr>
<td>2 Nature of leaf margin</td>
<td>Smooth, Sinuate</td>
</tr>
<tr>
<td>3 Fruit color</td>
<td>Purple, red</td>
</tr>
<tr>
<td>4 Leaf shape</td>
<td>Ovate, lanceolate, Diamond</td>
</tr>
</tbody>
</table>

3.3 DNA extraction

Fifty milligrams of leaf material from each individual collected was dried in snap-top bag containing silica gel before extraction of the DNA using CTAB protocol (Sue et al., 1997). Leaves were crushed using pestle in liquid nitrogen, 9.0ml of warm CTAB buffer (65°C) was added and mixed gently by inversion, and the centrifuge tubes were incubated at 60°C for 60min with continuous rocking in a water bath. Tubes were removed from the water bath and 4.5ml of chloroform/isoamyl mix (24:1) added followed by spinning in a centrifuge at 1500 revolutions per minute for 10min at room temperature. The supernatant was removed and a repeat chloroform extraction done. The top aqueous layer was pipetted into new 15ml tubes and 6.0ml of isopropanol added and then mixed gently by inversion. The samples were cooled overnight at 4°C followed by
spinning at 1500 revolutions per minute for 10min and supernatant discarded. 1ml of sterile distilled water and 50µl sodium acetate was added. DNA was precipitated with 100% and 70% ethanol in succession. DNA was spooled out, air dried for 2hrs and resuspended in 200µl of sdH2O before storage at -20°C. Final DNA concentration was estimated through electrophoresis in 1% agarose at 80V for 30min in TAE against a known standard of 1kb.

3.4 SSR marker analysis

DNA was used to screen for highly polymorphic primers previously used in Solanum species. PCR was carried out with 7 SSR primers as used by Angeline et al. (2010) for Solanaceae and cross species (Table 3.2). DNA amplification was performed on total reaction volume of 25µl containing; 5µl 10x PCR buffer[20mM Tris-HCL (pH 8), 50mM KCL], 17.875 µl ddH2O, 1 µl (10pMole) of primer set, 0.125 µl Taq polymerase and 50ng genomic DNA template. Amplifications were performed using thermocycler with following conditions; 5 minutes denaturing steps at 94°C, followed by 35 cycles of 1min at 95°C, 45°C (Annealing temperature) for 2min and 72°C for 2min. In the final PCR cycle the extension time at 72°C was increased to 10min. The optimum conditions were different for various primer pairs with annealing temperature of 45°C and 50°C for the seven primer sets.
Table 3.2: Primers used in the study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' 3'</th>
<th>Tm (°C)</th>
<th>Repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB4-32</td>
<td>fw-ctcggcggttagacagtcacrv-gcccatagcacagacacacacagccc</td>
<td>59</td>
<td>(AG)$_{15}$</td>
</tr>
<tr>
<td></td>
<td>fw-gcatatgacgctgctrv-cttccaaagtaaagacacacatca</td>
<td>60</td>
<td>(AG)$_{19}$</td>
</tr>
<tr>
<td>SB6-57</td>
<td>fw-acagggctttaggaatctrv-ccatcaccctggtcacctct</td>
<td>60</td>
<td>(AG)$_{18}$</td>
</tr>
<tr>
<td>SB6-84</td>
<td>fw-cgctctegggatgaatgarv-taacgggacactacaaatgatt</td>
<td>58</td>
<td>(AG)$_{14}$</td>
</tr>
<tr>
<td>TMS29</td>
<td>fw-ccttgcagttgagttgaattrv-tcaagctacacactacatactca</td>
<td>55</td>
<td>(CT)$<em>{3}$ (C)$</em>{14}$</td>
</tr>
<tr>
<td>TMS37</td>
<td>fw-ccttgcagttgagttgaattrv-tcaagctacacactacatactca</td>
<td>55</td>
<td>(GA)$<em>{21}$ (TA)$</em>{20}$</td>
</tr>
<tr>
<td>STWIN 12G</td>
<td>fw-tgtygattgttggtgataarv-tgtggagcgtgactgtga</td>
<td>46</td>
<td>(TGAAA)$_{2}$</td>
</tr>
</tbody>
</table>

After PCR amplification 5 µl aliquots of the product were mixed with gel loading dye and syber green stain and loaded on 2% agarose (Bioline) for separation by electrophoresis.

Electrophoresis was done for 1.5 hours at a constant voltage of 100volts in 0.5x TAE [0.438g/L Tris (pH 8), 0.11mL/L acetic acid and 0.029g/L EDTA]. A 1-kb ladder (Bioline) was used as a molecular marker to estimate fragment size. Finally polymorphic bands were visualized under UV trans-illuminator and photographed with digital camera. Each band was scored by comparison with the 1-Kb size ladder. The profiles were scored separately for each primer set used in such a way that each band at a defined migration position in the gel was a character and was coded as present ‘1’ or absent ‘0’ as used by Heather and Matthew (2012).
3.5 Data analysis

Data for *S. nigrum* traits assessed were log transformed to meet the assumptions of ANOVA. Tukey’s Honestly Significant Difference (HSD) test was then performed to calculate the HSD and to establish significant differences between the groups. All P-values ≤ 0.05 were considered statistically significant.

Band scores of the SSR profiles were used to calculate the genetic relatedness between samples and among populations. Data was entered into a matrix as discrete variables and subjected to analysis using Power Marker (ver 3.0) to determine Major allele frequency, Genetic diversity and Polymorphic information content (PIC) indices. The matrix was also exported to GenAIEx version 6.41 (Peakall and Smouse, 2010) to determine the banding patterns and Principle coordinates. POPGENE ver. 1.13 (Francis and Rong-Cai, 1999) was used to reveal percentage polymorphic loci in the samples. Genetic relationship was shown based on Nei’s genetic distances (Nei, 1987). Dissimilarity analysis was conducted using Unweighted Neighbor Joining with 1000 bootstrap and presented in dendogram.

3.6 Correlation of morphological and genetic data

Each of the morphological characters (Table 3.1) was separately correlated with genetic profile of each plant sample. Gel scores of SSR profiles for each sample were used to determine the Principle coordinates and compare the relationship between morphological characteristics and genetic diversity.
CHAPTER FOUR

RESULTS

4.1 Morphological characteristics

In each of the five farms A, B, C, D and E plants displayed a range of berry colours with some having purple while others had red berries. All unripe berries were green colour (Figure 4.1). Leaf margins varied from entire to sinuate in black nightshade samples assessed. Leaf shapes varied from ovate to lanceolate to diamond, while the leaf surface was either hairy or smooth (Figure 4.2)

According to Tukeys (HSD) test there was a high significant difference in the means of characteristics observed in S. nigrum samples in the four regions. The P-value ranged from <0.001 to 0.009 at α 0.05 (Table 4.2). Variations on the leaf surface, leaf margin and fruit colour were highly significant. However, while ovate and lanceolate leaf shapes exhibited significant variation diamond leaf shape did not reveal any significant variation among the populations assessed.
Figure 4.1: Mature fruits of *S. nigrum* samples collected from the three regions. (a) Large purple fruits (b) unripe fruits green in colour (c) Small red ripe fruits (d) Small purple ripe fruits
Figure 4.2: Mature leaves of *S. nigrum* sampled collected, (a) sinuate margin (b) lanceolate with entire margin (c) ovate with entire margin

Table 4.1: Mean number of *S. nigrum* with observed characteristics.

<table>
<thead>
<tr>
<th>Morphological Characters</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>Location</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kipkaren</td>
<td>Makuyu</td>
<td>Matanya</td>
<td>Mauche</td>
<td></td>
</tr>
<tr>
<td>Lsurface Smooth</td>
<td>4.8±0.2</td>
<td>5.2±0.4</td>
<td>4.2±0.4</td>
<td>6.0±0.0</td>
<td>0.009</td>
</tr>
<tr>
<td>Hairy</td>
<td>1.2±0.2</td>
<td>0.8±0.4</td>
<td>1.8±0.5</td>
<td>0.0±0.0</td>
<td>0.009</td>
</tr>
<tr>
<td>Lmargin Entire</td>
<td>4.4±0.2</td>
<td>4.8±0.2</td>
<td>3.2±0.2</td>
<td>4.4±0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Serrate</td>
<td>1.6±0.2</td>
<td>1.2±0.2</td>
<td>2.8±0.2</td>
<td>1.6±0.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Lshape Ovate</td>
<td>5.0±0.3</td>
<td>5.0±0.0</td>
<td>6.0±0.0</td>
<td>4.4±0.4</td>
<td>0.004</td>
</tr>
<tr>
<td>Lanceolate</td>
<td>1.0±0.3</td>
<td>1.0±0.0</td>
<td>0.0±0.0</td>
<td>1.2±0.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Diamond</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.4±0.2</td>
<td>0.083</td>
</tr>
<tr>
<td>Fcolour Red</td>
<td>6.0±0.0</td>
<td>4.4±0.2</td>
<td>1.4±0.2</td>
<td>3.6±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Psmall</td>
<td>0.0±0.0</td>
<td>0.4±0.2</td>
<td>4.6±0.2</td>
<td>2.4±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plarge</td>
<td>0.0±0.0</td>
<td>1.2±0.4</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Data of morphological traits assessed in the four regions (Mean ± SE). Mean values followed by the same small letter(s) within the same row do not differ significantly from one another (One-way ANOVA, Tukeys studentized range (HSD)-test, α= 0.05). L-leaf, F-fruit
4.2 Genetic characterization

After the 7 primers were screened, primer sets SB4-32, and TMS-29 showed amplification with annealing temperature of 45°C. When the annealing temperature was raised to 50°C, Primers SB6 - 84 and SB6 - 57 resulted in good amplification. Only the 4 primer sets that showed amplification among the tested samples were used on all the genomic DNA samples.

PCR carried out using the 4 primer pairs: Sb4-32, Sb6-57, Sb6-84 and Tms-29 gave a total of 63 alleles. Fragment sizes ranged from 50bp to 3000bp, primer Tms-29 was most polymorphic resulting in 17 alleles, whereas primer Sb6-57 yielded 14 alleles (Table 4.2; Figure 4.3).

Table 4. 2: SSR markers used, number of allele and annealing temperature

<table>
<thead>
<tr>
<th>Marker</th>
<th>Allele No.</th>
<th>Annealing temp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sb4 32</td>
<td>16</td>
<td>45</td>
</tr>
<tr>
<td>Sb6 57</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Sb6 84</td>
<td>16</td>
<td>50</td>
</tr>
<tr>
<td>Tms 29</td>
<td>17</td>
<td>45</td>
</tr>
<tr>
<td>Average</td>
<td>15.75</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3: PCR products amplified with primer Sb6-57 and visualized under UV light. Kipkaren population K17-K30, M: molecular marker, hyper ladder 1kb (Bioline). Polymorphic bands shown by arrows.

4.2.1 Genetic diversity estimates

The major allele frequency was lowest at 0.8164 (primer Sb4-32), and highest at 0.8783 (primer Tms-29) with a mean of 0.8506. Number of alleles per primer ranged from 14 to 17 with a mean of 15.75 alleles per primer. Genetic diversity ranged from 0.1429 for primer Tms-29 to 0.2324 for primer Sb4-32 with a mean of 0.2083. The average polymorphism information content was 0.1704. The highest PIC value revealed by POPGENE ver 1.13 was 0.1855 for primer Sb6-57 and the lowest 0.1429 for primer Tms-29 (Table 4.3).

### Table 4.3: Allele frequency, allele number, genetic diversity and PIC for SSR primers used in DNA amplification

<table>
<thead>
<tr>
<th>Marker</th>
<th>Major Allele frequency</th>
<th>Allele No.</th>
<th>Genetic Diversity</th>
<th>PIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sb4 32</td>
<td>0.8164</td>
<td>16</td>
<td>0.2324</td>
<td>0.1827</td>
</tr>
<tr>
<td>Sb6 57</td>
<td>0.8513</td>
<td>14</td>
<td>0.2230</td>
<td>0.1855</td>
</tr>
<tr>
<td>Sb6 84</td>
<td>0.8619</td>
<td>16</td>
<td>0.2044</td>
<td>0.1703</td>
</tr>
<tr>
<td>Tms 29</td>
<td>0.8783</td>
<td>17</td>
<td>0.1429</td>
<td>0.1429</td>
</tr>
<tr>
<td>Average</td>
<td>0.8506</td>
<td>15.75</td>
<td>0.2083</td>
<td>0.1704</td>
</tr>
</tbody>
</table>

PIC—polymorphic information content
Bands unique to population were observed in all populations. The number of unique bands ranged from 2 to 6. The percentage of polymorphic loci observed was 61.90% (Kipkaren) and a high of 73.02% (Makuyu), with mean of 68.25% and SE of 2.46%. The mean expected heterozygosity ($H_e$) ranged from 0.131 and 0.150 with Standard error of 0.015 and 0.019, respectively (Table 4.4, Figure 4.4). Size range between the smallest and largest band for any given primer was 50bp and 3000bp.

### Table 4.4: Total band patterns, % polymorphism and Mean heterozygosity of *S. nigrum* collected from four study areas.

<table>
<thead>
<tr>
<th>Population</th>
<th>Unique bands</th>
<th>% Polymorphic loci</th>
<th>Mean Heterozygosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kipkaren</td>
<td>2</td>
<td>61.90</td>
<td>0.135</td>
</tr>
<tr>
<td>Makuyu</td>
<td>6</td>
<td>73.02</td>
<td>0.144</td>
</tr>
<tr>
<td>Matanya</td>
<td>2</td>
<td>71.43</td>
<td>0.131</td>
</tr>
<tr>
<td>Mauche</td>
<td>3</td>
<td>68.25</td>
<td>0.150</td>
</tr>
<tr>
<td>Mean</td>
<td>3.25</td>
<td>68.25</td>
<td>0.137</td>
</tr>
</tbody>
</table>

**Figure 4.4**: Band patterns of the populations showing the number of bands, bands with frequency $\geq 5\%$ and mean heterozygosity in each population.
4.2.2 Partitioning genetic variation

AMOVA analysis was performed in GenAIEx which revealed that 86% of the total genetic diversity occurred within populations and 14% among populations (Table 4.5). All molecular variations were highly significant (p<0.001).

Table 4.5: Analysis of molecular variance (AMOVA) within and among S. nigrum population

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Ss</th>
<th>ms</th>
<th>Est var.</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among regions</td>
<td>2</td>
<td>72.245</td>
<td>36.122</td>
<td>0.000</td>
<td>0%</td>
<td>0.001</td>
</tr>
<tr>
<td>Among populations</td>
<td>3</td>
<td>37.518</td>
<td>37.518</td>
<td>1.061</td>
<td>14%</td>
<td>0.001</td>
</tr>
<tr>
<td>Within pops</td>
<td>109</td>
<td>754.098</td>
<td>6.794</td>
<td>6.794</td>
<td>86%</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>114</td>
<td>863.861</td>
<td>7.864</td>
<td>7.864</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

df. – degree of freedom, SS – sum of squares, MS – mean squares, p – level of significance,

4.2.3 Population cluster analysis

Results of the PCoA done using GenAIEx showed PC1 accounted for 23.35% of the total population, PC 2 accounted for 21.15% (as shown in figure 4.5). However, the efficiency of PCoA was moderate based on the percentage of observed pattern on the axis. Makuyu samples clustered in the 1st axis while Kipkaren and Matanya samples clustered in the 2nd axis.
Figure 4.5: Clustering of *S. nigrum* population as revealed by Principal Coordinate Analysis. Axis 1 and 2 account for 44.65% of the variance in the population distribution

Similarity matrix was created from binary data. The genetic relationship among the four populations was revealed by Nei’s genetic distance (Nei, 1987). Values ranged from 0.010 to 0.020, the smaller values indicating a closer relationship. The highest similarity (0.010) was observed between Kipkaren and Matanya populations. The lowest similarity (most diverse) was observed between *S. nigrum* populations Makuyu in Central region and Mauche in Rift valley revealing high genetic diversity between the two populations. Dice Unweighted Neighbor Joining analysis showed phylogenetic tree (Fig 4.6), based on dissimilarity matrix cluster analysis.
Figure 4. 6: Phylogenetic relationship among *S. nigrum* populations. Unweighted Neighbor Joining with 1000 bootstrap values in DARwin 6.0.5 (Perrier and Jacquemoud, 2006)
The pairwise distance for all populations of black nightshade revealed close association between samples of each population. Minimum dissimilarity distance was 0.09 while the maximum was 1. The edge distance between initial tree and bootstrapped tree was 0.8345.

4.3 Correlation of morphological characters and genetic data

Samples with sinuate and entire leaf margin did not cluster in any particular axis. PCoA did not reveal particular clustering pattern (Figure 4.7). Axis 1 accounted for 23.35% and axis 2, 21.15%.

![Figure 4.7](image)

**Figure 4.7**: Principle coordinates analysis showing the relationship of the leaf margin and genetic data. PC 1 and 2 account 44.50% of the variance in *S. nigrum* samples

Correlation between nature of leaf surface and genetic data showed some clustering (Figure 4.8) Samples with hairy leaf surface show a close genetic relationship as they clustered together with a few outliers. Axis 1 revealed 23.35% while axis 2 revealed 21.15% of the variation in the selected samples.
Figure 4.8: Principle coordinate analysis of leaf surface against genetic data. Axis 1 and 2 account for 44.50% of the variance in *S. nigrum* samples.

Leaf shape on the other hand did not show distinct clustering patterns. Axis 1 explained 23.47% while axis 2 explained 20.83% of the variation (Figure 4.9).

Figure 4.9: Principle coordinates analysis of leaf shape against genetic data. Axis 1 and 2 account 44.30% of the variance in *S. nigrum* samples collected.
Small purple fruits clustered in one axis while the rest did not show any distinct clustering (Figure 4.10) Axis 1 accounted for 23.15% and axis 2 19.67% of the variation.

**Figure 4.10:** Principle coordinates analysis of fruit colour against genetic data. Axis 1 and 2 account 42.52% of the variation in *S. nigrum* samples.
CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Considerable morphological variations were observed in the *S. nigrum* samples that were assessed. Morphological characters revealed variations in the nature of leaf surface, leaf margin and fruit colour at $p = 0.009$, $0.005$ and $<0.001$ respectively, which is in agreement with Nandhini *et al.* (2014). However, while ovate and lanceolate leaf shapes showed significant variation there was no detectable variation among diamond leaf shape $p = 0.083$ postulated by Manoko (2007). The frequent movement of seed material and subsequent adaptation to agro climatic condition may be responsible for such variation. Qualitative characters are more reliable in determining genetic relationship among nightshades compared to quantitative characters (Olet, 2004).

A total of 63 polymorphic bands, ranging between 14-17 bands per primer were observed. Studies carried out in South Africa in 2012 using SSR primers resulted in 232 bands from 7 primers, ranging from 1 to 13 bands. RAPDs revealed 65 polymorphic bands, 6-13 bands per primers with band size of up to 3000bp (Stracke *et al.*, 2008). Elena *et al.*, (2010), identified 90 SSR bands ranging from 2-12 bands per primer, with an average of 6.0. In the same study, allozymes used revealed 2-5 bands per loci with a mean of 2.8 bands. As observed in Angeline *et al.*, (2010), problems were experienced with spurious high-molecular weight bands (up to 3000bp). These bands would have
been a result of non-specific priming, or large inserts in the flanking sequence. The inserts would have been responsible for large products because the primers were designed for other species than those of *Solanum nigrum* complex (Angeline *et al.*, 2010). SSR markers revealed a total of 13 unique bands. A high amount of these population-specific bands was identified with Makuyu samples. In this case SSR markers were more reliable in revealing unique bands among the populations of *S. nigrum* grown in Kenya.

The major allele frequency varied from 0.8164 (Marker Sb4 32) and 0.8783 (Marker Tms 29) with average of 0.8506. Allele frequency depicts the amount of genetic diversity at the individual, population and species level. It defines the relative proportion of all allele of a gene that is of a designated type. Genetic diversity estimates ranged from $H = 0.1429$ (Marker Tms 29) to $H = 0.2324$ (Marker Sb4 32) with average of $H = 0.2083$. The values were relatively low but in agreement with findings by Péter and Hyvönen. (2011). Selective breeding by farmers for desirable traits omitting undesirable ones can be associated to low genetic diversity. This leads to nearly genetically identical plants. Little or no genetic diversity makes crops unable to adapt to changing environmental conditions and extremely susceptible to widespread diseases (Stracke *et al.*, 2008). PIC is the measure of polymorphism among varieties for marker loci used in linkage analysis. PIC value for each marker, which can be evaluated on basis of its allele, varied from 0.1429 (Marker Tms 29) and 0.1855 (Marker Sb6 57). PIC is used to
measure the usefulness of a genetic marker in genetic diversity studies. The information level depends on the set of primers used and sample plants evaluated.

The highest percentage polymorphic loci for SSR marker was 73.02% (Makuyu) and lowest 61.90% (Kipkaren). SSR used by Elena et al., (2010), showed 80% polymorphic loci among S nigrum populations in Atacama Desert of Peru and Chile. AFLP study revealed 91.7% polymorphic band in S. nigrum populations (Manoko et al., 2008). Polymorphism levels observed in this study are high, this could be associated to polyploidy nature of S. nigrum (Patricia et al., 2011). Each individual may present several alleles in one locus due to high reproducivity of SSR markers. Recombinations between ancient polyploids and diploids has resulted in high level of genetic polymorphism (Péter and Hyvönen, 2011), enabling them succeed in their new environment. Polyploidy and hybridization is often reported as an important source of diversity in S. nigrum.

A more appropriate measure of variation is average heterozygosity. The average heterozygosity was $H_e=0.1370$. This results relate to previous finding of $H_e=0.1450$ by Elena et al. (2010). Genetic markers revealed low levels of heterozygosity, this could be as a result of inbreeding a commonly encouraged by farmers to retain particular traits of agronomic importance.

AMOVA was used to partition the genetic distance among the populations and test whether there was any hierarchy of variation among individuals. Genetic variance was
high among the individuals within a population than among groups and regions. AMOVA carried out with SSR marker showed a high variance (86%) within individuals in a population. Studies done by Ganapathy *et al.* (2012), on pepper using SSR revealed a 59.51% variation between individuals and only 6.55% within regions. High levels of variation within individuals is an indicator that *S. nigrum* populations are reproductively and genetically isolated from each other as postulated by Poczai *et al.* (2010). Variation within populations may be associated to cross pollination (Sarah, 2009), common phenomenon in *S. nigrum*.

Cluster analysis was done independently for SSR markers, and the resulting PCoA demonstrated distinct patterns of SSR marker variation. Two major axis of differentiation (PCoA 1 and PCoA 2) explained 44.50% of the variation in SSR. The distance between samples represents their genetic variation. Individuals close together were interpreted as similar while those far apart interpreted to be different or distantly related (Ojiewo *et al.*, 2013). Lack of population clustering could have been the result of gene flow within the populations or random selection of DNA markers. This leads to combination of two gene pools reducing genetic differentiation between the groups. These results reflect the outcome of a study by Manoko *et al.* (2008). A similar clustering was reported between Uganda, Indonesia and European materials (Olet, 2004). Lack of geographic clustering is an indicator of lacking genetic variation. *S. nigrum* complex is a group of plants that are closely related as observed by (Angeline *et al.*, 2010)
The genetic distance varied from 0.010 and 0.020. Genetically Kipkaren and Matanya samples are closely related while Makuyu and Mauche samples are most distant. Low genetic distance was in agreement with previous results by Angeline et al. (2010), which showed a genetic distance of between 0.33 and 0.55. This is a strong indicator of how close the populations are to each other. Mwai (2007), reported low genetic distance in contrast with Schippers (2002), that reported very high distance among *S. nigrum*. The low genetic distance could be as a result of self-pollination among members.

Several samples from one region clusted together indicating their close genetic resemblance.

Morphological characteristics and genetic data showed varied clustering patterns. Variation between leaf margin and genetic data did not show distinct patterns. Both sinuate and smooth leaf margin lied on similar axis of the coordinates. Lack of clustering could be related to low genetic distance observed in the samples. Nature of the leaf surface revealed some degree of clustering where samples with hairy leaf surface clustered on one axis a strong indicator of their genetic resemblance. Leaf shape did not reveal distinct clustering pattern which means the sample under study were genetically closely related. However, fruit colour showed some level of clustering where Small purple fruits clustered together. This shows that samples with small purple fruits are genetically closely related, while the comparison between leaf margin and leaf shape
with genetic data did not reveal any observable correlation, significant correlation was observed between leaf surface and fruit colour with genetic data.

This lack of clear relationship is an indicator that leaf margin and leaf shape have low taxonomic value at species level as observed by (Dehmer and Hammer, 2004). Similar results were observed with *Solanum villosum* Mill (Manoko, 2007).

In some cases these results correlate with the morphological classification, whilst in other cases show that there are markedly different genotypic verses morphological classifications. Random selection for SSR and environmental dependence of morphological traits (Manoko et al., 2008). One character may be genetically controlled in one species but phenotypically plastic in another as observed in Katarina. (2003). *Solanum nigrum* may not be predominantly self pollinating hence variation between individuals in the same species.

Based on the results obtained, it appears there were no significant differences between morphological and genetic characteristics in samples used. Lack of congruency between morphological and genetic differences can be attributed to selection by farmers for different plants types.
5.2 CONCLUSION

- Considerable morphological variation was observed in *S. nigrum* samples assessed during the study. Levels of variation observed could make contribution to the genetic characterization of *S. nigrum*.

- There is low genetic diversity in *S. nigrum* species collected in the selected regions in Kenya.

- High variations were observed within *S. nigrum* populations than among populations and regions in the study.

- There is no correlation between morphological characteristics observed and genetic variation in *S. nigrum* complex.

- Results of the study shows variation in agro ecological zone does not imply variation in the *S. nigrum* species grown.

- SSR Markers systems could have the advantage of distinguishing closely related members and facilitate breeding practices.

- Samples from Mauche are genetically separated from all other populations.

- *S. nigrum* complex is a group closely related plants.
5.3 RECOMMENDATIONS

- Genetic diversity is necessary for survival of a specie hence there is need to expand the study to wild varieties of *S. nigrum*.

- Germplasm collection strategies should be aimed at preserving the overall genetic diversity by expanding sampling to other parts of Kenya.

- Samples from Mauche are genetically separated from other population. There is therefore need for further studies to determine this uniqueness.
REFERENCES


