Genetic Identification of Kikuyu Grass (*Pennisetum clandestinum*) Cultivars by RAPD and ISSR Techniques

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Abstract

Kikuyu grass (*Pennisetum clandestinum*), originally from tropical and subtropical African countries, is one of the most important pasture grasses in the higher rainfall regions of South Africa (of utmost importance in agriculture, to the economy and to the industry). However, different cultivars are poorly characterised on a morphological basis and diagnostic genetic markers are missing, which could substantially support successful breeding and could be of crucial importance for rural dairy farmers who largely depend on low cost pasture crops. This study is aimed at finding genetic markers for cultivars of kikuyu grass based on two different PCR based methods.

Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) marker systems were employed to detect polymorphism and identify genetic relationships among forty (40) kikuyu lines from Cedara, Department of Agriculture near Hilton, KwaZulu-Natal. Thirteen (13) RAPD primers amplified a total of 144 reproducible bands of which 80 were polymorphic and fourteen (14) ISSR primers amplified a total of 90 markers of which 56 were polymorphic. The percentage of polymorphic bands detected by ISSR and RAPD was fairly similar (62.22 and 55.56 %, respectively).

Band scoring was analysed and FastTree dendrograms were constructed using the raxmlGUI1.3 and viewed using FigTree v1.4.0 analysis programs. Cluster analysis of the thirteen (13) informative RAPD primers produced an unrooted tree which grouped the forty cultivars into 8 distinct clusters (comprised of 3 larger clusters and 5 smaller ones) and 9 independent branches. The cluster analysis of the fourteen (14) informative ISSR primers produced an unrooted tree which grouped the forty cultivars into 4 distinct clusters (made of 2 larger clusters and 2 smaller ones) and 6 independent branches.

Some cultivars were elucidated to share common clusters in dendrograms of both techniques and authenticate their genetic relationship among other cultivars. Cultivars sharing the same cluster (cluster 1) in both, the ISSR dendrogram and the RAPD dendrogram were cultivar 30, cultivar 40, cultivar 35, cultivar 39, cultivar 38, cultivar 34, and cultivar 36. Moreover, cultivar 11, cultivar 20, cultivar 19, cultivar 16, cultivar 14, cultivar 13, cultivar 15, and cultivar 12 showed the same grouping in both dendrograms (cluster 6) and (cluster 4), with cultivar 13 and 14 sharing more similarity than any other cultivar within the cluster.

The cluster analysis results for both molecular techniques showed that Kikuyu grass cultivars tested here harbour considerable genetic variation, as was expected from the results of other preliminary research work focussing on physiological characters.

Keywords: *Pennisetum clandestinum*; polymorphism; genetic relationships; ISSR; RAPD; dendrograms; cultivar identification

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Chapter 1

Introduction

Currently in South Africa or at least in KwaZulu-Natal kikuyu pastures form relatively small parts (but highly producing) of dairy systems due to their specified growing season, low nutritional quality and poor yield distribution throughout the year in comparison to ryegrass (Goodenough *et al*. 2012). The majority of farmers are taking out kikuyu in favour of ryegrass. Due to this reason, this research study hopes to re-instate kikuyu as a sole pasture crop by identifying polymorphic cultivars that will eventually be used in forage making systems.

1.1. Background on pasture systems

The accelerating daily temperatures and elevated atmospheric carbon dioxide (CO_2) concentration, and the change in annual rainfall for future climates will influence the quantity and quality of pasture, as conditions may become more favourable for warm season (C4) rather than cool season (C3) grass species (Howden *et al.* 2008). These predictions for warmer, wetter winters and hotter, drier summers, with increasing frequency of extreme weather patterns (Harrison *et al.* 2001; Humphreys *et al.* 2006; Cullen *et al.* 2009) in future have posed a growing interest in the performance of more heat tolerant and deep rooted subtropical (C4) pasture species such as kikuyu (*Pennisetum clandestinum*) (Bell *et al.* 2011). Pasture systems need to be adaptable and withstand both the inter-annual variability in climate and the longer term projected climatic changes (Cullen *et al.* 2009) that might affect the quality and quantity of pasture. However, the challenge with tropical grasses is that their high fibre content makes them to be less digestible than temperate species when consumed by ruminants (Minson & McLeod 1970; Howden *et al.* 2008).

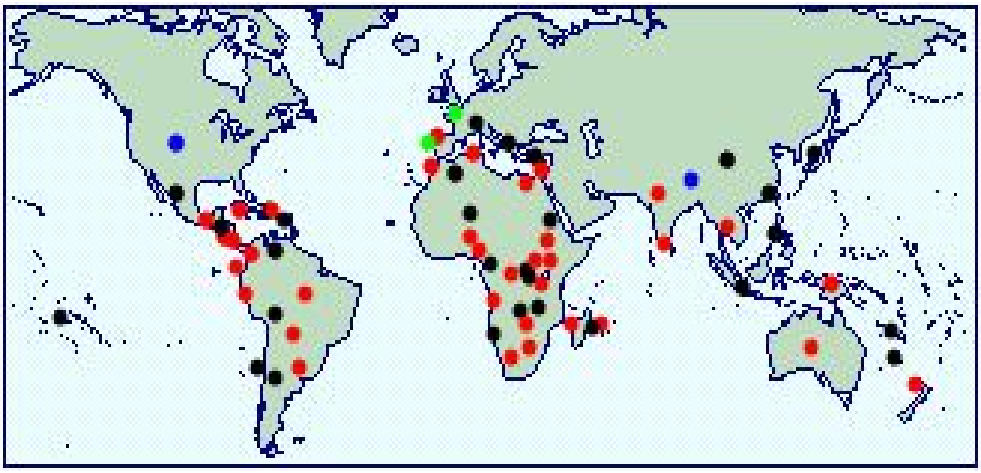
1.2. Kikuyu yield and climate change

Differing abilities of kikuyu grass species or ecotypes to withhold or release water, and the effects they have on soil structure and stability, can play a significant part in abilities to withstand climate extremes (Sanford *et al.* 2003). Generally, kikuyu yield ranges between 9 and 30 t DM/ha depending on nitrogen (N) fertilization, climate and soil type. The grass responds very well to fertilization (Mears 1992). In a study conducted to test the effect of

water (irrigation) on the growth and production of kikuyu, it was demonstrated that in extreme conditions of water shortage (33% less water than optimal irrigation), kikuyu provided the highest yield significantly higher with 17 t DM/ha/year when compared with 15 other perennial forages (Neal *et al.* 2011). These results suggest the possible utilization of kikuyu grass in environments where the survival of other fodder species is markedly reduced (Muscolo *et al.* 2004). For instance, this grass can provide forage throughout the Mediterranean summer when there are high temperatures and low rainfall, when cool-season grasses become less productive. This grass species, *Pennisetum clandestinum* provided the suitable combination of agronomic and yield characteristics which were similar to those of alfalfa (*Medicago sativa*) when compared with seven other grasses in a study conducted by Gherbin *et al.* (2007) in Southern Italy (Gherbin *et al.* 2007).

1.3. Origin and history of kikuyu grass

Kikuyu (*Pennisetum clandestinum*) is not indigenous to South Africa but originates from East/Central Africa i.e. in Kenya, Ethiopia, Tanzania, Uganda, Ruanda, Zaire and the Congo (Skerman & Riveros 1990). The grass species was named after the Kikuyu tribe in Kenya and, it has been introduced widely around the world including Southern Africa (Rumball 1991; Herreroborgonon *et al.* 1995; Mears 1970).



Source: CAB International (http://www.cabi.org/isc/datasheet/39765)

KEY:

- Black = Present, no further details
- Blue = Evidence of pathogen
- Red = Widespread
- Purple = Last reported
- Yellow = Localised
- Light blue = Presence unconfirmed
- Brown = Confined and subject to quarantine
- Orange = See regional map for distribution within the country
- Green = Occasional or few reports

Figure 1. Geographical distribution kikuyu grass (*Pennisetum clandestinum*) on the African map.

1.3.1. Kikuyu introduction into South Africa

A literature review on kikuyu grass (Mears 1970) reported that in 1910 Forbes collected kikuyu roots at Lake Naivasha (1884 m asl) in Kenya which was sent to the Botanic Gardens in Pretoria (Cameron 1960). It is assumed that this particular non-seeding, male-sterile kikuyu ecotype got spread by root cuttings from farm-to-farm throughout South Africa in subsequent years. However, in Wilbur Smith's book "Power of the Sword" (W.) it was reported that General Jan Smuts brought back shoots of kikuyu to South Africa "from his East African campaign in 1917 and it had flourished all over the country" (Doodenough *et al.* 2012). A third kikuyu type to be introduced into South Africa in the 1970's was the seeding variety "Whittet" which got spread and was initially established on Mr Gerrie de Jong's farm just outside Howick, South Africa, then subsequently at various other sites and further establishments at various other sites were recommended by the late Dr Pierre Theron, then Head of Pasture Science at Cedara (Doodenough *et al.* 2012).

Besides the above mentioned means of kikuyu's introduction into South Africa, Goodenough (1993) reported a set of 41 different kikuyu ecotypes which were discovered at the Kaikohe Research Station in North Island, New Zealand in 1993. A single rooted stolon of each of the 41 ecotypes had been sent to South Africa where they were kept at Roodeplaat just outside Pretoria before being sent to Cedara for re-establishement. It is assumed and highly possible that the seeding kikuyu varieties Whittet, Noonan and Breakwell were among these ecotypes sent to South Africa. Whittet was originally collected at Kitale, Kenya, at 1890 m asl; Breakwell possibly originates from Congo while Noonan is a single-plant selection made at Grafton, New South Wales, Australia (2006).

Even though kikuyu is no longer used as a sole pasture crop in KwaZulu-Natal or rather South Africa (but ryegrass instead) however, it is still one of the most common pastures currently under cultivation within the Eastern Cape area of agriculture (DeRidder 2005-Ongoing/Long-term). Many dairy farmers depend merely on kikuyu as a pasture during the summer growing season (its growth period). Under favourable conditions, kikuyu will outperform most grass species under high fertilized management (DeRidder 2005-Ongoing/Long-term). However, the value of kikuyu pasture cannot be over-emphasized and on-going research to increase the production capability of this pasture is of the utmost importance.

A research study carried out at Döhne research station, near Stutterheim, Eastern Cape, South Africa demonstrated that some kikuyu ecotypes perform better than others and some even better than the local kikuyu strain in terms of production values (DeRidder 2005-Ongoing/Long-term). These observations bring back a hope that kikuyu can still be the sole pasture species. This would be beneficial to farmers, especially dairy farmers since they depend on permanent pastures to utilize as the bulk of their animal feed source. Such situations are especially true within the rural community areas where most dairy producers cannot afford high cost pasture crops. A permanent and cheap pasture such as kikuyu is therefore more suited for the rural dairy farmer. The farmers involved have already shown interest towards kikuyu, as a pasture they intend to use for their dairy in future although it still needs careful management to realize the full economic potential (DeRidder 2005-Ongoing/Long-term).

1.4. The Importance of kikuyu grass

Approximately 20% of the Earth's land surface is covered with grasses which are used for feeds for domestic animals. The feed may be consisting partly or wholly of pasture grasses (Kellogg 2001). The grass family therefore plays a very essential role in agricultural production, economy and environmental sustenance (Kellogg 2001).

1.4.1. Agricultural importance

The fast and aggressive reproduction and spread of kikuyu grass made it a weed in numerous agricultural and recreational areas (Wilen *et al.* 1995; Wilen & Holt 1996; Morris 2004). Therefore, much money is spent in weed control and management programs. However, such characteristics could be an advantage for reclamation of salt-affected sites. Kikuyu grass is also known to be tolerant to drought, and water logged environments (Whiteman 1990; Morris 2004) thus, this species seems to be a good candidate for planting and use in such habitats.

1.4.2. Economic importance

The marketing strategy needs to be considered when deciding on which animal breed to utilise on the pasture (Tainton 1999). When deciding for relatively heavy stocking rates (5 cow and calf pairs/ha and heavier), the herd is very vulnerable to droughts in summer which will cause serious fodder shortages. Cows and suckling calves are not easily transported and marketed during periods of drought. Therefore, when operating at heavy stocking rates it is more crucial than at light stocking rates, to have a reliable fodder bank (Clatworthy & Price 1980; Tainton 1999). Kikuyu pastures are known to be tolerant to constant heavy grazing and trampling provided they are well fertilized and managed (Fukumoto & Lee 2003; Partridge 2003; Mears 1992; Cook *et al.* 2005) thus they are suitable for heavy stocking rates.

The main problems experienced when relying only on kikuyu grass pastures for production is that the winter and spring production is low since its optimal growing period is in summer and autumn (Botha *et al.* 2008; Bell *et al.* 2011). This crop species is considered a fodder of low quality for dairy cows and the exclusion of legumes make it dependent on nitrogen which increases the input cost (FAO 2010; Tainton 1999). Diseases associated with kikuyu grass such as kikuyu "yellows", nitrate poisoning and bloat also have negative impact to the country's economy (ABARE 2007; Bell *et al.* 2011). Kikuyu yellows (caused by the oomycete fungus *Verrucalvus flavofaciens*) is particularly prevalent in the low-veld regions of KwaZulu-Natal and Mpumalanga, South Africa also in Australia only through late spring

to early autumn, normally after periods of rainfall (Tainton 1999). The viral infection causes a leaf-spot surrounded by a yellow halo, and results in some leaf death, but is not of economic importance in a well-managed and fertilized pasture (Skerman & Riveros 1990; FA0 2010). As yet there is no economic control for kikuyu “yellows” in kikuyu grass (FAO 2010) but, returning the area to cultivation of cash crops for a few years can help get rid of the virus (Cunningham & Bartholomew 2005). While it is assumed that kikuyu yellows does not play a major role in inhibiting animal production but, it is recognized that it can affect palatability of the pasture (FAO 2010). Nitrate poisoning and bloat disease contribute negatively to the economy by causing animal loss in severe cases (Moore *et al.* 2006). By keeping pastures short, well fertilized and well managed, the incidence of "yellows" and of course other diseases is minimized (Cunningham & Bartholomew 2005).

A renewable biofuel economy is projected as a pathway to reduce reliance on fossil fuels, reduce greenhouse gas emissions, and enhance rural economies (McLaughlin *et al.* 2002). High lignocellulosic biomass from agro-food industries such as kikuyu grass serves as an alternative for chemical and biofuel production (Trebbi 1993). These feedstocks require fewer agricultural inputs than annual crops (i.e. maize) and can be grown on agriculturally marginal lands (McLaughlin *et al.* 2002). It is also reported that high concentrations of nitrogen in kikuyu grass reduce the effectiveness and chemical output of thermochemical conversion systems (Agblevor *et al.* 1992) which is an advantage to the environment.

1.4.3. Industrial importance

Because of its rapid growth and degradable biomass characteristics, kikuyu grass has a potential for bioenergy production and conversion to alcohol or methane (Trebbi 1993; Muscolo 2011). Research study by the Railway’s Research Lab (Muscolo 2011) has reported several crop plants as new plants species for renewable energy production at railway stations. These plant species include kikuyu (*Pennisetum clandestinum*), *Jatropha curcas* and *Vetiver*. The research project reported that these plant species are able to reduce about 40% of the energy cost thus increasing the environmental sustainability. It was also found that these plant species have high fibre content useful for biogas production and that these new renewable grass species can restore soil along railways. Most often kikuyu is used as a cover crop, especially on airstrips and as an erosion control measure on river banks and newly constructed roadside embankments (Morris 2004; Fulkerson 2007; FAO 2010; Mears 1992). Besides that, kikuyu has also been used in the treatment of acid mine drain age (AMD) and

other industrial effluents (Greben-Wiersema 2007). It is also used in erosion control and as ornamental turf (Cook *et al.* 2005).

1.5. The nature and morphology of kikuyu grass

Kikuyu grass (*Pennisetum clandestinum*) used to be confused with *Pennisetum longistylum* Hochst (Whittet 1921). The two species have been adequately described and illustrated on several occasions (Whittet 1921; Breakwell 1923; Cameron 1960). Kikuyu occurs at elevations of between 1950 and 2 700 m (Skerman & Riveros 1990). It is however important to take note that an elevation of 2000 m at the equator is not equivalent, in terms of plant growth, to South African conditions at 2000 m at a higher latitude (32 °S) (Doodenough *et al.* 2012).

Its mean minimum and maximum temperatures ranging from 2 to 8 °C (Mears 1970) and 16 to 22 °C (Russell 1976) i.e. somewhat lower than most other tropical species. The growth declines noticeably on temperatures below 7 °C and responds poorly to high temperatures (Russell 1976) mainly, perhaps, because of disease problems under such conditions. The species grows well in areas with annual rainfall ranges from 1 000 to 1 600 mm (Mears 1970). However, Russell and Webb (1976) reports a presumably rainfall/irrigation requirement of 1 269 ± 632 mm while Whiteman (1980) regards 850 mm to be reasonable for kikuyu.

Kikuyu is tolerant of low soil pH and of high aluminum (Al) and manganese (Mn) content and tolerant of salinity (Mears 1992). It is invasive (advantage of controlling weeds and preventing soil erosion) and well known to grow well on uneven landscape which cannot be cultivated. However, several factors reduce its nutritive value. Its digestibility potential is relatively low (due to high fibre content) and the plant species is particularly low in readily digestible carbohydrates. The plant is also deficient in sodium and produces oxalic acid, which binds calcium, rendering it largely unavailable to the grazing animal thus negatively affecting the animal performance (Clark & Wilson 1993).

1.6. Nutritive value of kikuyu grass

Kikuyu grass is palatable to cattle and mainly used for fodder as permanent dryland irrigated pasture, hay or silage (FAO 2010; Mears 1992). Most existing kikuyu pastures are monospecific. Pure kikuyu pastures, top-dressed with nitrogen, are usually more productive than grass/legume mixtures (FAO 2010). In some countries kikuyu grass is used as a pasture

plant because of its acceptable nutritive properties compared to other grass species i.e. buffalo grass (Marais *et al.* 1992). Various studies have been conducted to test the performance of kikuyu and whether the grass species can be used as a future forage crop. Moir *et al.* (Moir *et al.* 1979) studied dairy cows grazing kikuyu pasture and found that high milk producing animals were firstly limited by the energy concentration of the grass rather than other dietary attributes. The production/performance of ruminant livestock is generally limited by energy or protein present in their diet, but in most cases animals meet their metabolisable protein requirement from grazed forages (Kerley & Lardy 2007). Exceptions to this may be growing animals or grazing dairy cows that respond to supplementation of protein due to a greater demand for amino acids to support protein synthesis for lean growth or milk production (Kerley & Lardy 2007).

Like most warm season grasses, kikuyu has a high fibre content but can be managed (Clark & Wilson 1993) to keep a metabolisable energy (ME) content of the leaf above 9MJ/kg DM (Morris 2004). Kikuyu requires high soil fertility for good growth (Morris 2004). Highly productive kikuyu pastures play a vital role in the dairy (Cunningham & Bartholomew 2005; Fulkerson 2007) and beef (Ouda *et al.* 2001) industries by providing a nutritional feed for animals in summer and autumn, its period of predominant growth. However, the grass is deficient in sodium (Na) and calcium (Ca) so these minerals need to be provided as supplements to dairy cows grazing kikuyu pastures (Marais 2001; Fulkerson 2007). Reports indicate that cows grazing well managed kikuyu pastures (but with sodium and calcium supplements) can produce up to 14-15 L milk/cow/day (Fulkerson 2007) and beef animals produce over 400 kg/ha/yr (Ouda *et al.* 2001). Due to its popularity, today kikuyu represents the base pasture species for over 80 % of all dairy farms in New South Wales (NSW) alone in Australia (Morris 2004).

Even though kikuyu grass is valued for its nutritive properties, energy is a major nutritional limitation in kikuyu due to its high cell wall content and low digestibility of structural components (Marais & Figenschou 1990). The concentration of metabolizable energy is as low as 8.5 MJ/kg DM, as calculated from an organic matter digestibility (OMD) of 65 % (Marais 2001). Feedipedia (Heuzé *et al.* 2013) reports the OMD of kikuyu to vary between 47 and 73 %, depending on the regrowth stage. Due to the large difference between leaf and stem tissue, the nutritive value appears to be optimized at 4.5 leaves per tiller growth stage (Marais 2001).

The composition of crude protein varies from 8.5 to 25.6 % dry matter (DM) (Murtagh 1990) and is higher than that of other tropical grasses. However, the high nitrogen levels in kikuyu induce a poor protein metabolism and thus a low animal production (Marais & Figenschou 1990; Hanna *et al.* 2004; Carvalho *et al.* 2010). High concentration of nitrogen in young kikuyu plants may result in high rumen ammonia which is largely lost as urea via urine (urea poisoning) (Marais 2001) since kikuyu grass lacks condensed tannins which could reduce ammonia formation in the rumen (Jackson *et al.* 1996; Marais 2001). The concentration of neutral detergent fibre (NDF) ranges from 58.1 to 74.1 % DM which is comparatively low in comparison to that of temperate species, but digestibilities are similar to those of tropical species (Marais 2001). A negative correlation between cell wall content (NDF) and digestibility exist (FAO 2010; Heuzé *et al.* 2013).

1.7. Grazing kikuyu grass

Kikuyu is very resistant to constant heavy grazing and trampling provided it is well fertilized (Fukumoto & Lee 2003; Partridge 2003; Mears 1992; Cook *et al.* 2005). It should be grazed down to 5 cm height and allowed to re-grow to 15 cm height to preserve forage quality and palatability (FAO 2010). Furthermore, as a fodder plant, kikuyu possesses the capability to regenerate rapidly following repeated mowing which is a highly important trait of fodder plants. This important trait makes kikuyu suitable to be a fodder plant and to be grown under saline conditions (Radhakrishnan *et al.* 2006).

1.8. Animal production on kikuyu grass

1.8.1. Dairy cattle

For grazing dairy cows, kikuyu grass is still preferred after ryegrass in South Africa, in Australia it comes after prairie grass and before white clover despite the grass's drawbacks such as its low nutritional value and specified growth period (Fulkerson 2007; Horadagoda *et al.* 2009). Even though kikuyu grass silage is palatable to dairy cattle, the digestibility of the silage is about 19.5 units lower than freshly-cut grass (Horadagoda *et al*. 2009). Due to its high DM yield, kikuyu supplemented with energy sources has been used for pasture by dairy cattle in many studies worldwide, for example in a study conducted by Heuze *et al*. (2013) in Columbia. In Friesian cows, milk production was restricted to 13-16 L/d even on well-managed kikuyu pasture (Reeves *et al.* 1996; Marais 2001) (Hamilton *et al.* 1992; Carvalho *et al.* 2010), this compares agreeably with 14-15 L milk/cow/day reported by Fulkerson

(Fulkerson 2007). The low milk protein content (less than 3.0 %) indicates low nitrogen efficiency utilization (average 17.4 %) for the synthesis of milk proteins whereas the high milk content of conjugated linoleic acid (20.0 mg/g lipids) shows the high linoleic and linolenic acid content of kikuyu (Correa *et al.* 2008). The supplementation of dairy cows grazing kikuyu rotationally with energy concentrates is sufficient for the mid and late lactation, without requiring protein supplementation.

In a survey of 229 dairy farms in Colombia, 33 % of the farms had cows feeding on kikuyu pastures producing 16 to 20 L/day (Osorio 2004; Heuzé *et al.* 2013). The total milk production may be increased when kikuyu is over-sown with annual ryegrass (from 3.9 T milk/ha to 8.1 T milk/ha) or with clover (7.3 T milk/ha). But the mean annual grazing capacity has to be considered to evaluate the effective gain of milk production per hectare. Indeed, the milk produced may vary between seasons, being higher during spring and summer (15 kg/d and 14.4 kg/d) than during the autumn (12.1 kg/d) (Botha *et al.* 2008).

1.8.2. Beef cattle

In a research study conducted by Clatworthy & Price (1980) it was elucidated that by adjusting the loading or the ratio of days grazed per day rested and taking into account the grazing period, it is possible to improve the carcass weight of heifers grazing on irrigated kikuyu mixed with white clover. Otherwise, animals can be grazed on pure kikuyu pastures provided the animals are subjected to energy, sodium (Na) and calcium (Ca) supplements while feeding on kikuyu since the grass lacks these minerals and need to be provided as supplements (Marais 2001; Fulkerson 2007). Satisfying beef production records have been reported on kikuyu pastures (Ouda *et al.* 2001).

1.8.3. Sheep

It has been seen that milling and pelleting kikuyu leaves for feeding sheep results in a live-weight increase three times as compared to that of sheep fed on the unmilled leaf ration (Barnes & Dempsey 1993; FAO 2010). Moreover, it was discovered that the use of tree leaves can help to improve the nutritive value of kikuyu hay. In a research study conducted by Sanford *et al.* (Sanford *et al.* 2003) it was found that a mixture of kikuyu pastures with trees of Tasmanian blue gum (*Eucalyptus globules*) can lead to a significant increases in the clean wool production of Merino sheep (75 kg/ha) compared to kikuyu alone. In another study conducted in Chiapas (Mexico) kikuyu hays were mixed with the leaves of *Buddleia skutchii*, a common multipurpose fodder tree, without decreasing the DM intake and digestibility (Camacho *et al.* 1999). However, this experiment found that the inclusion of

Buddleia leaves maintained daily weight gain as well as wool (in sheep) and manure production, but the dry matter intake (DMI) and digestibility of the feed were decreased thus resulting in lower economic benefits than with kikuyu grass alone (Nahed *et al.* 2003).

1.8.4. Other ruminants

Kikuyu grass is also known to support other ruminants such as rabbits (Singh *et al.* 1997), donkeys and horses (Stevens *et al.* 2002) worldwide where kikuyu grass grows but commonly in South Africa and Australia (Heuzé *et al.* 2013).

1.9. Toxicity and diseases of kikuyu grass

Kikuyu is sensitive to a number of diseases but these only really become a problem under hot, humid tropical lowland conditions (Turgeon 1980). In New Zealand, serious toxicity occurs spasmodically on kikuyu pastures after rainfall in excess of 20 mm, grass temperatures above 14°C and invasion of pasture by army-worms. The toxin is unknown (Skerman & Riveros 1990). Excessive application of nitrogen can cause and it promotes diseases (Skerman & Riveros 1990). Some of the effects associated with high nitrogen levels include nitrate poisoning (Cook *et al.* 2005) and bloat (Said 1971). Nitrates are non-toxic but their conversion into ammonia, within the rumen, produces toxic nitrites that bind with haemoglobin and prevent blood from binding with oxygen, resulting in oxygen starvation of the tissues and animal death in the most severe cases (Marais 2001).

1.10. Registered cultivars

Currently, four registered varieties of kikuyu grass exist and are well recognised worldwide, namely Whittet, Breakwell, Crofts and Noonan (Morris 2004). “Whittet”, a seeded kikuyu cultivar from Kenya, was released in 1969 but registered in 1970. This is a taller, coarser, more broad-leaved and vigorous plant that survives better than common kikuyu under less fertile conditions (Mears 1970). The cultivar Whittet is the common cultivar among others due to its high seeding tendencies (only cultivar grown for commercial seed production in Australia) (Morris 2004; Morris 2009). ‘Breakwell’ was produced as a result of natural selection in northern NSW, Australia and the variety was registered in 1971. ‘Crofts’ is also a product of natural selection but with a preference for cooler weather. The variety was collected at Camden, London and registered in 1983. ‘Noonan’, is a product of open pollination between ‘Whittet’ and ‘Breakwell’. This cross has been found to be resistant to “Kikuyu Yellows” disease over a period of 10 years by Dr. Percy Wong and was registered

in 1983. The three cultivars Breakwell, Crofts and Noonan have seen little use within the agricultural or horticultural industries. Attempts to establish Noonan in large scale seed farm production have not become successful due to its inconsistent seeding habits (Wilson 2005).

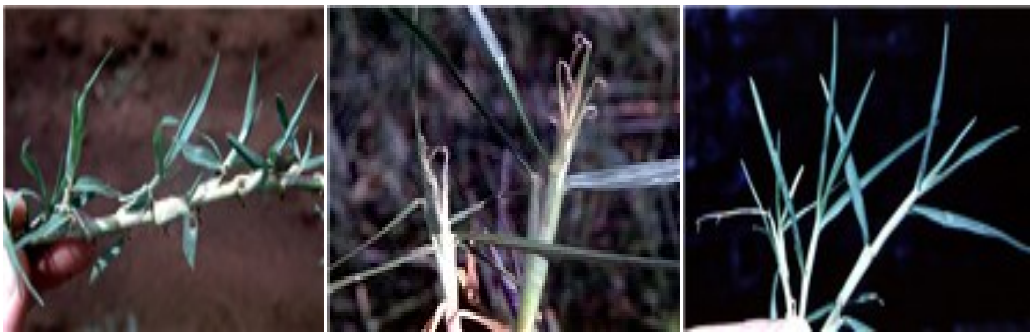


Figure 2. Different developmental stages of Kikuyu grass.
Image A: *Pennisetum clandestinum*, vegetative runner (source: FAO, Rome)
Image B: Flowering shoots showing exserted styles (source: FAO, Rome)
Image c: Flowering shoots showing exserted stamens (source: Chris Parker/CAB International)

The three cultivars i.e. Whittet, Breakwell and Noonan were also recognised in a spaced-plant nursery established at Cedara Department of Agriculture near Hilton, KwaZulu-Natal. It was noted that Noonan and Breakwell, both of which are used as turf grasses in Australia, are generally more prostrate and less vigorous than Whittet. Moreover, a high degree of morphological variation and vigour between and within the various spaced-plants of these three varieties (namely Whittet, Breakwell and Noonan) was witnessed (Goodenough 2011). To account for morphological variation observed this may typically be due to cross-pollination although conflicting evidence has been reported on the mode of reproduction in kikuyu (Mears 1970).

1.10.1. Background on the forty cultivars/varieties under study

The forty kikuyu lines used in this study came from the forty-one (41) kikuyu lines which were originally sent from New Zealand to Roodeplaat just outside Pretoria, then to Cedara (Doodenough *et al.* 2012). Duplicate collections were also established at Outeniqua (George, Western Cape) and Döhne (Eastern Cape) Research Stations. The preliminary evaluations undertaken by Marais *et al.* (2000) at Cedara on the chemical composition in terms of nutritional value of the New Zealand collection, the forty-one lines demonstrated a great variance indicating that these lines may be genetically variable and respond differently to the environmental influence. Therefore, it was expected that these lines would appear

differently even on molecular level unless they have been overgrown by the wild type or the local kikuyu strain. The latter scenario did happen (twice) at another research station, Outeniqua, George, Western Cape where the duplicate collections were established (Marais *et al.* 2000). This had cost the breeding lines to be replanted twice as a result it was concluded that the local kikuyu strain "is a stronger grower than the imported cultivars and once it had invaded the plots it was impossible to keep it out"(Botha 2011). This resulted in no further research being conducted on the ecotypes at this research farm.

Relatively little has been done on kikuyu grass since the last registration of a kikuyu cultivar (Noonan) in 1983. But since then this grass species has continued to be used extensively across the world resulting in it being planted in a wide range of environments. Even though this grass species showed to survive various and even extreme environmental conditions, what is still unclear however, is its genetic background especially of the kikuyu being planted today (Morris 2009). This creates problems when organizing germplasm resources as well as in maintenance of grass breeding programs.

Furthermore, not much research has been conducted to improve kikuyu for pasture production apart from the selection trials based on dry matter yield and leaf to stem ratio by Australian workers, which gave rise to the seeding variety, Whittet (Marais *et al.* 1992; Marais *et al.* 2000). More research still needs to be done to identify more nutritious kikuyu landraces which are also better adapted to climatic conditions and fodder flow regimes than existing kikuyu pastures in South Africa. The fact that kikuyu can grow over a broad ecological range with respect to many environmental gradients and stresses may indicate that kikuyu is genetically variable in terms of its chemical composition (nutritional value) and its response to environmental factors.

1.11. Genetics and reproduction

Kikuyu (*P. clandestinum)* is a tetraploid with somatic chromosome number, 2n, of 36 (Meredith 1955). Bisexual and male-sterile races exist. The Rongai strain is female-fertile. Youngner (1961) confirmed the existence of bisexual and male-sterile races of kikuyu. Male sterile kikuyu can be identified by the absence of filaments and stamens protruding above the canopy, with only a feathery stigma being produced.

Contradicting results have been reported on the mode of reproduction in kikuyu grass. It has been suggested that apomictic reproduction occurs (Mears 1970). Narayan (1955) tried and explained the limited number of pollination he observed on female-fertile Rongai strain as that apomictic reproduction may have occurred from the formation of haploid aposporic embryo sacs. However, Narayan's conclusion was disputed by Carr and Ng in favour of the hypothesis that some strains are genetically male-sterile and can produce seeds when fertilized with viable pollen (Carr & Eng 1956). In some cases, kikuyu is clonally propagated (via stolons and rhizomes), can also spread by seeds to multiply and increase stock and to also observe any changes in flowering patterns. Furthermore, since kikuyu exists only as a tetraploid (2n=36), traditional hybridisation techniques may not result in hybrids which fall along the classical Mendelian inheritance, however, useful in the transfer of desirable dominant genes from elite germplasm (Morris 2004).

1.12. Species identification and characterization

The establishment of cultivars or species or varieties identification techniques is essential and has great importance in cultivars or species or varieties certification, studying genetic diversity and determining the genetic relationships between and among them. This could provide valuable information in organizing germplasm resources as well as in maintenance of breeding programs (Monte-Corvo *et al.* 2002). Traditionally, characterisation and identification of cultivars has been conducted according to morphological and physiological aspects. However, the problem with these studies is that they require a large set of phenotypic data and are error-prone due to environmental variations, epistatic interactions and pleitropic effects (Monte-Corvo *et al.* 2002; Sozen 2010).

1.12.1. Taxonomic tree of P. clandestinum (Meredith 1955)

Domain: Eukaryota
Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Monocotyledonae
Order: Cyperales
Family: Poaceae
Genus: Pennisetum
Species: *Pennisetum clandestinum*

1.13. Molecular markers

The emergence of PCR-based molecular markers has created the opportunity to directly analyse plant genomes, allowing a successful means for studying genetic diversity and for cultivar identification. The common techniques include randomly amplified polymorphic DNA (RAPD; (Williams *et al.* 1990; Botta *et al.* 1998; Oliveira *et al.* 1999)), simple sequence repeats (SSR; (Akkaya *et al.* 1992)), amplified fragment length polymorphism (AFLP; (Monte-Corvo *et al.* 2000; Vos *et al.* 1995)) and inter simple sequence repeats (ISSR; (Zietkiewicz *et al.* 1994; Monte-Corvo *et al.* 2002)). Each technique has different advantages and drawbacks. Most of these DNA markers have been used and shown to be powerful tools for characterization and genetic diversity estimation among accessions in forage grasses and other crop species (Xu *et al.* 1995; Hayward *et al.* 1998; Jones *et al.* 2002a; Harris *et al.* 2009; Bert *et al.* 1999; Chotiyarnwong *et al.* 2007). Molecular characterization of cultivars is also useful to evaluate potential genetic erosion, i.e., a reduction of genetic diversity along breeding processes (Rafalski & Tingey 1993; Weising *et al.* 1995).

Apart from the above mentioned molecular techniques, more advanced molecular techniques such as the single nucleotide polymorphism (SNP; Ganal *et al*. 2009), diversity array technology (DArT; (Jaccoud *et al*. 2001; Wenzl *et al*. 2004)), etc. have been invented and extensively employed in various studies including the identification and analysis of quantitative/qualitative trait loci (QTLs) and their positioning on linkage maps; cloning of genes for desirable traits based on the molecular linkage maps; gene pyramiding and marker-assisted selection (MAS); the determination and analysis of genetic diversity within germplasms and other plant collections and analysis of genome structures for several crop plants (Mohan *et al*. 1997; Bagge *et al*. 2007; Peleman & Voort 2003; Deschamps *et al*. 2012).

DArT is a microarray hybridization-based technique that enables the simultaneous typing of several hundred polymorphic loci spread over the genome without the need of prior sequence information (Jaccoud *et al*. 2001; Wenzl *et al*. 2004). The technique is also high-throughput, quick, and highly reproducible.

Although numerous advanced molecular techniques have been invented but microsatellite markers remain a standard for map construction, as they are highly polymorphic even between closely related lines; require a small amount of DNA; can be easily automated; allow high-throughput screening; can be exchanged between laboratories; and are highly

transferable between populations (Gupta *et al.* 1999). AFLP offers a high level of utility for various purposes and has been used to generate large numbers of markers for the construction of high-density genetic maps (Barrett & Kidwell 1998; Huang *et al*. 2000; Chalmers *et al.* 2001).

As applied in breeding programs, the molecular markers play a crucial role in accelerating the incorporation of genes that control or contribute to the variation of the target traits and also provide reliable information of kinship and phylogeny between species (Ranade & Yadav 2014). The molecular marker research has existed for more than three decades now with continuous progress and considerable achievements since the very first molecular marker application as a RFLP was reported by Botstein *et al*. (1980). These molecular markers vary in their resolution power, genome coverage and linkage or otherwise to loci controlling traits of relevance to the breeder. Moreover, these markers have varying levels of complexities of experimental designs, ease of field level application and the need for advanced skill sets and resources for successful application in breeding strategies (Ranade & Yadav 2014).

Breeding programmes are currently searching for improved turf grass cultivars therefore molecular research into the backgrounds of selected types may aid in the discovery and production of improved cultiars. Moreover, this may assist in identifying and tracking the spread of kikuyu grass across South Africa. Until now, research into kikuyu grass has primarily been focused on protein and dry matter production, rather than on genotypes and population structures (Morris 2009).

1.14. Aims and hypothesis of the study

The aims of this study were to:

i) Identify the usefulness of RAPD and ISSR markers in differentiating among and between selected kikuyu grass cultivars;
ii) Identify and analyse the degree of relatedness between selected cultivars under study.

The hypothesis is that even though the kikuyu cultivars used in the study originated from the same source (sharing a common ancestor) over large time periods due to their evolutionary history imply that they are now highly genetically diverse.

Chapter 2

Genetic characterization of *Pennisetum clandestinum* varieties/cultivars using RAPD marker system

2.1.Background on the random amplified polymorphic DNA (RAPD)

Random amplified polymorphism DNA (RAPD) markers (Williams *et al.* 1990) have shown to be a very useful marker system intensively applied in various molecular departments, more specifically in plant research such as phylogenetic studies, population genetic studies, genome mapping, as well as in cultivar identification and germplasm management (Schnell *et al.* 1995; Loureiro *et al.* 1998; Qian *et al.* 2001; Bandelj *et al.* 2002). Several advantages are associated with this molecular technique including the simplicity of use, the use of a small amount of plant material and low cost (Fritsch & Rieseberg 1996) and the fact that it does not require prior sequence information. However, this technique possesses several limitations including dominance, reproducibility, and uncertain locus homology, sensitivity to the reaction conditions, and reliability from lab to lab (Williams *et al.* 1990).

RAPD fingerprinting has been successfully used to distinguish genetic diversity amongst a range of turfgrasses including perennial ryegrass (*Lolium perenne* L.) (Bolaric *et al.* 2005); buffalo grass [*Buchloë dactyloides* (Nutt.) Engelm.] (Huff *et al.* 1993); Kentucky bluegrass (*Poa pratensis*) (Huff 2001); and couch grass (*Cynodon* spp.) (Karaca *et al.* 2002). Furthermore, RAPDs have been used in the differentiation of olive cultivars (*Olea europaea* L.) (Cresti *et al.* 1996; Khadari *et al.* 2003; Martins-Lopes *et al.* 2007), to study inter- or intra-cultivar genetic diversity (Wiesman *et al.* 1998; Belaj *et al.* 2002; Roselli *et al.* 2002; Gemas *et al.* 2004), to establish genetic relationships between cultivars (Besnard *et al.* 2001a; Belaj *et al.* 2002; Khadari *et al.* 2003), and to study genetic differentiation in the olive

complex (Besnard *et al.* 2001b). Concluding from the extensive and successful use of RAPDs in the identification and/or differentiation of crop species including turfgrasses, a study was conducted with an aim to differentiate between forty kikuyu grass cultivars using RAPD markers. Moreover, to identify and analyse the degree of relatedness (genetic relationships) between the cultivars using a phylogenetic software, raxmlGUI1.3.

2.2.Materials and method

2.2.1. Plant material

The grass samples (leaves) were collected from the Agricultural Plant Breeding Institute, Cedara, Howick, South Africa from a total of forty (1-40) planted kikuyu lines/cultivars. These breeding lines are duplicates of the forty-one (41) kikuyu lines which were originally sent from New Zealand and were planted in 1 x 3m plots in the nursery at Döhne research station, Eastern Cape, South Africa during the 1998 planting season. Analyses on total production and digestibility of the cultivars were conducted from a period of 1999 to 2000 by De Ridder, 1999. Any additional information regarding the cultivars was kept confidential by the Agricultural Plant Breeding Institute from which the samples were provided.

Table 2.1 Forty kikuyu lines (cultivars) used in the study and their nutritional ratings in terms of digestibility.

Cultivar	**Sample identity number**	**Digestion value**	**Final digestion value (x 0.7997) (%)**	**Rating**
Cultivar 1	1	80.4	64.29588	16
Cultivar 2	2	80.2	64.13594	18
Cultivar 3	3	79.6	63.65612	23
Cultivar 4	4	72	57.5784	37
Cultivar 5	5	82	65.5754	9
Cultivar 6	6	74.8	59.81756	33
Cultivar 7	7	76.4	61.09708	30
Cultivar 8	8	82	65.5754	10
Cultivar 9	9			
Cultivar 10	10	74.6	59.65762	34
Cultivar 11	11	79.8	63.81606	20
Cultivar 12	12	73.2	58.53804	35
Cultivar 13	13	68	54.3796	41
Cultivar 14	14	69.6	55.65912	40
Cultivar 15	15			

Cultivar 16	16	77.8	62.21666	27
Cultivar 17	17	80.4	64.29588	14
Cultivar 18	18	82.4	65.89528/	6
		76.4	61.09708	31
Cultivar 19	19	76	60.7772	32
Cultivar 20	20	80.2	64.13594	17
Cultivar 21	21	82.6	66.05522	4
Cultivar 22	22	82.4	65.89528	7
Cultivar 23	23	87	69.5739	1
Cultivar 24	24	83.6	66.85492	3
Cultivar 25	25	79.6	63.65612	22
Cultivar 26	26	71.4	57.09858	38
Cultivar 27	27	77.2	61.73684	28
Cultivar 28	28	72	57.5784	36
Cultivar 29	29	79.4	63.49618	24
Cultivar 30	30	84.2	67.33474	2
Cultivar 31	31	79.8	63.81606	21
Cultivar 32	32	80.8	64.61576	13
Cultivar 33	33	77	61.5769	29
Cultivar 34	34	81.6	65.25552	12
Cultivar 35	35	82.2	65.73534	8
Cultivar 36	36	71.2	56.93864	39
Cultivar 37	37	80.4	64.29588	15
Cultivar 38	38	78	62.3766	26
Cultivar 39	39	82	65.5754	11
Cultivar 40	40	80	63.976	19

According to digestibility ratings above, cultivar 23 appeared to be highly digestible (rates position 1 with 69.57 %), followed by cultivar 30 (rates position 2 with 67.33 %), 24 (rates position 3 with 66.85), and 21 (rates position 4 with 66.06 %). Cultivars 13 and 14 occupy the last positions, 41 (54.34 %) and 40 (55.66 %) respectively.

2.2.2. Template DNA isolation

Healthy young leaf material was collected from each plant (forty cultivars) placed into a plastic pocket and stored in a deep freezer for quite some time (at least 3 months) before the establishment of the research study. At that point, all materials and equipment necessary for experimentation was being gathered. Prior to DNA extraction, frozen samples were defrosted and approximately 200 mg wet weight per sample disrupted in liquid nitrogen using a mortar and pestle, put in two separate microcenrtifuge tubes and stored in a freezer.

DNA was extracted using the DNeasy Plant Mini Kit from Qiagen (Southern Cross Biotechnology (Pty) Ltd, Cape Town, South Africa) according to the manufacturer's recommendations. Following extraction, DNA concentration was determined by NanoDrop

2000 Spectrophotometer and was checked for integrity on a 1 % agarose gel. All DNA samples were diluted to7.5 ng/µL with sterile MQ water before proceeding to PCR.

2.2.3. Primer selection

A total of thirteen decamer primers (Operon Technologies, Inc., Alameda, California) were selected based on studies in *Cynodon* spp. by Ho *et al.* (1997) and Karaca *et al.* (2002). All the thirteen primers provided reproducible banding patterns and were used in the statistical analysis, Table 2.2.

Table 2.2. RAPD Primers used for the study.

Primer	**Sequence (5' – 3')**
OPAA	GTGGGTGCCA
OPAE	CTGAAGCGCA
OPM20	AGGTCTTGGG
K07	AGCGAGCAAG
A17	GACCGCTTGT
K17	CCCAGCTGTG
P19	GGGAAGGACA
M01	GTTGGTGGCT
OPK20	GTGTCGCGAG
OPO06	CCACGGGAAG
OPA11	CAATCGCCGT
OPB15	GGAGGGTGTT
OPB17	AGGGAACGAG

Source: Morris (2009).

2.2.4. PCR Amplification

Amplification was conducted via the polymerase chain reaction (PCR) technique. The 12.5 µL reaction mixture, Table 2.3, consisted of 10x KAPA *Taq* Buffer (KAPA Biosystems, Cape Town, South Africa), 2.5 mM dNTP, 25 mM $MgCl_2$, 10 µM primer, 5 U/µL of Ampli*Taq* DNA Polymerase (Inqaba Biotechnical Industries (Pty) Ltd., Hatfield, South Africa) or KAPA *Taq* DNA Polymerase (KAPA Biosystems, Cape Town, South Africa) and 7.5 ng/µL of the template DNA with the remainder consisting of sterile MQ water. Controls consisted of 2.5 µL sterile MQ water instead of template DNA. After an initial denaturation cycle of 94°C for 4 min the reaction mix was subjected to 45 cycles of 94°C for 15 sec (denaturation), 40 - 47°C (depending on a specific primer) for 30 sec (annealing), and 72°C for 90 sec (extension). Final extension was at 72°C for 4 min, before holding at 4°C, Table 2.4.

Table 2.3. PCR reaction mixture

PCR Reaction Mixture	Per 12.5 µL reaction
Sterile MQ water	6.65 µL
10x KAPA *Taq* Buffer	1.25 µL
dNTP (2.5 mM)	1.25 µL
MgCl2 (25 mM)	0.5 µL
Primer (10 µM)	0.25 µL
DNA (7.5 ng/µL)	2.5 µL
Ampli*Taq* Polymerase (5 U/µL)	0.1 µL
Total	**12.5 µL**

Table 2.4. PCR cycling times and temperatures

Cycle	Temperature	Time
Initial denaturation	94°C	4 min
45 cycles of:		
Denaturation	94°C	15 sec
Annealing	40 - 47°C	30 sec
Extension	72°C	90 sec
Final extension	72°C	4 min
Hold	4°C	

2.2.5. Visualisation

Amplified products were mixed with 4 µL of DNA loading dye (6X) (0.1 % SDS, 60 mM Tris-HCl (pH 8.0), 60 mM EDTA, 0.125 % Orange G, 0.025 %, xylene cyanol and 60 % glycerol) and separated on horizontal 2 % agarose gels in 1x TBE buffer (0.5 M EDTA (pH 8.0), Boric acid, Tris (hydroxymethyl) aminomethane and sterile water) stained with ethidium bromide, alongside KAPA Universal ladder or 1 kb ladder (KAPA Biosystems, Cape Town, South Africa). The gels were electrophoresis in 1x TBE buffer at 100 V for 2 - 3 h before images were captured using a Gel Doc-It imaging system (UVP Bioimaging Systems, Upland, California) under UV light.

2.2.6. Statistical analysis

The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands (banding pattern) across all primers and matrices of the different RAPD profiles were assembled and used in the statistical analysis. The fragments were only considered when

reproducible bands were obtained, implying that for each primer PCR reaction was repeated at least two times with the same result. Band scoring was analysed and FastTree dendrograms constructed using the raxmlGUI1.3 and viewed using FigTree v1.4.0 analysis programs (Silvestro & Michalak 2012).

2.3.Results and discussion

2.3.1. DNA Purity and quantity

DNA extractions were successful using the DNeasy Plant Mini Kit from Qiagen. The genomic DNA extracted was of fair quality with an A260/A280 average ratio of 2.20, and solid bands were produced on agarose gels. The A260/A280 average ratio is in fairly agreement with findings of other studies, for example, Morris (2009) got an average ratio of 1.91 with kikuyu grass having the lowest compared to buffalo (*Stenotaphrum secundatum* – A_{260}/A_{280} ratio average: 2.17) and couch (*Cynodon dactylon* - A_{260}/A_{280} ratio average: 2.07) samples extracted with the same method (ABI PRISM 6100 Nucleic Acid Preparation Station extraction protocol from Applied Biosystems, Foster City, California) at the same time but for other study purposes.

A DNA concentration average of 37.1 ng/uL was achieved. Usually, the quantity of DNA extracted from kikuyu is less than other grass types, partly due to the coarseness of the leaf blade when sampling a small amount even when using fresh leaf tissue. This was confirmed when Morris (2009) compared kikuyu with a similar coarse leaf such as buffalo and got an average total of 76.2 ng/µL extracted from kikuyu compared to an average of 108.2 ng/µL from buffalo.

2.3.2. Primer banding pattern images

A total of thirteen decamer primers of arbitrary nucleotide sequences were selected and used until the end of the study, Table 2.2. Examples of banding patterns of the thirteen primers used in the study are found following as Figures 2.1 – 2.5.

L 1 2 3 4 5 6 7 8 9 10 11 12 L

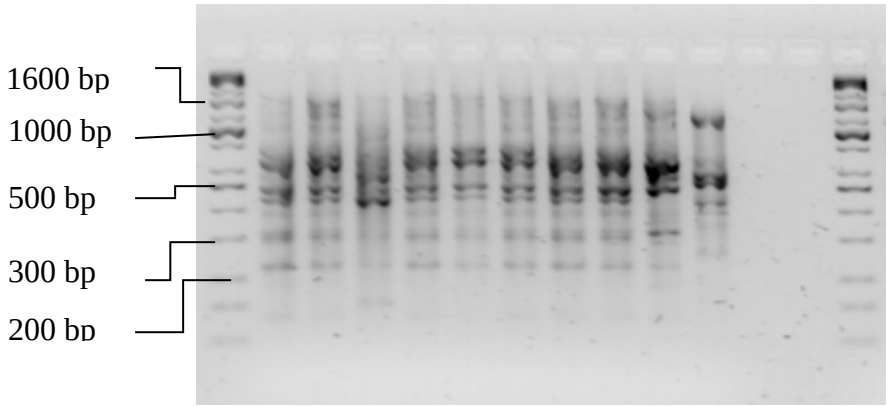


Figure 2.1. RAPD DNA banding patterns of kikuyu grass with RAPD primer 9 (0PK20) on cultivars 1 – 10 respectively. (L) represents the Universal ladder, (1) cultivar 1, (2) cultivar 2, (3) cultivar 3, (4) cultivar 4, (5) cultivar 5, (6) cultivar 6, (7) cultivar 7, (8) cultivar 8, (9) cultivar 9, (10) cultivar 10, (11) no-template control (NTC), (12) empty lane, and (L) Universal ladder.

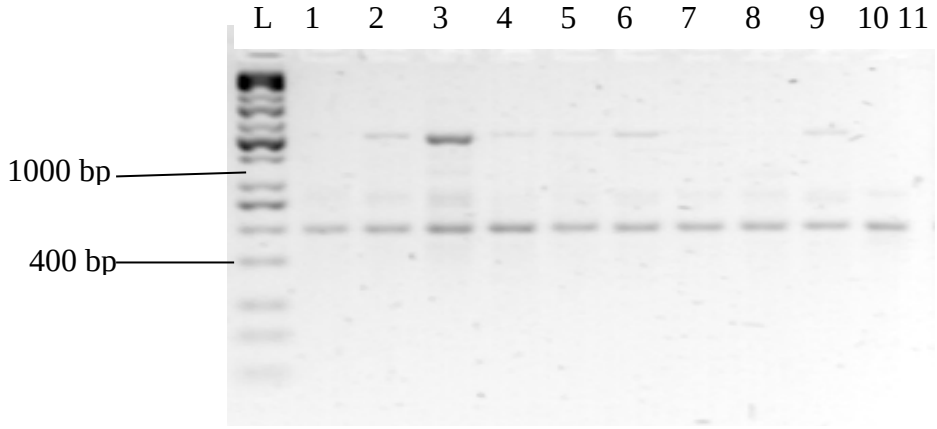
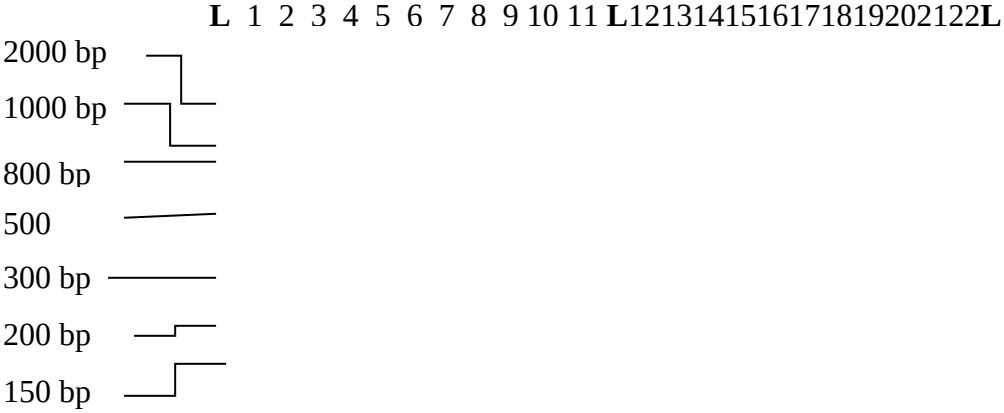


Figure 2.2. RAPD DNA banding patterns of kikuyu grass with RAPD primer 5 (A17) on cultivars 11 – 20 respectively. (L) represents the Universal ladder, (1) cultivar 11, (2) cultivar 12, (3) cultivar 13, (4) cultivar 14, (5) cultivar 15, (6) cultivar 16, (7) cultivar 17, (8) cultivar 18, (9) cultivar 19, (10) cultivar 20 and, (11) no-template control (NTC).



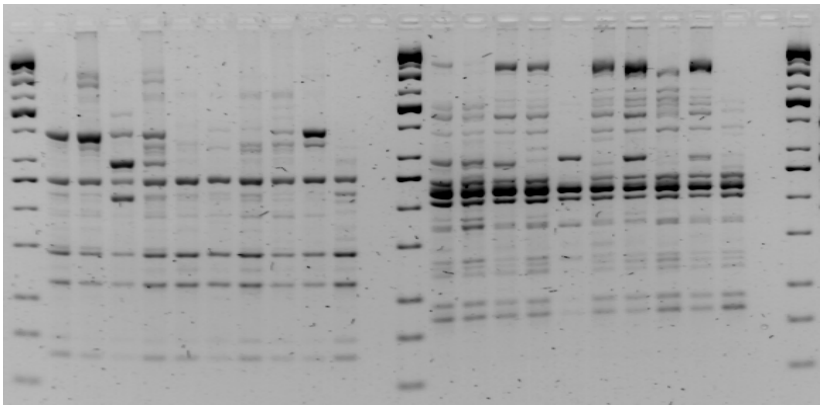


Figure 2.3. RAPD DNA banding patterns of kikuyu grass with RAPD primer 8 (M01) & 9 (0PK20) on cultivars 11 – 20 respectively. (L) represents the Universal ladder, (1) and (12) cultivar 11, (2) and (13) cultivar 12, (3) and (14) cultivar 13, (4) and (15) cultivar 14, (5) and (16) cultivar 15, (6) and (17) cultivar 16, (7) and (18) cultivar 17, (8) and (19) cultivar 18, (9) and (20) cultivars 19, (10) and (21) cultivar 20 and, (11) and (22) no-template control (NTC).

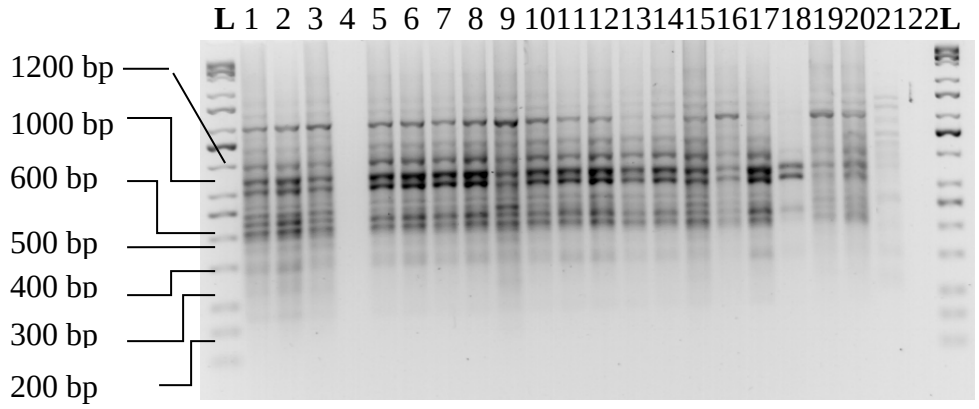
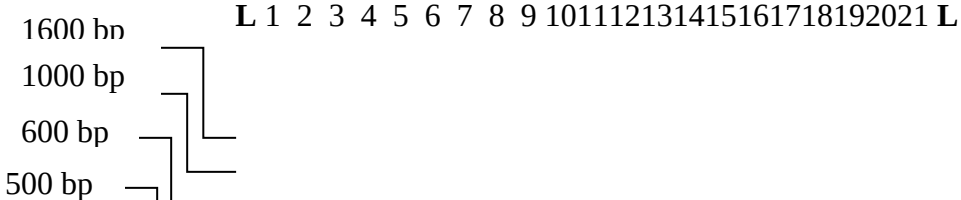


Figure 2.4. RAPD DNA banding patterns of kikuyu grass with RAPD primer 4 (K07) on cultivars 21 – 40 respectively. (L) represents the Universal ladder, (1) cultivar 21, (2) cultivar 22, (3) cultivar 23, (4) empty lane, (5) cultivar 25, (6) cultivar 26, (7) cultivar 27, (8) cultivar 28, (9) cultivar 29, (10) cultivar 30, (11) cultivar 31, (12) cultivar 32, (13) cultivar 33, (14) cultivar 34, (15) cultivar 35, (16) cultivar 36, (17) cultivar 37, (18) cultivar 38, (19) cultivar 39, (20) cultivar 40, (21) no-template control (NTC) and, (22) empty lane.



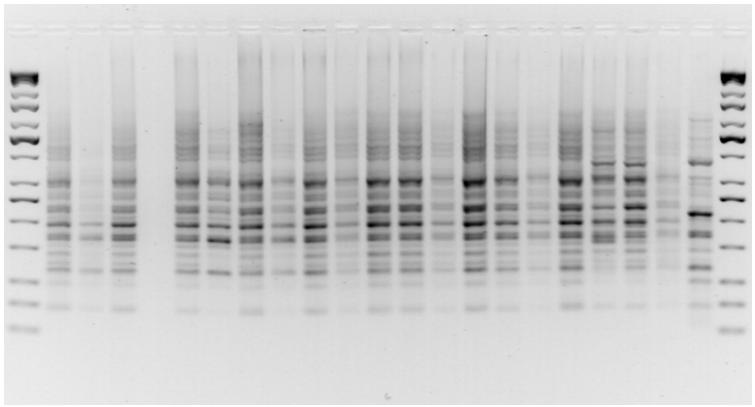


Figure 2.5. RAPD DNA banding patterns of kikuyu grass with RAPD primer 8 (M01) on cultivars 21 – 40 respectively. (L) represents the Universal ladder, (1) cultivar 21, (2) cultivar 22, (3) cultivar 23, (4) empty lane, (5) cultivar 25, (6) cultivar 26, (7) cultivar 27, (8) cultivar 28, (9) cultivar 29, (10) cultivar 30, (11) cultivar 31, (12) cultivar 32, (13) cultivar 33, (14) cultivar 34, (15) cultivar 35, (16) cultivar 36, (17) cultivar 37, (18) cultivar 38, (19) cultivar 39, (20) cultivar 40 and, (21) no-template control (NTC).

The banding pattern images above illustrate examples of different banding patterns produced by the RAPD primers used in the study. Each primer produce a different pattern from which the cultivars can be distinguished. However, some primers were monomorphic (could not differentiate between the cultivars) and thus non-informative, for example, RAPD primer 5 (A17) (figure 2.2).

2.3.3. DNA Amplification banding patterns

The thirteen RAPD primers yielded a total of 144 loci from the forty kikuyu cultivars under the study. The primers produced an average range of between 5 (primer 12, OPB15) and 19 (primer 8, M01 and primer 9, OPK20) loci per primer. Band size ranged from 100 bp to 2.0 kb. Of these, 80 (55.56 %) were polymorphic and 64 (44.44 %) were monomorphic, Table 2.5. None of the primers only produced polymorphic markers. The percentage of polymorphic fragments ranged from as little as 8 % (OPA11) to 80 % (OPB15).

These results show that the forty cultivars of kikuyu grass are moderately polymorphic (55.56 %) using the RAPD fingerprinting technique analysis. It is also shown that the primers used for the study were informative as they could discriminate between the cultivars.

Table 2.4. RAPD primers and marker results for RAPD profiling on kikuyu grass cultivars.

Primer no.	Primer	Sequence	No. of scorable bands	No. of polymorphic bands	No. of monomorphic bands	% Polymorphism

1	OPAA	GTGGGTGCCA	8	6	2	75.0
2	OPAE	CTGAAGCGCA	8	3	5	37.5
3	OPM20	AGGTCTTGGG	10	7	3	70.0
4	K07	AGCGAGCAAG	13	7	6	53.8
5	A17	GACCGCTTGT	7	5	2	71.4
6	K17	CCCAGCTGTG	9	6	3	66.7
7	P19	GGGAAGGACA	9	3	6	33.3
8	M01	GTTGGTGGCT	19	10	9	52.6
9	0PK20	GTGTCGCGAG	19	11	8	57.9
10	OPO06	CCACGGGAAG	13	8	5	61.5
11	OPA11	CAATCGCCGT	12	1	11	8.3
12	OPB15	GGAGGGTGTT	5	4	1	80.0
13	OPB17	AGGGAACGAG	12	9	3	75.0
Total			**144**	**80(55.56)**	**64(44.44)**	

Ten of thirteen primers used in this study were able to differentiate over 50 % of the cultivars tested. The other three primers, primer 11 (OPA11), primer 7 (P19) and, primer 2 (OPAE) were able to differentiate as little as 8, 33 and, 38 % of the cultivars respectively. Some primers displayed a high level of similarity (monomorphism) among the cultivars tested i.e. with primer 11 (OPA11) cultivars could not be distinguished from each other. The majority of the primers were able to differentiate the majority of the cultivars. This includes primer 2 (OPAE), primer 5 (A17), primer 6 (K17), primer 7 (P19), primer 10 (OPO06) and primer 12 (OPB15). Cultivars with closest similarity as shown by the cluster analysis were only separated by a single marker. RAPD primer 1 (OPAA), primer 3 (OPM20), primer 4 (K07), primer 8 (M01) and primer 9 (0PK20) and primer 13 (OPB17) were able to discriminate among all cultivars under study.

2.3.4. Cluster analysis

Cluster analysis of the thirteen (13) informative primers was able to produce an unrooted tree with raxmlGUI1.3 analysis providing a grouping of 8 distinct clusters (comprised of 3 larger clusters and 5 smaller ones) and 9 independent branches, Figure 3.

Cluster 1 (pink coloured) is the second largest cluster of them all and is comprised of 7 cultivars: cultivar 30, cultivar 40, cultivar 39, cultivar 38, cultivar 34, cultivar 36, cultivar 35. In this cluster, the following cultivar pairs: 34 and 36, 30 and 40, were found to be more closely related than all other cultivars as shown by the cluster analysis. The following cultivars were also found located on the same cluster (cluster 1) in the ISSR FastTree

dendrogram, figure 4: cultivar 30, cultivar 40, cultivar 35, cultivar 39, cultivar 38, cultivar 34, and cultivar 36.

Cluster 2 (purple coloured) is a smaller cluster comprised of only two closely related cultivars, cultivar 25 and cultivar 28. Following is another smaller cluster (lime coloured) also consisting of 3 related cultivars, cultivar 26, cultivar 22 and cultivar 32 with cultivar 22 and 26 sharing some similarity than cultivar 32. Cultivar 33 and 27 formed another mini-cluster (dark-green coloured). Cluster 5 (light-blue coloured) consisted of 3 cultivars: cultivar 23 and 21 which formed a pair alongside with cultivar 29.

Cluster 6 (blue coloured) is the largest cluster of them all and is made up of 8 cultivars: cultivar 15, cultivar 20, cultivar 16, cultivar 11, cultivar 19, cultivar 12, cultivar 14 and, cultivar 13. In this cluster, the following cultivar pairs: 12 and 14, 15 and 20, were found to be more closely related than all other cultivars within the cluster as shown by the cluster analysis. The following cultivars were also found located in one cluster (cluster 4) in the ISSR FastTree dendrogram, figure 4: cultivar 11, cultivar 20, cultivar 19, cultivar 16, cultivar 14, cultivar 13, cultivar 15, and cultivar 12; with cultivar 13 and 14 sharing more similarity than any other cultivar within the cluster.

Cluster 7 (red coloured) consist of 4 cultivars: cultivar 6, cultivar 8, cultivar 9 and cultivar 5 with cultivar 6 and 9 being closely related than other cultivars within the cluster. Cultivar 6 and 9 existed as separate members of two different but closely located mini-clusters (cluster 2 and cluster 3) in the ISSR FastTree dendrogram, figure 4. Lastly, cluster 8 (brown coloured) is a mini-cluster made up of only two closely related cultivars: cultivar 24 and cultivar 4.

When linking the cluster analysis results to the nutritional rating data in terms of percentage digestibility (Table 2.1), the two aspects fairly correlated since some of the cultivar pairs disproved this. The two aspects showed a strong correlation with cultivar pair 27 and 33 (dark-green coloured, mini-cluster 4). The two cultivars occupy consecutive positions 28 (61.73684 %) and 29 (61.5769 %) particularly. Cultivars 23 and 21 were found located on the same cluster, cluster 5 (light-blue coloured) and their ratings on percentage digestibility are positions 1 (69.5739) and 4 (66.05522) respectively. Cultivar 12 and 14 (cluster 6) were shown to share more similarity than any other cultivar within this larger

cluster and, on digestibility ratings the two cultivars occupy positions 35 (58.53804 %) and 40 (55.65912 %) respectively.

2.3.5. Conclusion

Polymorphism between and among species can result from different events and is of much importance in plant breeding programs (Bornet & Branchard 2004). The results obtained from cluster analysis demonstrated a positive correlation between the chemical composition (percentage digestion) and the genetic constitute of the kikuyu grass cultivars under study. The findings of the study using RAPD marker system indicated that kikuyu grass is a polymorphic species (80 markers (55.56 %) from 13 informative primers). This also showed that RAPD assay is still an effective method for identifying significant genetic variation within kikuyu grass. Also, the cluster analysis results proved that these kikuyu grass cultivars are highly genetically variable as expected.

Chapter 3

Genetic characterization of *Pennisetum clandestinum* varieties/cultivars using ISSR marker system

3.1. Background on the inter simple sequence repeats (ISSR) molecular technique

Inter simple sequence repeat (ISSR) is an alternative PCR based molecular technique invented to study polymorphism based on the presence of microsatellites throughout genomes (Zietkiewicz *et al.* 1994). Polymorphism between and among species can result from different events such as mutations and is of much importance in plant breeding programs (Bornet & Branchard 2004). This molecular technique amplifies regions (100 - 3,000 bp) between inversely oriented closely located microsatellites, and it is preferred in comparison to the RAPD technique even though much better and advanced molecular techniques such as the single nucleotide polymorphism (SNP), diversity array technology (DArT), etc. exist. (Zietkiewicz *et al.* 1994). Like common simple sequence repeats (SSR), ISSRs primers may

be dinucleotide, trinucleotide, tetranucleotide or pentanucleotide repeats (Zietkiewicz *et al.* 1994).

ISSR markers possess similar advantages to RAPDs (Fang & Roese 1997) but the ISSRs are more informative, more reproducible and have been reported to produce more complex marker patterns than the RAPD assay (Chowdhury *et al.* 2002), which is advantageous when differentiating closely related cultivars. These conclusions were drawn from various studies i.e Nagaoka and Ogihara (Nagaoka & Ogihara 1997) observed that ISSR amplification was much more informative than RAPDs for genetic diversity evaluation in wheat (*Triticum aestivum*); Korbin *et al.* (2002) observed the same in fruit plants and Galvan *et al.* (2003) observed it with common bean (*Phaseolus vulgare*). ISSR markers are considered to be more reproducible than RAPD markers due to high annealing temperature (Bornet & Branchard 2001; Chowdhury *et al.* 2002).

ISSR markers have been employed in numerous studies and turned out successful. They have been used to measure genetic diversity in barley (Fernández *et al.* 2002) and rice (Joshi *et al.* 2000), also for cultivar identification in maize (Pejic *et al.* 1998), wheat (Nagaoka & Ogihara 1997), potato (Prevost & Wilkinson 1999) and bean (Métais *et al.* 2000), peanut (Raina *et al.* 2001), strawberry (Arnau *et al.* 2003) and cicer (Sudupak 2004). The successful use of this marker technique in previous studies has motivated the conduction of the present study with an aim to differentiate between forty kikuyu grass cultivars using the same technique, ISSR markers. The study also aimed to identify and analyse the degree of relatedness (genetic relationships) between the cultivars using a phylogenetic software, raxmlGUI1.3.

3.2.Materials and methods

Same DNA material from the previous chapter (chapter 2, 2.2.1 – 2.2.2) was used for PCR amplification.

3.2.1. Primer selection

A set of sixteen oligonucleotide primers were selected and used to obtain specific molecular markers based on various grass studies. Primers were adapted from various papers by Arslan *et al*. (2011), Farsani *et al*. (2012), de Lima *et al*. (2011), Poulin *et al*. (2005) and Reddy *et al*. (2009). Out of sixteen only fourteen primers provided reproducible banding

patterns and were selected for the final study, Table 3.1. ISSR primer 3 and ISSR primer 16 failed to amplify and were omitted from the final analysis.

Table 3.1. ISSR Primers used for the study.

Primer	Sequence
ISSR1	$((GA)_9C)$[1]
ISSR2	$(GA)_8$
ISSR3	$((GATA)_4)$[2*]
ISSR4	$((AC)_8CG)$[3]
ISSR5	$((GA)_8T)$[3]
ISSR6	$((GA)_8TA)$[2]
ISSR7	$((TG)_8C)$[3]
ISSR8	$(CA(GT)_8)$[3]
ISSR9	$((AC)_8GA)$[4]
ISSR10	$(T(AG)_9)$[4]
ISSR11	$(GA(CA)_8)$[4]
ISSR12	$((CAC)_3GC)$[5]
ISSR13	$((CTC)_3GC)$[5*]
ISSR14	$((GTG)_3GC)$[5]
ISSR15	$((AG)_8CTA)$[6]
ISSR16	$((GACA)_4)$[6]

* ISSR primers omitted from the final analysis.

Source: [1]Arslan *et al*. (2011); [2]Reddy *et al*. (2009); [3]Farsani *et al*. (2012); [4]Al-Humaid *et al*. (2011) and; [5]de Lima *et al*. (2011).

3.2.2. PCR Amplification

Amplification was conducted via the polymerase chain reaction (PCR) technique. The 12.5 µL reaction mixture, Table 3.1, consisted of 10x KAPA *Taq* Buffer (KAPA Biosystems, Cape Town, South Africa), 2.5 mM dNTP, 25 mM MgCl2, 10 µM primer, 5 U/µL of KAPA *Taq* DNA Polymerase (KAPA Biosystems, Cape Town, South Africa) and 7.5 ng/µL of the template DNA with the remainder consisting of sterile MQ water. Controls consisted of 2.5 µL sterile MQ water in place of template DNA. After an initial denaturation cycle of 94°C for 4 min the reaction mix was subjected to 45 cycles of 94°C for 15 sec (denaturation), 38.9* – 55.16°C* (depending on a specific primer) for 30 sec (annealing), and 72°C for 90 sec (extension). Final extension was at 72°C for 4 min, before holding at 4°C, Table 3.2.

*Indicates an annealing temperature 5°C below each primer's melting point temperature (Tm).

Table 3.2. PCR reaction mixture

PCR Reaction Mixture	Per 12.5 µL reaction
Sterile MQ water	6.65 µL
10x KAPA *Taq* Buffer	1.25 µL
dNTP (2.5 mM)	1.25 µL
MgCl2 (25 mM)	0.5 µL
Primer (10 µM)	0.25 µL
DNA (7.5 ng/µL)	2.5 µL
KAPA *Taq* Polymerase (5 U/µL)	0.1 µL
Total	**12.5 µL**

Table 3.3. PCR cycling times and temperatures

Cycle	Temperature	Time
Initial denaturation	94°C	4 min
45 cycles of:		
Denaturation	94°C	15 sec
Annealing	38.9* – 55.16°C*	30 sec
Extension	72°C	90 sec
Final extension	72°C	4 min
Hold	4°C	

3.2.3. Visualisation

Amplified products were mixed with 4 µL of DNA loading dye (6X) (0.1% SDS, 60 mM Tris-HCl (pH 8.0), 60 mM EDTA, 0.125 % Orange G, 0.025 %, xylene cyanol and 60 % glycerol) and separated on horizontal 2 % Agarose gels in 1x TBE buffer (0.5 M EDTA (pH 8.0), Boric acid, Tris (hydroxymethyl) aminomethane and sterile water) stained with ethidium bromide, alongside KAPA Universal ladder or 1kb ladder (KAPA Biosystems, Cape Town, South Africa). The gels were electrophoresis in 1x TBE buffer at 100 V for 2 - 3 h before images were captured using a Gel Doc-It imaging system (UVP Bioimaging Systems, Upland, California) under UV light.

3.2.4. Statistical analysis

The PCR fragments were scored for the presence (1) or absence (0) of equally sized bands (banding patterns) across all primers and matrices of the different ISSR phenotypes were assembled and used in the statistical analysis. The fragments were only considered when reproducible bands were obtained, implying that for each primer the PCR reaction was repeated at least two times with the same result. Band scoring was analysed and FastTree dendrograms constructed using the raxmlGUI1.3 and viewed using FigTree v1.4.0 analysis programs (Silvestro & Michalak 2012).

3.3.Results and discussion

3.3.1. Primer banding pattern images

A total of sixteen decamer primers of arbitrary nucleotide sequences were selected and only fourteen were finally used until the end of the study, Table 3.1. Examples of banding patterns of the final primers used in the study are found following as Figures 3.1 – 3.5.

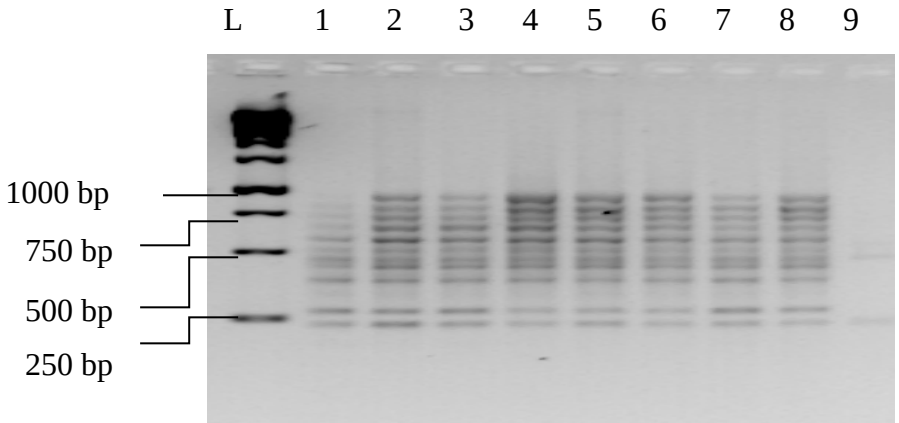


Figure 3.1. ISSR DNA banding patterns of kikuyu grass with primer ISSR15 on cultivars 2 – 9 respectively. (L) represents the 1kb ladder, (1) cultivar 2, (2) cultivar 3, (3) cultivar 4, (4) cultivar 5, (5) cultivar 6, (6) cultivar 7, (7) cultivar 8, (8) cultivar 9 and, (9) no-template control (NTC).

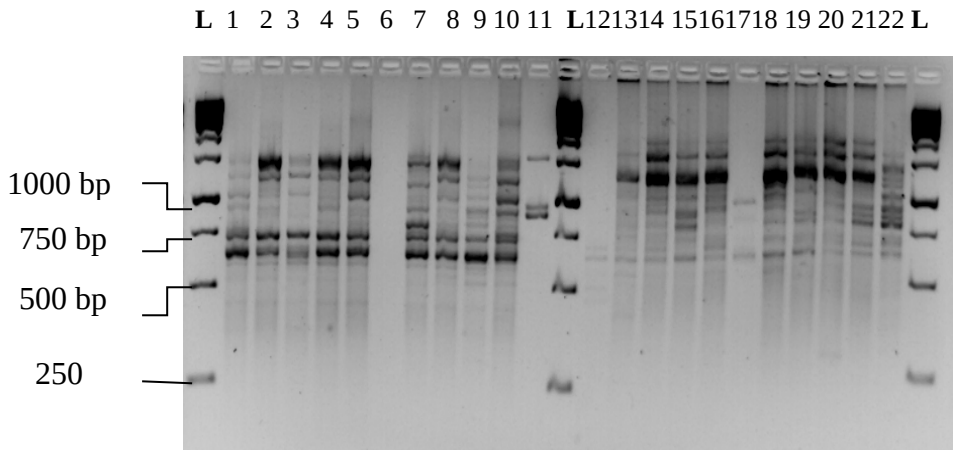


Figure 3.2. ISSR DNA banding patterns of kikuyu grass with primer ISSR1 & ISSR4 on cultivars 11 – 20 respectively. (L) represents the 1kb ladder, (1) and (13) cultivar 11, (2) and (14) cultivar 12, (3) and (15) cultivar 13, (4) and (16) cultivar 14, (5) and (17) cultivar 15, (6) and (18) cultivar 16, (7) and (19) cultivar 17, (8) and (20) cultivar 18, (9) and (21) cultivars 19, (10) and (22) cultivar 20 and, (11) and (12) no-template control (NTC).

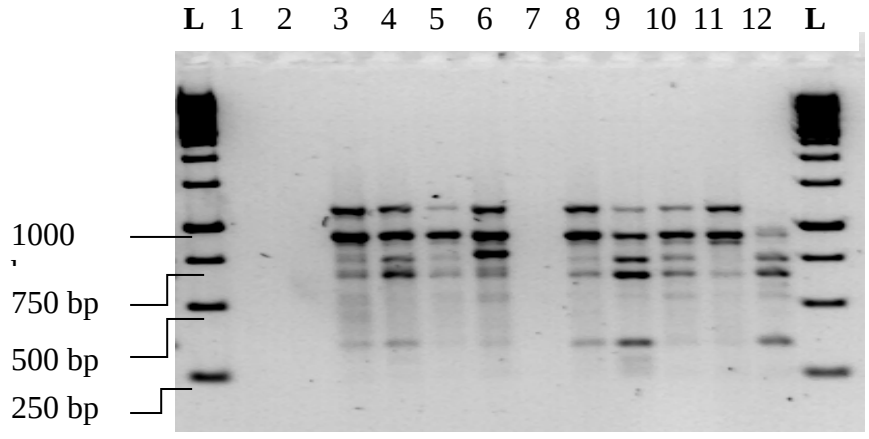


Figure 3.3. ISSR DNA banding patterns of kikuyu grass with primer ISSR15 on cultivars 21 – 30 respectively. (L) represents the 1kb ladder, (1) and (2) empty lanes, (3) cultivar 21, (4) cultivar 22, (5)

cultivar 23, (6) cultivar 25, (7) cultivar 26, (8) cultivar 27, (9) cultivar 28, (10) cultivar 29, (11) cultivar 30 and, (12) no-template control (NTC).

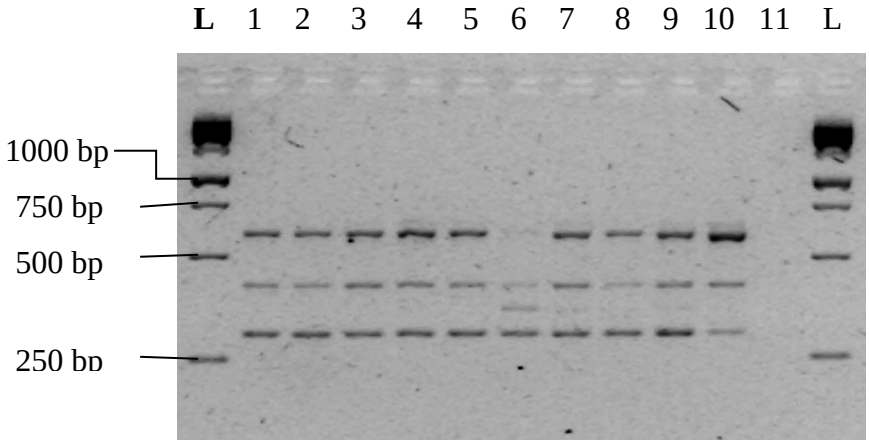


Figure 3.4. ISSR DNA banding patterns of kikuyu grass with primer ISSR11 on cultivars 21 – 30 respectively. (L) represents the 1kb ladder, (1) cultivar 21, (2) cultivar 22, (3) cultivar 23, (4) cultivar 25, (5) cultivar 26, (6) cultivar 27, (7) cultivar 28, (8) cultivar 29, (9) cultivar 30, (10) no-template control (NTC) and, (11) empty lane.

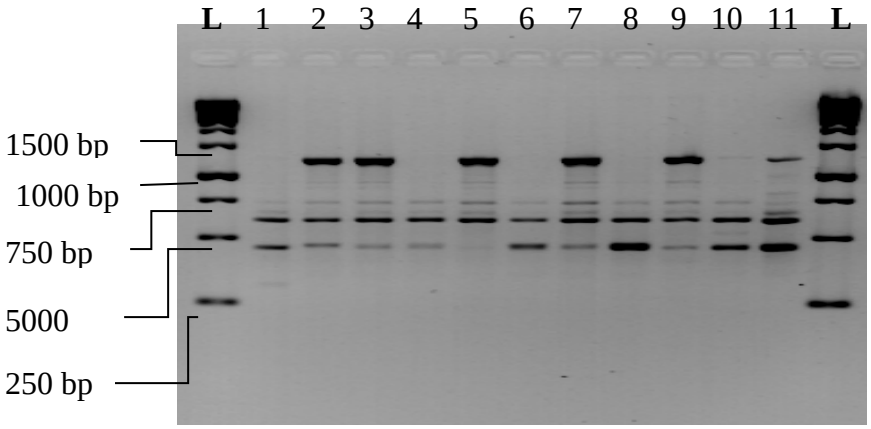


Figure 3.5. ISSR DNA banding patterns of kikuyu grass with primer ISSR12 on cultivars 31 – 40 respectively. (L) represents the 1kb ladder, (1) cultivar 31, (2) cultivar 32, (3) cultivar 33, (4) cultivar 34,

(5) cultivar 35, (6) cultivar 36, (7) cultivar 37, (8) cultivar 38, (9) cultivar 39, (10) cultivar 49 and, (11) no-template control (NTC).

3.3.2. DNA Amplification banding patterns

ISSR3 and ISSR13 failed to amplify some cultivars and were omitted from the study. Fourteen ISSR primers, including 11 di-nucleotide repeats, 2 tri-nucleotide repeats, and 1 tetra-nucleotide repeats yielded a total of 90 loci from the forty kikuyu cultivars tested. The primers produced an average range of between 4 (ISSR11 and ISSR11) and 11 (ISSR14) marker loci per primer. Band size ranged from 250 bp to 2.5 kb. Of these, 56 (62.22 %) were polymorphic and 34 (37.78 %) were monomorphic, Table 3.4. None of the primers only produced polymorphic loci. The percentage of polymorphic fragments ranged from 36 (ISSR14) to 83 % (ISSR4).

Table 3.4. ISSR primers and average marker results for the ISSR profiling on forty kikuyu grass cultivars.

Primer	Sequence	No. of scorable bands	No. of polymorphic bands	No. of monomorphic bands	% Polymorphism
ISSR1	$(GA)_9C$	10	7	3	70.0
ISSR 2	$(GA)_8$	5	3	2	60.0
ISSR 4	$(AC)_7ACCG$	6	5	1	83.3
ISSR 5	$(GA)_8T$	7	4	3	57.1
ISSR 6	$(GA)_8TA$	6	4	2	66.7
ISSR 7	$(TG)_8C$	4	2	2	50
ISSR 8	$CA(GT)_8$	6	4	2	66.7
ISSR 9	$(AC)_8GA$	5	3	2	60.0

ISSR10	$TA(GA)_8G$	7	4	3	57.1
ISSR11	$GA(CA)_8$	4	3	1	75.0
ISSR12	$CA(CCA)_2CGC$	6	4	2	66.7
ISSR14	$GT(GGT)_2GGC$	11	4	7	36.4
ISSR15	$(AG)_8CTA$	7	5	2	71.4
ISSR16	$(GACA)_4$	6	4	2	66.7
	Total	**90**	**56(62.22)**	**34(37.78)**	

Thirteen of fourteen primers used in this study were able to differentiate over 50 % of the cultivars tested. Only one primer (ISSR14) was able to differentiate 36 % of the cultivars. Some primers displayed a high level of similarity (monomorphism) among the cultivars tested i.e. with ISSR5, ISSR6, ISSR7 and ISSR14 cultivars could not be distinguished from each other. The majority of the primers were able to differentiate the majority of the cultivars but not all of them. This includes ISSR8, ISSR10 and ISSR12. Cultivars with closest similarity as shown by the cluster analysis were only separated by a single marker. ISSR1, ISSR4 and ISSR15 were able to discriminate among all cultivars under study.

3.3.3. Genetic variation of kikuyu grass and cluster analysis

Although kikuyu grass originated in the cooler highlands of eastern Africa, it has adapted to a wide range of environments across South Africa. Unlike other grasses such as couch (*Cynodon dactylon*) which have been the focus of intense breeding activity and has given rise to improved cultivars for particular regions and various purposes, kikuyu has received relatively little attention. The genetic variation detected among kikuyu grass cultivars (figure 4) was expected and is consistent with the findings of Marais *et al.* (2000) who also regarded these cultivars as genetically diverse.

Cluster analysis of the fourteen (14) informative primers was able to produce an unrooted tree with raxmlGUI1.3 analysis providing a grouping of 4 distinct clusters (made of 2 larger clusters and 2 smaller ones) and 6 independent branches, Figure 4. Cluster 1 (pink coloured) is the largest cluster of them all and is comprised of 18 cultivars: cultivar 25, cultivar 31, cultivar 23, cultivar 27, cultivar 30, cultivar 26, cultivar 40, cultivar 29, cultivar 21, cultivar 22, cultivar 35, cultivar 39, cultivar 38, cultivar 34, cultivar 33, cultivar 32, cultivar 37 and cultivar 36. In this cluster, the following cultivar pairs: 25 and 31, 27 and 30, 29 and 40, 35 and 39, were found to be more closely related than all other cultivars as shown by the cluster analysis.

Cluster 2 (lime coloured) is a smaller cluster comprised of only two closely related cultivars, cultivar 8 and cultivar 9. Following is another smaller cluster (red coloured) also consisting of two closely related cultivars, cultivar 6 and cultivar 7. Cluster 4 (blue coloured) is the second large cluster and is comprised of 12 cultivars: cultivar 11, cultivar 18, cultivar 20, cultivar 19, cultivar 24, cultivar 26, cultivar 16, cultivar 14, cultivar 13, cultivar 15, cultivar 12, and cultivar 17. In this cluster, cultivar pairs: 12 and 15, 13 and 14, 24 and 26, and 11 and 18, were found to be more closely related than all other cultivars within the cluster.

When linking the cluster analysis results to the nutritional rating data in terms of percentage digestibility (Table 2.1), the two aspects were found to be in strong correlation. Cultivar 13 and 14 (cluster 4) were shown to be closely related on the FastTree dendrogram (the two actually branched from the common node), figure 4, and on digestibility ratings the two cultivars occupy consecutive positions 41 (54.3796 %) and 40 (55.65912 %) respectively. Cultivars 18 and 19 were found located on the same sub-cluster of cluster 4 and their ratings on percentage digestibility are positions 31 (61.09708 %) and 32 (60.7772 %) respectively.

According to cluster analysis results, cultivars 6 and 7 were found located on the same mini-cluster (cluster 3, red coloured) implying a close relationship, and in terms of digestibility rating the two cultivars occupied fairly close positions, 33 (59.82 %) and 30 (61.10 %) respectively. Moreover, cultivars 25 and 31 (cluster 1) were found on the same cluster and they appeared to be closely related than any other cultivar. On the digestibility ratings the two cultivars occupy positions 22 (63.66 %) and 21 (63.82 %). Finally, cultivars 35 and 39 located on cluster 1 (sharing a common node) rated positions 8 (65.73 %) and 11 (65.57 %) particularly. From the results obtained from cluster analysis it was clearly shown that a positive correlation exists between the chemical composition (percentage digestion) and the genetic constitute of the kikuyu grass cultivars under study. Moreover, the preliminary evaluations undertaken by Marais *et al.* (2000) on the chemical composition (nutritional constitution) of the forty kikuyu lines demonstrated that they are genetically variable and probably they would respond differently to the environmental influence.

3.3.4. Conclusion

Polymorphism between and among species can result from different events and is of crucial importance in plant breeding programs (Bornet & Branchard 2004). The findings of the study using ISSR marker system indicated that kikuyu grass is a polymorphic species (56 markers (62.22 %) from 14 informative primers). This also showed that ISSR assay is an effective method for identifying significant genetic variation within kikuyu grass. Furthermore, the cluster analysis results proved that these kikuyu grass cultivars are genetically variable as expected.

Chapter 4

General conclusions and recommendations

This is the first study using PCR-RAPD and PCR-ISSR techniques to examine genetic relationships between kikuyu lines in South Africa, except for other studies done in Australia i.e. Morris (2009). An average percentage polymorphism of 55.56 % was obtained with RAPD analysis system while a slightly higher value of 62.22 % (56 marker loci) was registered with the ISSR analysis system in forty cultivars of kikuyu under study. This proved the ISSR marker system to be more effective and informative than RAPDs in identifying significant genetic variation within kikuyu grass and many other plant species (Nagaoka and Ogihara, 1997; Korbin *et al*. 2002; Galvan *et al*. 2003). Furthermore, a higher number of marker loci (but lesser polymorphism) was generated with RAPDs, a total of 144 in which 80

of them were polymorphic (55.56 %) in contrast to ISSRs whereby a total of 90 marker loci was generated and 56 of them were polymorphic (62.22 %).

The cluster analysis results for both molecular techniques proved that these kikuyu grass cultivars are genetically variable. The genetic variation detected among kikuyu grass cultivars was expected and is consistent with the findings of Marais *et al.* (2000) who also regarded these cultivars as genetically diverse. The evidence obtained from cluster analysis demonstrated that a positive correlation exists between the chemical compositions (percentage digestion) and the genetic constitute of the cultivars under study. In terms of the hypothesis proposed in Section 1.14, the hypothesis stating a high genetic diversity among the cultivars was supported by the evidence obtained from the molecular techniques, RAPD and ISSR marker systems.

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