Genetic Characterisation of Yam (*Dioscorea* Species, Dioscoreaceae) using Microsatellite Markers

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ABSTRACT

The influence of asexual propagation on the genetic diversity of yams is poorly understood. In tropical and subtropical regions, farming practices had unintended consequences and made it often impossible to distinguish wild yams from domesticated counterparts. This project aimed to investigate the ennobled yams, and specifically farmers' impact on genetic divergence of *Dioscorea alata, Dioscorea bulbifera,* and *Dioscorea cayenensis* species. Forty-eight accessions were collected from Southwestern Ethiopia and South-west Malay Peninsula. Eighteen microsatellite markers developed by Tostain *et al.* (2006) were utilized to amplify marker loci from the leaves and tubers of yam accessions from Malaysia and Ethiopia (both cultivars and ennobled). The optimized annealing temperatures (T_a) and $MgCl_2$ concentrations for the PCR amplification were determined. Eighteen primers succeeded in amplifying *Dioscorea cayenensis* accessions. However, Da1D08 primers failed in amplifying *Dioscorea alata,* and Dpr3B12, Da1D08, and Dab2E09 primers from the eighteen primers were not successful in amplifying *Dioscorea bulbifera* samples. *Dioscorea cayenensis* species had the best record in optimum annealing temperature (T_a) compared to *Dioscorea alata* and *Dioscorea bulbifera* species. The fragment size produced by Ym30, Da1A01, Da3G04, and Dpr3D12 markers incorporated with M13 FAM amplifying *D. alata, D. bulbifera*, and *D. cayenensis* species deployed in characterisation of the *Dioscorea* species that revealed a minor divergence between the three species. Moreover, the $MgCl_2$ and M13 FMA influence on the PCR products was investigated and documented where the optimal annealing temperature (T_a) and fragment size of some *Dioscorea* species specimens were altered by the $MgCl_2$ different quantity in the PCR protocol.

In addition, a discussion of the yam's physical characterisation, and the impact of traditional farming management in different ecological and ethno-linguistic regions are included.

Keywords: *Dioscorea* sp., ennobled, divergence, Microsatellite markers, asexually propagated crops.

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OBJECTIVES

This study will create a crucial opportunity to study the wild genetic diversity within the yam species. Screening for the optimal annealing temperature (T_a) for the eighteen microsatellite markers will assist in determining the specificity of the PCR products. The amplification from across *D. alata, D. bulbifera,* and *D. cayenensis* species are going to be deployed for grouping the markers into transferable and non-transferable classes across the *Dioscorea* species where the possibility of transferring a gene of interest between the species of yam.

The microsatellites allele scores can be employed to estimate genetic distance and the microsatellites information content, and to investigate divergence between *Dioscorea alata, Dioscorea bulbifera,* and *Dioscorea cayenensis* species.

Incorporating M13 FAM, tailed primers with Ym30, Da1A01, Da3G04, and Dpr3D12 to amplifying *Dioscorea* primer sets will facilitate introgression of desired genes into yam species for better characterisation. In addition to that, the production of the incorporation of the M13 FAM will be used to analyse the variation of the fragment lengths that will assist in decoding the linkage between *D. alata, D. bulbifera,* and *D. cayenensis* species.

The $MgCl_2$ role in the PCR productivity will be examined to investigate the impact on optimum annealing temperature and the fragment length of the *Dioscorea* species.

An understanding of variation within *Dioscorea* species can assist in an investigation of nutritional quality, size, and adaptability aiming to explore the potential to cultivate superior yams.

The amplified fragment sizes can be used to provide an accurate genotype for individual lines for future research related to *Dioscorea* species.

Dioscorea alata, Dioscorea bulbifera, and *Dioscorea cayenensis* are the species employed in this study, which will generate an initial comparison and create an opportunity to study and conserve the wild genetic heritage of yams.

1 Chapter 1: INTRODUCTION

The specific characteristics and similarity to the ancestors of a species of any crop are determined by the genetic factors, the selection that occurred during domestication, and farming systems. Mutations might occur which can be observed in the physical characteristics of the offspring. The majority of crop characteristics are a combination of the results of the genotype interaction with the environment to produce the phenotypes. The variation of the morphological traits, which can be found, or occurs between different species, can be utilized to distinguish these species from each other (Asiedu *et al.*, 1997; Egesi *et al*., 2006).

The domestication of wild species has been conducted for thousands of years, and Yam is one of these prehistoric species that has been ennobled (to better character) by farmers to serve as a staple crop. Yam tubers to be consumed as a staple crop the year-round are carefully stored prior to their use (Ikediobi and Oli, 1983) to minimise the tubers physiological alteration. The descendants of the Yam native to the Southeast Asian regions has become one of the widely cultivated tuber crops in West Africa, the Caribbean, Indo-China, and the South Pacific regions for starch sources in recent years, according to Sayed *et al*., (2006).

In the moist woodland of the Southwest of Ethiopia, Hildebrand (2003, and 2007) documented traces of a *Dioscorea cayenensis* yam cultivar with morphological alterations dated to the fourth millennium of the "Calibrated years before the present" (cal-BP) (Marshall and Weissbrod, 2011). Hildebrand (2003) stated that since the ancient time (Holocene period) farmers in Southwest Ethiopia have been transferring wild yams to their home gardens. *Dioscorea rotundata, Dioscorea cayenensis,* and *Dioscorea dumetorum* were the first yam species ennobled to have better characters in West and Central Africa, whilst *Dioscorea alata* was the first species cultivated in Southeast Asia (Bhattacharjee *et al*., 2011).

It was stated that the continuation of out-crossing between the domesticated and the wild yam, in addition to the carelessly packing yam tubers into harvesting-baskets during the tuber harvesting time, or by uprooting the tubers by the farmers might have caused the morphological alteration in the yam tubers (Marshall and Weissbrod, 2011).

In spite of having a wide range of genetic pool size, many yam species are gradually disappearing because of poor inquiry about the causes, and this has resulted in a major genetic loss (Baco *et al*, 2007; Siqueira *et al*., 2011).

The continuation of the ennoblement process where the cultivar is subjected to carry better characters of the wild yam has significantly resulted in potential diversities in the intraspecific and interspecific traits of the new crop (Mignouna and Dansi, 2003; Scarcelli *et al.* 2006; Tamiru *et al*., 2007b).

1.1 TAXONOMY AND CLASSIFICATION

Kingdom:	Plantae, Plants
Subkingdom:	Tracheobionta, Vascular plants
Division:	Magnoliophyta, Flowering plants
Class:	Liliopsida, Monocotyledons
Subclass:	Liliidae
Order:	Liliales
Family:	Dioscoreaceae, Yam family
Genus:	Dioscorea L., yam

Contains 30 Species and 15 accepted taxa overall

Species:	*Dioscorea alata L*., water yam
Species:	*Dioscorea bulbifera L*., air yam
Species:	*Dioscorea cayenensis Lam*., yellow guinea yam (USDA, 2016)

Yam is the common name of the plant species of the *Dioscorea* genus of the *Dioscoreaceae* family (Obidiegwu *et al*., 2009a). Crop species have a wide range of divergence and differences in their genetic materials, and individuals within a same species have variations in their genomes, and yam is one these crops. Moreover, species are generally classified according to the differences of the physical characteristics and manifestation of their traits.

Although yam is believed to be heterogeneous species, there are numerous overlaps of the morphological, biochemical and physiological characteristics between the species. Yams are characterised as polyploid-based on the chromosome number 9, or the number 10. The number 10 chromosomes are observed in all Asian yam species, while African and American species have ten chromosomes in 52% and 13% of the samples analysed

respectively (Bousalem *et al*., 2006). Moreover, environmental factors might stimulate yams' vegetative parts causing constant change in their phenotypes and creating a great level of plasticity, presenting challenges in classifying the cultivar into distinct taxonomical groups relying only on the morphological similarity (Egesi *et al*., 2006). A classification mainly based on morphological, or agronomic characteristics will conceal significant genetic variation within yam species (Zannou *et al*., 2009).

The morphological traits of the roots and tubers can be beneficial tools for classifying plants into categories according to the source of the origin vegetative parts initially being utilised for breeding (Hather, 1994). Dumont *et al*., (2006) classified yams into dioecious monocotyledonous plants, but they might develop into hermaphrodite species because of the domestication and the ennoblement processing, and farmers' attempt to have the desired characters to be combined into their home garden. Moreover, the embryonic shoot might emerge from either the hypocotyls or epicotyls. The significance of this is that in numerous cases, a combination of the hypocotyls and epicotyls might form the internode regions of the yam plant vines, or might produce the base stem that yields the edible part of the yam tubers that regrow every year (Hather, 1994; Lebot, 2009). A number of dicotyledonous characteristics such as netted veins can help distinguish the *Dioscoreaes* cultivar's species, and the cultivar vascular system arranged in a circular way in the stem. The pistil where the pollen is germinated is laterally positioned, showing a second undeveloped cotyledon. The *Dioscoreaceae* family species are commonly propagated by the tubers vegetative parts (Martin and Degras, 1978; Mignouna *et al*., 2008). Moreover, *Dioscorea* species might have evolved independently out of the plant-kingdom evolution form that took place prior to the distinction of monocotyledons and dicotyledons (Tamiru, 2006). Yams are winding or climbing herbs; developing annually or perennially. Yams have stems with or without spines. The plant's fleshy shoots are distinguished by their wings, which are disordered in their dispersion, or reduced to be absent in some species (Martin and Sadik, 1976). Yam flowers are dioecious. They are small with an unequal number of male and female inflorescences. The auxiliary parts of the female inflorescences resemble the male in its supporting functions. *Dioscorea* seeds are either winged or wingless, and they are carried on

locules. The seeds are located in triangles, or in deep tri-lobed capsules, which are dehisced into three valves (Miege and Demissew, 1997). The *Dioscorea* seeds are small, and the seeds with wing spread easily to the nearby field because of their lightweight. *Dioscorea* seeds sprout rapidly because they lack dormancy. However, it takes several ages for the seedling to grow at a size when it draws the farmer attention (Mckey *et al*., 2012).

In a study of 45 white yam cultivars classified into 22 morphotype groups, the use of Simple Sequence Repeat (SSR), Amplified Fragment Length Polymorphism (AFLP), and Random Amplified Polymorphic DNA (RAPD) techniques revealed a divergence in an average of 0.39 Gl, 2.56 Gl, and 0.35 Gl respectively. Notably, in terms of genetic characteristics of individual yams categorised as a single group according to their forms and structure, these frequently varied genetically, and this lack of resolution emphasises the necessity for yam germplasm DNA profiling when classifying the cultivar (Mignouna *et al*., 2003a).

Remarkably, the ennoblement of wild yams led to morphological changes in size, tuber shape, and fibrous quantity; these transformations might have been caused by mutation during cultivation (Tamiru *et al*., 2007a). The tubers' shapes have been used in the past to estimate the genetic variation of *Dioscoreaceae* species (Martin and Sadik, 1976; Sayed *et al.,* 2006).

1.2 HISTORY AND ORIGIN

Species of the *Dioscorea* genus of *Dioscoreaceae* family are one of the most important roots/tuber crops as a starch source after potatoes and cassava. The species plays a primary role in food security of many Southeastern Asians, Africans, and tropical Americans because it contains vitamins and amino acids to sustain growth and development. Moreover, the crop has a high concentration of polyphenols and tannins that bind to and precipitate proteins and amino acids (Gan *et al*., 2014). The crop contains a great deal of vitamin C and B_6, potassium and magnesium, but it is poor in the

saturated fat and sodium (Kwon *et al*., 2015) making it the core ingredient for a rich-carbohydrate diet. Moreover, *D. alata, D. bulbifera,* and *D. cayenensis* species are considered the main tuber crops used as staple food globally and a source of some drugs (Lebot, 2009). Medically, yams are used for antifungal activities, lowering blood glucose (Hashimoto *et al.*, 2009), and treating cardiovascular diseases (Qin *et al.*, 2009; Kwon *et al.*, 2015).

Candolle 1885, and Burkill 1951 related the presence of *Dioscorea alata* in a hybrid form at the present time as the species is evolved from *Dioscorea hmiltonii* and *Dioscorea persimilis* species (Martin and Sadik, 1976).

From the 600 *Dioscorea* species, only 11 species of yams are primarily cultivated in Ethiopia. However, in the Malay Peninsula, six collections of *Dioscorea alata* are recognised, and classified locally into white and purple yam. Yam is mainly cultivated in the east coast and northern rural communities in Malaysia (Sayed *et al.* 2006). The yam cultivar is grown in the dwellings' backyard doorsteps in Southeast Asia (Martin and Sadik, 1976). Conversely, in Ethiopia, yam species are regularly grown with crops of *Ensete ventricosum*, and *Colocasia esculenta* (Westphal, 1975).

Interestingly, farmers for generations have been contributing to the genetic diversification of yams through planting wild materials in their home-gardens (Frankel *et al*., 1995; Hildebrand, 2003; and Dumont *et al.,* 2006) and this process has contributed to the evolution of the crop species in the regions where farmers have settled. Martin (1974) stated that *Dioscorea* species of both continents of Africa and Asia morphologically vary from each other, and the only species that is safe to consume on both continents is *Dioscorea bulbifera*.

Based on the result of DNA analysis using (RAPD) markers, it was concluded that Malaysian *Dioscorea alata* species showed dispersal and reasonable levels of genetic variation in their descendants. In addition, the species exhibited variation in morphological characteristics that offers a range of different traits that can be used to improve the crop (Sayed *et al.* 2006).

1.3 BOTANY AND ETHNOBOTANY

A cultural link connects Southeast Asians and West Africans to yam crop beyond it is being simply a source of nutrition where they are having a special spiritual ceremony celebrating the crop-harvesting season (Martin and Sadik, 1976). Ethnobotanical studies revealed that *Dioscorea alata* leaves and *Dioscorea cayenensis* tubers have the capability to treat fever and diarrhoea (Aiyeloja and Bello, 2006).

The Sheko people of Ethiopia in Maji were amongst a few regions of the globe to domesticate both *Ensete ventricosum* and wild yams of *Dioscorea cayenensis* (Hildebrand, 2003, and 2007). Interestingly, this ennoblement of stimulating the plant to have a better character of their origins that contributed to the diversification of the genetic heritage of the farmers' home-gardens (Dumont *et al*., 2006).

Anecdotally, the origin of yams among Bench farmers of Ethiopia is as follows. The farmers were searching for *Liana* in the woodland to build huts when they saw the wild yam stems and chopped them down for construction purposes. When they noted the yam tubers, they consumed the starchy tuber and planted the root parts. Remarkably, yam production in Sheko district is highly sacred and many young farmers consider it a privilege to cultivate yam species. According to Tamiru *et al.,* (2007b) microsatellite study of the Ethiopian yams that this cultivation approach of the wild yam into the farmers' home garden has contributed to a high diversity of the genetic material in the yams grown in Sheko district.

Yam can survive a harsh environments and a long drought, and revive quickly after rains have resumed. In addition, the tubers can be preserved in the absence of refrigeration. Some of the wild yam species possess even greater superiority in terms of size, nutrition, and adaptability to be domesticated as varieties of other staple root crops ((Tamiru *et al.,* 2007b; Sanginga and Mbabu, 2015). The quality of this tuber crop is significant for its wide nutritional properties of tropical inhabitants where it exceeds 95% of the world production (FAO., 1991; Mwirigi *et al.*, 2009).

The farmers' intervention in the sexual reproduction process of the wild and pre-domesticated yams through the ennoblement procedures has stimulated an alteration and

expanded the genetic diversity of the *Dioscorea* species in Benin, West Africa (Scarcelli *et al.*, 2006).

The test of a successful ennoblement is to taste the flesh of the tuber. If the breeder is satisfied with the taste, the cultivar is harvested. The newly domesticated plant is propagated in a customised environment where the land is well regulated to accommodate the ennobled plant. This regulation contributes to environmental adaptation allowing the tubers to develop (Lebot, 2009).

1.4 CULTIVATION OF YAM

Yam breeders have selected heterogeneous of the wild species of the *Dioscorea* with different kinds of hybrids for ennobling procedures after drastic genetic alterations of the propagules has been observed in the plant. It has been stated that African farmers domesticated wild yams from *Enantiophyllum*, which produced the *Dioscorea cayenensis-rotundata* complex after the ennoblement processing has occurred, and the statement has been backed by genetic studies (Mignouna and Danis, 2003b). Commonly, young yam plants are grown in rows with wooden poles for support, whereas tubers collected from the wild are propagated near trees for support (Tamiru *et al.* 2007a). Because of its high propagation potential compared to other vegetative parts, the tuber's apex is used.

Yams vary in their edible parts and size of their tubers. For yams producing aerial bulbils from their leaf axils, portions of the bulbils can be propagated, or entire little tubers can be deposited back into the soil for species producing multiple small tubers (Hather, 1994). During a dry season of up to six months, the tubers of both the annual and perennial yams survive through the drought period as tubers in the field (Lebot, 2009).

Dioscorea abyssinica and *Dioscorea praehensilis* used for pre-domestication were wild yam species brought from the wild uncultivated areas (Dumont and Vernier, 2000; Mignouna and Dansi, 2003; Tostain *et al.,* 2003; Scarcelli *et al.,* 2006).

Domesticated yam cultivation varies according to the number of harvesting seasons per year. They might have one (single) harvesting season, or two (double) harvesting seasons a year. Throughout the two-harvesting seasons, the yield varies according to tuber types. The two harvesting times crop yields multiple tubers after the first harvest (Onwueme and Charles, 1994). The two harvesting yields one to two bulky and heavy weight tubers. Moreover, the bulky and heavy tubers produced after the premature tubers are being consumed, and they are harvested before the plant's vegetation life cycle ends. The single harvesting crop yields once a year at the end of the plant vegetation life cycle. The harvested tubers can be stored for 3 to 6 months (Tamiru *et al.,* 2007a).

The wild yam, brought into the home garden, with the cultivated yam unexpectedly sometimes produce a hybrid plant (Scarcelli *et al.*, 2006), which improves the genetic variation of the ennobled yams for having better characters (Kwon *et al*., 2015).

African farmers assess genotypes of the wild yam species of *Dioscorea abyssinica, Dioscorea praehensillis,* and *Dioscorea burkilliana*, and the plant's capability to be domesticated by placing an obstacle on the mound beneath the seedling of the propagules of the plant that is directly collected from the wild field. This procedure is to stimulate an alteration in the maturation of the tubers. The outcome of the process determines the fate of the selected genotype of the yam plant, whether it can be domesticated, or not (Lebot, 2009).

To capitalize on potentially superior characteristics of wild tubers, Sheko farmers have planted wild yams in their home-gardens introduce a new variable species into their cultivation system; farmers in Benin have followed this approach. When farmers in Benin wish to ennoble yams, they test their transformation ability for potential domestication by placing an obstacle on the apex of the propagated parts of the tubers to twist the propagules' routine to grow deeper into the soil instead of a shallow growing (Lebot, 2009). While other yam farmers place pieces of pottery beneath the propagules to force the plant to grow shallowly into the soil, forcing the plant to yield bulky tubers that can easily be harvested. Interestingly, after generations, the plant adapts and produces tubers without the need for manipulation (Mckey *et al*, 2012).

Interestingly, classifying yam landrace based on the variation of the agro-morphological characters, and environmental adaptation has brought insight regarding the gender, and the responsibility in the family. The species *Hatuma boye* (male yam), which grows vigorously, can cope with harsh environments, and produce bigger tubers. In contrast, *Macha boye* (female yam) species forming the early harvest, consumed as a family staple, requires better conditions to grow. The cultivar produces the best quality, and yields multiple sweet tubers in their second yield (Tamiru *et al.,* 2007b).

The cultivars' genotype juvenility governs the ennoblement procedure periods. The yam farmers subject the selected genotype for domestication processing in which the plant is stimulated to go through evolutions in order to acquire the favoured characters. When the superiority of cooking flavour is obtained, the domestication process that takes three to five years is terminated. At the end of the domestication, the ennobled yam tubers will have undergone morphological and biochemical alterations as a result of the intensive vegetation replication, and selection over the different periods of domestication. The ennobled yams are either similar to the farmer's familiar cultivars, or are defined as entirely new cultivars based on their structural activity changes. The newly ennobled yam will have potential in the yam-breeding process, and potentially benefiting the farmers' income. Some African yam farmers practise ennoblement to generate a novel cultivar and to have more diversity in their field, while others are curious to assess the tale about the wild origin of their plants (Mignouna and Dansi, 2003b).

Throughout the ennoblement process of hybridisation of the wild yam tubers, the yam breeders attempt to achieve changes in the shape, size, and flavour of the pre-domesticated tubers by exposing them to go under stress over the course of the three years of cultivation cycles until changes are obtained. If the breeder approves the changes, the modified tubers are mixed with similar tubers, or categorised into a new class (Dumont and Vernier 2000; Tostain *et al.*, 2003; Vernier *et al.*, 2003; Scarcelli *et al.*, 2006). In order to avoid the genetic alteration, the yam breeders use vegetative propagation because no genetic modification occurs throughout the ennoblement procedures if the cultivar is vegetatively propagated. Moreover, the ennobled cultivar

returns to its wild characteristics if it is not cultivated continuously (Dumont and Vernier, 2000; Scarcelli *et al.*, 2006).

1.5 OBSTACLES FACING YAM CULTIVATION

The yam breeders' desire for novel elite genotypes adaptable to the local environment and resistant to local pests and diseases, requires widespread genetic variation (Norman *et al.*, 2012b). The evolution of new genotypes of yams with enhanced characters yielding a steady harvest complying with the trading trait criteria is needed to reduce the risk of hereditary loss within the assembled *Dioscorea* germplasm. However, the propagation of these enriched qualities of genotypes has been hampered because of the landraces structure (Asiedu *et al.*, 1997; Norman *et al.*, 2012a).

Disease and storage pests are preventing African farmers from harvesting a greater yield, which is a problem for a crucial food staple source in Africa. Relatively, different ploidy levels of intraspecific and interspecific variability are limiting progress for genetic breeding of yams (Ngo-Ngwe *et al.*, 2014). A preliminary identification of disease tolerance would be achievable when specific genotypes, which were resistant, are paired up with the genotyping of the DNA samples. This method identified Yam Mosaic Virus, which jeopardises the stability of yams production worldwide. *D. alata* is considered the most vulnerable species of *Dioscorea* to the Yam Mosaic Virus and anthracnose disease (Mignouna *et al.*, 2003a & b; Sheela and Rajmohan, 2006).

Storage conditions alters the biological state of yam tubers considerably and stimulates an alteration in physiological characters of the tubers by instigating a deformation in the soluble tubers' protein profiles (Asemota *et al.*, 1992; Mignouna *et al.*, 2003a). In addition to the genetic alteration, recent domestication of yam has altered the flavour's quality and the fresh tuber appearance (Dumont *et al.*, 2006). In fact, deterioration in the physiological conditions of yam tubers causes an alteration in the tuber soluble protein profile and the cultivars' traits (Norman *et al.*, 2012a).

It was claimed that the on-going ennoblement of *Dioscorea* species in Southwest Ethiopia led to taxonomic misinterpretation of Guinea yams caused by farmers' collections and by interspecific interbreeding practice (Wilkin 2001; Mengesha *et al.*, 2012a). In addition, *D. cayenensis* plant leaves significantly differs in shape within one individual (Hildebrand, 2003); and the wild or hybrid of *D. praehensilis* and *D. abyssinica* can grow to resemble *D. rotundata* or *D. cayenensis* without any genetic alteration (Mignouna and Dansi, 2003; Mengesha *et al.* 2012a). Intensive vegetative propagation during the ennoblement process sparked morphological and biochemical alterations in the wild Guinea yam (Mengesha *et al.,* 2012a).

1.6 DIOSCOREA SPECIES

1.6.1 *Dioscorea alata*

Dioscorea alata L. (Water yam) is considered the most widespread cultivated yam species globally because of its adaptability and high production capacity, and storage lifetime compared to *D. cayenensis* and *D. rotundata* (Anokye *et al.*, 2014).

Dioscorea alata has a strongly twisted stem growing up to ten meters in length (Botanical-Online, 2014). The plant's branches are internodes with wings carried in their compound angles. The species produces aerial tubers in the leaves' axil, which can grow to a length of ten centimetres and have an uneven bumpy surface. In addition, the species is a dioecious plant producing unisexual inflorescences that hardly ever produce viable seeds. The male inflorescences and female inflorescences grow separately on two different plants (Martin and Sadik, 1976). The male inflorescences become mature after four weeks, growing in the plant's panicles up to 30 centimetres long while the female inflorescences, which are formed on smaller spines, become mature in three weeks (Lebot, 2009).

Dioscorea alata L. propagated vegetatively is a polyploidy species that appeared in the Melanesian region in Southeast Asia (Onwueme and Charles, 1994). Melanesia and neighbouring islands are believed to be the cradle in which diversification of *Dioscorea*

alata started (Siqueira *et al.*, 2013). The species is considered one of the most widely harvested yams of the humid tropical and subtropical regions (Lebot, 2009; Siqueira *et al.*, 2011).

Siqueira *et al.* (2013) indicated that *Dioscorea alata* tuber shapes could be classified into irregular, oblong, and round from field observations. Moreover, the tuber skin colour comes in three shades: from brown, to light brown, to dark brown. Considerable levels of variation in morphological and genetic features were observed between local variants and commercial cultivars within the investigated regions. Farmers' non-stop cultivation of the *Dioscorea alata* has caused morphological alteration of the cultivar (Martins and Oliveira 2009; Zannou *et al.,* 2009; Siqueira *et al.*, 2013). The use of microsatellites markers has detected a high level of genetic variation within *Dioscorea alata* (Obidiegwu *et al.*, 2009b; Siqueira *et al.*, 2013).

Martin and Sadik (1976) stated that the difference in tubers morphological traits might have resulted in classifying *Dioscorea alata* into several species. Lebot (1999) who commented that *D. alata* is one of the most ancient crops is broadly grown has backed this view. The Australoids people on the Sahul plate (present day Guinea) were first to ennoble the species around 60,000 years ago.

1.6.2 *Dioscorea bulbifera*

Dioscorea bulbifera is a species whose stems are twined anti-clockwise, climbing up to eight meters in height. The stems have simple big leaves alternating, or positioned opposite to each other. *Dioscorea bulbifera's* inflorescences are bigger than any other *Dioscorea* species. When the cultivar's female inflorescences coupled with the male inflorescences, they produce fertile seeds. *D. bulbifera* is also an invasive plant, which ends by being eradicated from the fields (Lebot, 2009). The tubers are irregular in shape, and the bulbils are smaller than three centimetres in diameter (Wilkin and Kew, 2001).

Dioscorea bulbifera L. a widespread yam species in Africa and Southeast Asia, has ten different selections with tubers yellow in the diagonal section. Three selections of

Dioscorea bulbifera var. bulbifera, Dioscorea bulbifera var. simbha, and *Dioscorea bulbifera var. heterophylla* are widely cultivated in China (Yifeng *et al.*, 2008).

1.6.3 *Dioscorea cayenensis*

Dioscorea cayenensis has a stem that might have spines, or are spineless. The simple leaves are alternating at the base to the right. The leaves vary from four to twenty centimetres in length, and they vary in the number of vines. The species grows to ten meters in height. The cultivars' stem grows vertically. *D. cayenensis* is a dioecious plant. Moreover, the inflorescences are produced at the early stage of vegetative cycling. The male plant is identified by having a high number of inflorescences compared to the female plant, which seldom produces inflorescences. The female inflorescences are produced on axillaries spines and having a length of 0.5 centimetres. The inflorescence perigonium is composed of two whorls of three sepals and three petals, lobbing over the ovary (Martin and Sidki, 1977).

Miege and Demissew (1997) stated that differentiating *Dioscorea cayenensis* and *Dioscorea rotundata* taxonomically is not possible because of the close interactions of the two species, thus they are classified as a complex species of *D. cayenensis;* with *D. rotundata, D. praehensilis,* and *D. abyssinia* proposed as one species. The transferring of yams from the lowland of Ethiopian forested savannah to home-gardens complicated distinguishing between the *D. cayenensis* species (Obidiegwu *et al.*, 2009a).

2 Chapter 2: MARKER ANALYSIS

Biotechnology has developed enhanced methods to reveal DNA–based polymorphism through numerous DNA marker systems, with the power of the Polymerase Chain Reaction (PCR) making it the favoured technique for laboratory-research, and for developing and applying DNA markers.

Oetting *et al.*, (1995) developed a technique of deploying an M13-sequence primer with fluorescence dyes for capillary electrophoresis detection, with a locus specified M13 Tagged primer sets. The utilization of a three-primer reaction allowed a generic M13 primer to be used in labelling specific Simple Sequence Repeat (SSR) products, without the need for fluorescent dyes to label all individual specific SSR primer set. This technique was used with four markers in this project to analyse the fragment sizes of *D. alata, D. bulbifera, and D. cayenensis.*

Gamiette *et al.*, (1999) evaluated 85 *Dioscorea alata, Dioscorea cayenensis-rotundata* and four of the linked wild species, and employed chromosomal counting and flowcytometry to determine the species chromosome numbers; the result indicated that accessions all contained 4x, 6x, and 8x chromosome sets. Surprisingly, the cytometer result of the *Dioscorea cayenensis-rotundata* and its wild linked species were identical. However, fusing gametes of diploid with haploid of *D. cayenensis-rotundata* species and its counterpart *D. alata* produced two distinctive species. Thus, it can be concluded that the *D. cayenensis-rotundata* and its wild species might be a part of the same genetic group. Norman et al., (2012b) admitted of having difficulty in counting *Dioscorea spp*. Chromosomes because of their small sizes and blurred visuals.

Mat-Isa (2000) utilized RAPD to investigate the genetic relationship between cultivated *Dioscorea sp*. within Malaysia, and the species harvested in the country of Japan; the result indicated differentiation among accessions of *D. alata* species, with similarity of 31.3%. It was concluded that the Malaysian accessions were distinct by 68.7% from their Japanese *Dioscorea* counterpart species, and the existence of some divergence within *Dioscorea alata* species.

Mignouna *et al*. (2003a) claimed that the ennoblement process expanded the genetic characteristics of the *Dioscorea*, and led to a number of novel cultivars because of

African farmers' desire to introduce more diversity into their landraces. In their experiment, Mignouna and Dansi (2003b) observed ennoblement of 57 Nago and Fon farmers' procedures in Benin where 68 recently ennobled accessions were studied for their morphology and biochemistry alterations.

Malapa *et al.*, (2005) claimed that morphological differences of colour and shape were exhibited in aerials, tubers, and roots of yams in the island of Vanuatu (an archipelago in the South-West Pacific). Their verdict based on the use of AFLP markers, which measured the genetic relationship between *Dioscorea alata* and nine other edible *Dioscorea* species.

Sayed *et al.,* (2006) stated that the collected data from 60 RAPD markers analysed by electrophoresis resulted in 95% polymorphism with obvious patterns from eight primers deployed, giving the same characteristic variation that was broadly seen in the Malaysian Yam. Conversely, there was similarity between 27 of the *Dioscorea alata* cultivars according to Jaccard's similarity coefficient, which varied in the range from 0.84 to 0.64. The success in developing markers from *Dioscorea* and related species and transferring them to other species has enriched the microsatellite stocks, which was a crucial step in enhancing food productions.

Tostain *et al.*, *(*2006) developed sixteen transferable microsatellite markers for *Dioscorea* species. Moreover, they found that the SSR markers were able to discriminate diversification in twenty-two accessions of *D. abyssinica*, *D. rotundata*, *D. praehensilis*, *D. cayenensis*, *D. alata*, *D. nummularia*, *D. bulbifera*, and *D. trifida.* The primers developed by Tostain *et al.* (2006) were deployed in this project to acquire an optimal-annealing temperature for a number of species, and to distinguish marker transferability between species of *D. alata*, *D. bulbifera,* and *D. cayenensis*. In addition, a fluorescence M13 tailed primer was incorporated into the forward sequence of five of the eighteen markers for fragment analysis appraisal.

Tamiru *et al.* (2007b) employed AFLP to examine the genetic variation of forty-eight *Dioscorea* accessions cultivated in Ethiopia, and to determine their relationship with the West African *Dioscorea*. They combined data from ten of AFLP primers that resulted in 97% polymorphism across the 900 fragments. Interestingly, the Ethiopian *Dioscorea*

species were distinguished from their counterpart West African cultivars, thus an Analysis of Variance (ANOVA) revealed 81% of the genetic variation was within the combined accessions of the Southern Ethiopian species. The report proposed the differences observed in Ethiopian *Dioscorea* might have been caused by a different evolutionary pathway distinguishing the Ethiopian yam from the broadly denoted centre of yam diverse in the West Africa.

Mwirigi *et al.* (2009) employed Principle Component Analysis (PCA) to study the Kenyan *Dioscorea* species based on their morphological characters, such as the cultivars' twining direction, the shoot colour, the spine and the leaf shape, and the presence or absence of male and female inflorescences. In addition, the tuber colour, shape, and rooting patterns were varied among the *Dioscorea* species. Agglomerative Hierarchical Clustering data revealed clustering of the four main groups of the 43 *Dioscorea* species. Furthermore, the research revealed the existence of a wide divergence among the Kenyan yams, which might enhance the potential for cultivating better yields and screening for pests and diseases resistance species.

Obidiegwu *et al.*, (2009b) used fifteen microsatellite markers to investigate the genetic variation between 219 accessions of *Dioscorea cayenensis/ D. rotundata* (Guinea yam). A high level of genetic diversification with a mean genetic distance of 0.677 was detected between the samples, with the average allele number per microsatellite observed was 8.06, with an average Polymorphic Information Content (PIC) value of 0.65. Heterozygote of Ho = 0.563 was consistent with Yams are dioecious, and naturally outcrossed.

Studies of *Dioscorea cayenensis*, and *Dioscorea rotundata*, using AFLP and SSR attempted to classify the *D. cayenensis* complex into different species in Western Africa.

Recently, a French team carried out a study in Benin on the *Dioscorea rotundata* complex and an additional twenty cultivars of *D. alata*. The RAPD analysis revealed significant variation between species, groups within a species, and between cultivars within groups (Zannou *et al*., 2009). The Chloroplast DNA primer sets were used to analyse genetic divergence of wild yams and domesticated yams from Benin along the

genetic maternal lineage, and to investigate if *D. rotundata* species contains similar chloroplast to their wild ancestors (*D. praehensilis* and *D. abyssinica*). The study investigated whether the wild species have more chlorotypes than the domesticated yams (Scarcelli *et al*., 2011a).

Obidiegwu *et al.* (2009b) claimed that a natural hybridisation occurred in the yams' ancestry, which transferred genetic variability into some of the accessions. Additionally, the farmers' selection of somatic mutants with phenotypic effects to improve their cultivars might have been the major cause of divergence of the *Dioscorea* species (Siqueira *et al*., 2012).

Mengesha *et al.* (2012b) utilized SSR markers to study the genetic diversification of *Dioscorea* species of 58 accessions from South and Southwest Ethiopia. The study proposed that the wild accessions and their relatives Guinea yams of *D. cayenensis* and *D. rotundata* were genetically diverse, and 64% of the whole population of the 58 accessions were heterozygous. It can be inferred from the results of this study that Guinea species and their relative wild species are highly varied with the wild species exhibiting higher levels of allelic diversity.

Wu *et al.*, (2014) reported an analysis of molecular variation for twenty-one domesticated yams from seven populations and discovered high levels of polymorphism for Inter Simple Sequence Repeat (ISSR) markers at 95.3%, and for Sequence-related Amplified Polymorphism (SRAP) at 93.5 %, per band. In addition, molecular diversity was 40.39% between accessions of the four species of *Dioscorea opposita*, *Dioscorea alata*, *Dioscorea persimilis*, and *Dioscorea fordii* cultivated in China. The twenty-one yam landraces were easily distinguished by the use of the ISSR and SPAR markers.

In spite of the initial steps of DNA markers used in yams toward the genetic characterisation of the species, further studies are needed to reveal and to illuminate the complex genetic ties between close species.

3 Chapter 3: MATERIAL AND METHODS

3.1 PLANT AND MATERIALS

Accessions used in this project were from two different tropical regions, from Africa and Asia. Sheko and Bench villages of Maji districts in South-western Ethiopia were the locale of forty accessions (eight *D. alata,* twelve *D. bulbifera,* twenty *D. cayenensis*), while Semenyih and Kajang of Selangor districts, in the Malay peninsula were the source of the eight Asian accessions (eight *D. alata*).

Young leaves and tubers were scientifically documented, and frozen at minus 80 Celsius. Eighteen primers developed by Tostain *et al.,* (2006) with various expected allele sizes were selected for the project. Maps 3.1 & 3.2 display the location from where the accessions were collected.

Map 3.1 Adapted from Google: Map showing Southwestern Ethiopia, Maji region, Sheko, and Bench villages where forty *Dioscorea sp*. samples were collected.

Map 3.2 Adapted from Google website: Map showing Southwestern of Malay Peninsula, Selangor region, Kajang, and Semenyih districts where eight *Dioscorea sp*. accessions were collected.

3.1.1 DNA Isolation

The i-genomic Plant DNA Extraction Mini Kit, and Genelute plant Genomic DNA Miniprep kit were used to isolate DNA from the collected specimens. Because of phenolic complexes, extremely thick polysaccharides, and DNA degrading endonucleases present in the tropical tuber crops' leaves, it was difficult to isolate a decent quality of DNA specimen from the yam cultivar's leaves. When phenolic oxidise during tissue homogenisation, the substance irreversibly adhered to proteins and the DNA (Sharma *et al.*, 2008). Moreover, the polysaccharides form abnormal re-association kinetics in the DNA specimens (Merlo and Kemp, 1976). Thus, the polysaccharides precipitate with the DNA during alcohol precipitation, forming highly sticky mixtures (Do and Adams, 1991), and altering the DNA efficacy. Thus, the DNA becomes inadequate for PCR amplification (Sharma *et al.*, 2008).

It was possible to increase the yield of nucleic acid between one and 250ng/µL by keeping the solution at minus 20^0C for thirty minutes to twenty-four hours after adding tRNA, or glycogen prior to centrifuging the mixture to precipitate (Mulhardt, 2007).

A purified DNA specimen was stored at 4^0C for immediate use, at minus 20^0C for long-term storage, and at minus 80^0C for an unknown period in order to avoid the DNA degradation (Wu *et al.*, 2008).

3.1.1.1 DNA extraction processing steps

Frozen young leaf tissues were used to extract the DNA deploying i-genomic Plant DNA Extraction Mini Kit for Malayan accessions while Genelute plant Genomic DNA Miniprep kit was deployed for Ethiopian samples. The flow-through of the extracted DNA templates was preserved at minus 20^0C.

3.2 DNA QUANTIFICATION

3.2.1 Spectrophotometers (Nano-Drops)

The isolated DNA templates were quantified by employing spectrophotometers (Thermo Scientific/NanoDrop 1000). The Ethiopian accessions were titled according to the place of the collection, (Sh) titled for Sheko and (Be) for Bench villages. However, the Asian accessions collected from Semenyih and Kajang in Southern of Malaysia were titled (M).

A spectrophotometer is an efficient method of quantifying DNA, and is comparable to the use of High Performance Liquid Chromatography (HPLC), Fluorometric, or any of other methods deployed in DNA estimation. The precision mainly depends on the DNA structure (Dell'Anno *et al.*, 1998). An accurate and precise result in genetic analysis or related experiments primarily depends on the accuracy of the DNA quantification. The genomic DNA purification was estimated by $A_{260/280}$ and $A_{260/230}$ ratios calculated from spectrophotometric measurements (Sharma *et al.*, 2008).

The standard measurement for a pure DNA specimen was 1.8 for its optical-density at 260nm, and 2.0 for its optical-density at 280 while less pure specimens measured

considerably diverse in their optical densities. The DNA specimens' absorption was interpreted in µg/µl for samples diluted 1:20 in water or a buffer (Ahn *et al.*, 1996).

Undoubtedly, spectrophotometer data were accurate for DNA concentration, but there was uncertain about how intact the DNA samples were. Thus, it was necessary to deploy an electrophoresis method to have a better estimation of the DNA quantity and to acquire a physical confirmation of the samples' qualities.

3.2.1.1 Spectrophotometers (Nano-Drop) processing steps

The extracted DNA samples of Ethiopia and Malay Peninsula were quantified deploying Thermo-Scientific spectrophotometer where Vivantis buffer VI-A (without $MgCl_2$) was used in blanking the instrument

3.2.2 Electrophoresis

Electrophoresis was utilised in measuring the magnitude of DNA molecules, in differentiating sequences of similar sizes of DNA strands, in dispersing and purifying DNA fragments, and in measuring the variation of allele expressions (Bartlett, 2003). Electrophoresis has two electric sides with negatively charged electrode and positively charged electrode. Ions oxidation occurs in the positive perimeter charged where electrons migrate from negatively charged anode into the positively charged anode direction. The ions movement toward the appropriate electrode rules the molecules migration in electrophoresis gel when electric current is applied. Moreover, size and numbers of ionic charges dictate the ions pace. Thus, small ions move faster compared to large ones, whereas ions with a single charge are slower compared to ions with two charges (Altria, 1996).

3.2.2.1 Electrophoresis components

The *D. alata*, *D. bulbifera,* and *D. cayenensis* samples of the two tropical regions were scanned on electrophoresis agarose gel to assess their DNA quantifications.

3.2.2.1.1 Agarose gel

The agarose gel is a mesh network containing pores with diameters of 100-300nm through which the negatively charged molecules can migrate toward the positively charged electrode when an electrical field is applied. Agarose melts at 65^0C. The pore size and the fragment lengths proportionally control the rate at which the molecules migrate toward the positive electrode. The pores sizes are subjected to the agarose intense in the gel. Relatively, the higher the intensity the smaller the pores are; small pores slow the DNA fragment migration through the gel. A gel of 1% agarose concentration was efficient in separating DNA fragments ranging between 400bp-8kb, whereas 2% agarose was efficient at separating smaller size DNA molecules between 100bp-3kb (Brown, 1998).

Large fragments migrate slower than small fragments due to the pores sieve-like form in the agarose gel forming less resistance to small fragments (Mulhardt, 2007).

Nowadays, agarose gel is favoured in many Electrophorese protocols because of its high, robust adequacy in separating PCR amplicons ranging from 200 to 50,000bp. It is safe and easy to prepare. Separating fragments ranged up to ten mega base pairs, a pulsed electric field is needed to be run through agarose gel (Bartlett, 2003).

A gel with intensity of 3% agarose can differentiate PCR products of 30-40bp long. However, the bands might lose their sharpness when low voltage runs throughout electrophoresis processing, particularly if the bands are shorter than 400-500bp (Henegariu *et al.*, 1997).

3.2.2.1.2 Electrophoresis buffers

TAE or TBE buffer provides a neutral pH and a conductivity field for the molecules to migrate when an electric current is applied in electrophoresis assay. The ionic power and buffer structure influence the DNA migration rate on the electrophoresis gel. In fact, favouring either TBE or TAE in electrophoresis run is subjected to the objective of the assay. TBE offers a great buffering capability for long runs throughout the electrophoresis processing with no need for refreshing or adding. DNA fragments migrate toward the positively charged electrode 10% faster in TAE compared to the same concentration of TBE buffer (Kumar and Garg, 2005). TAE is utilised to separate molecular larger than 2Mb. Because TBE has a high capacity and resolution in separating small fragments, it was deployed to separate fragments less than 2Mb long (Birren and Lai, 1993). Controversially, TAE is suggested to be used when the DNA needs to be retrieved from the gel because TBE borate ions intervene with enzyme manipulations used in purifying DNA. TAE, which is credited for great resolution in separating long fragments, might become fatigued throughout the electrophoresis long run. TAE was used for raw (non-denatured) DNA for high resolution of long fragments on the agarose gel.

3.2.2.1.3 DNA Staining dyes

SYBR safe is considered less hazardous compared to Ethidium bromide staining dye, and the stained samples can be viewed under a blue light, causing less damage to the nucleotides. Whereas the presence of Ethidium bromide dye causes stiffness and rigidity in the dsDNA, which breaks or nicks the nucleotides when exposed to UV light for longer than 30 minutes (Viljoen *et al.*, 2005). When the dye is applied to the gel, it intercalates between the dsDNA bases pairs causing expansion of the strand. Notably, the dye migrates in the opposite direction of the DNA migration on the electrophoresis gel, which will delay nucleotides migration about 15% (Farrell, 2010). The intercalated staining dyes have a strong attraction for GC base pairs (Butler, 1997).

3.2.2.1.4 Gel loading dyes

Prior to loading the DNA samples into the gel-wells, the samples were mixed with loading dye comprised Bromophenol blue and Xylene cyanol FF to track the sample's migration throughout the electrophoresis processing. The buffer contained sucrose and glycerine, which intensified the samples' concentration, and helped settle the samples at the bottom of the gel-wells; likewise, it made loading samples into the wells visible. Moreover, the blue dye migrates in the same direction of the DNA migration (Kumar and Garg, 2005).

3.2.2.2 Agarose-Gel preparation steps

A weight of 0.6g of Agarose powder was mixed with 60ml of 1X TAE buffer at pH8.0 to prepare 1% agarose gel for electrophoresis DNA quantification. 1.2g of agarose powder was mixed with 120ml of 1X TBE in preparation of 2% agarose gel for electrophoresis visualisation of the PCR products by adapting Sambrook Molecular Cloning Laboratory Manual protocol.

3.2.2.3 Electrophoresis processing steps for DNA quantification

A different concentration of a standard amount of Thermo-Scientific lambda DNA ladder 500bp/1µg/µl was diluted into 250µg/µl, 200ng/µl, 150ng/µl, 100ng/µl, and 50ng/µl measurement. 5µl of Fermentas GeneRuler 1kb DNA Ladder 0.5ug/µl, 50ug ladder (GeneRuler DNA ladder- Thermo Scientific) was loaded into the first and last well. Then, 20µl of the diluted lambda of 250ng/µl, 200ng/µl, 150ng/µl, 100ng/µl, and 50ng/µl were used as DNA standard rulers. 20µl of all DNA samples were loaded after being mixed with 6X loading dye buffer (Blue dye). The ratio was 4:1:1 (4µL SDW: 1µL ladder: 1µL Blue-dye). The Electrophoresis gel was run at 90V for 60 minutes using a buffer of 1X TAE. The gel was transferred to the transilluminator (AlphaInnotech), (FluorChem HD2) for visualisation under the UV, and a photo was taken and saved for

the record. The DNA concentration was estimated by comparison with the DNA quantification standards.

3.3 POLYMERASE CHAIN REACTION

Polymerase chain reaction is a technique with the capability to amplify a nanogram of nucleotides into multiple copies with complementary bases of a single strand of nucleotides. Nevertheless, for efficient products, a sufficient amount of template is required. PRC is a repetitive process of three elements proceeding with the denaturation of the template by high temperature, annealing of the oligonucleotides (primers) to the single stranded DNA (ssDNA) targeted regions, and extension of the synthesised nucleotides by the Taq DNA polymerase.

To proceed with the reaction, the PCR mixture was heated up to 94^0C for three minutes to unwind the double stranded DNA (dsDNA) template. In order to denature a double helix of DNA, the hydrogen bonding “hydration Shell”, the bases arrangement, and the interaction forces stabilising the DNA had to be overpowered. Subsequently, the double strands came apart because of ionisation of the bases when the strands were heated. Moreover, the ionisation altered the donor and acceptor of the hydrogen bond properties and the bases causing disruption of the A-T and C-G hydrogen bonds. It is known that alteration in solutions’ temperatures alters the solutions’ conductivity. Therefore, an increase in the solution temperatures decreased the solution intensity, and accelerated the ion movements. Likewise, the high temperature increased the solution ions numbers due to dismantling of the molecules. Hence, heating up the PCR solution escalated its conductivity causing alteration in the nature of the hydrogen atom’s electrical charge. The high heat broke the hydrogen bonds and damaged the DNA hydration shell resulting in dismantling of the forces linking the two DNA strands to each other (Sinden, 1994). Once the templates were denatured into ssDNA, a second wave of high heat at 94^0C for one-minute was applied to ensure a complete denaturation of the DNA templates was achieved. By breaking the hydrogen bonds, the ssDNA was ready to be hybridised by the primers.

The pre-programmed thermocycler lowered the temperature to 50^0C for one-minute allowing hybridisation of identified sequenced primers to their matching sequence of the DNA template. The primers' Melting Temperatures (T_m) govern the annealing stage, which occurs at low temperature between 50^0C to 65^0C where 50% of the DNA template were split into ssDNA (Nelson *et al.*, 2014).

In the PCR solution, the two designed short nucleotides outnumbered the denatured DNA templates presence, thus the two oligonucleotides effortlessly hybridised to their complementary bases on the DNA templates offering an initiating point to start synthesising antiparallel strand of the DNA targeted region.

The Forward primer hybridised to the downstream strand while the reverse primer hybridised to the upstream strand pairing up with the complementary bases of the DNA templates. The moment the two primers succeed in hybridising into the DNA templates instantly non-covalent bonds were constructed linking the complementary bases of the primers and the DNA templates generating short dsDNA. Likewise, cooling down the temperature of the solution stimulated the ionisation phenomena and the hydrogen bonds to link the primers' bases into their complement bases (G to C) and (A to T) on the DNA templates where the annealing process occurred. When Taq DNA polymerase collided into the primers, the polymerase attached itself near the primer's 3' end to be able to read the DNA template's code, and to ensure only complementary dNTPs building blocks were added to the hydroxyl groups on the primer 3' terminus.

The temperature was elevated to 72^0C for two minutes to continue elongating and synthesising the complementary strands of the targeted DNA. Hence, Taq polymerase molecules disassociate themselves after certain numbers of dNTPs were added, and fresh polymerase molecules continued adding the dNTPs building blocks to the newly synthesised strands. To elongate a polynucleotide, a covalent phosphodiester-bond was formed to link the primer hydroxyl groups on the 3' prime carbon to the incoming nucleotide phosphate groups on the 5' prime carbon forming the backbone holding the nucleotides (Minkoff and Baker 2004).

Remarkably, complete matching of the primer 5' terminus to the DNA template might not be as critical as it is on the 3' terminus because the thermostable DNA polymerase

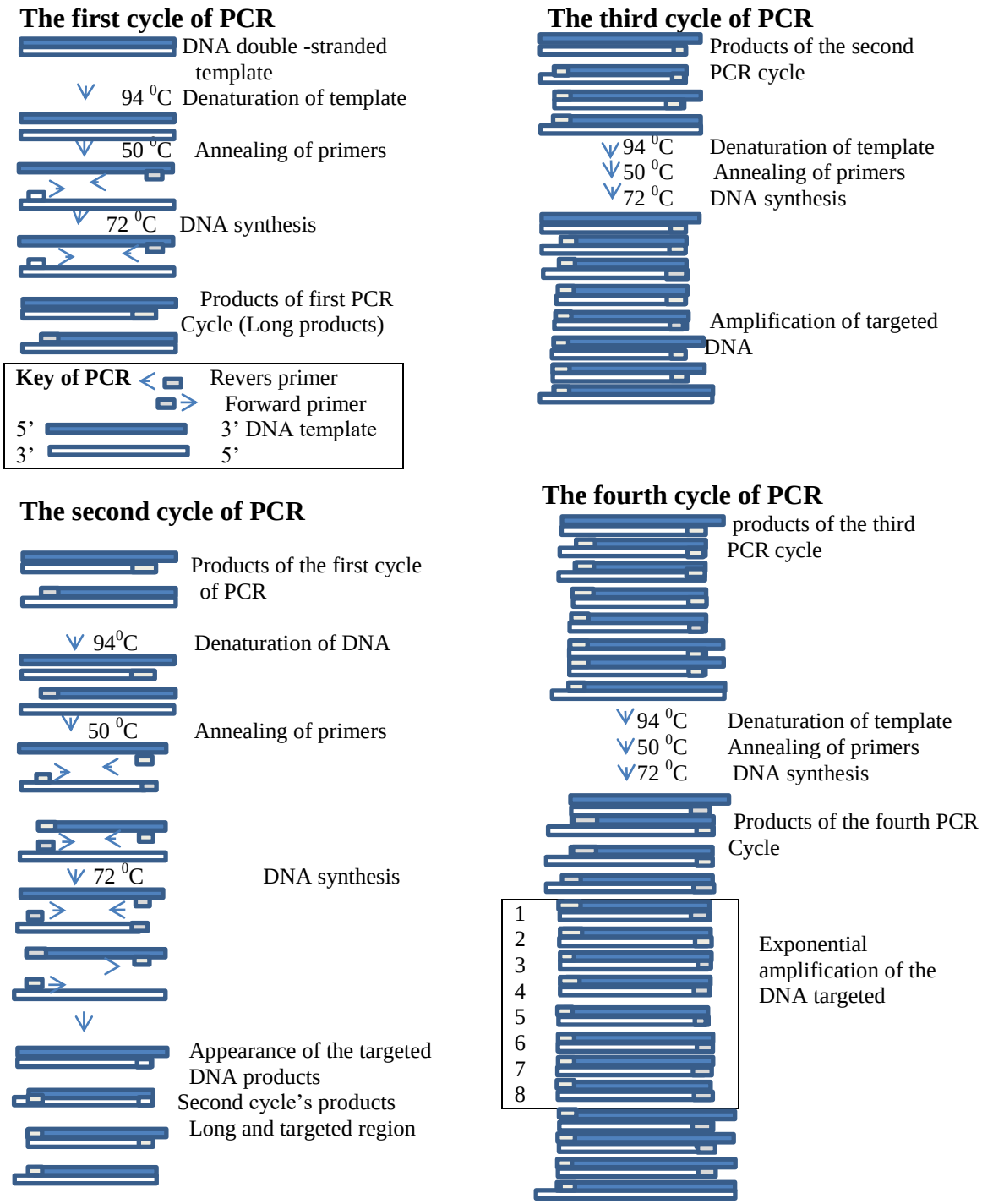
cannot initiate synthesis of the complementary strand during the extension stage of PCR unless a complement matching between the primer 3' terminus and the DNA targeted is formed (Onodera, 2007).

The newly synthesised complementary strands proceeded to copying the targeted DNA to the end of the template and only halted when the temperature was increased for the denaturation step of the PCR's second cycle.

A heat wave of 94^0C is deployed for one minute to denature the heteroduplex DNA comprised the original DNA template and the newly synthesised DNA that was generated in the first round of PCR. The two strands, the original template and the strand defined by the primers starting position acting as templates for the upcoming round of PCR (McPherson and Moller, 2006). The heteroduplex split into two single strands and hybridised by the forward and reverse primer amplifying the targeted regions of DNA when the temperature was cooled down. The extension stage proceeded when the temperature was elevated to 72^0C. Furthermore, the polymerisation proceeded from one primer position and far down the DNA strand where the opposite primer started. By the end of the second cycle, the PCR amplicons contained two reasonably short fragments of ssDNA whose sizes were exactly matched the position of the forward and reverse primers. The short amplicons and the genomic DNA were functioning as templates for the subsequent PCR cycles (Oliver, 2009). By the end of the third cycle, the first double stranded of the targeted DNA with an accurate length was generated (McPherson and Moller, 2006).

The PCR concluded with seven minutes final polymerisation step at 72^0C to ensure that all amplicons were elongated to their accurate sizes. With successive PCR cycles, the long dsDNA PCR products were outnumbering the excessive number of short dsDNA PCR products. In an ideal PCR, the number of amplified dsDNA was doubled with every cycle. In subsequent PCR cycles passing the third, the target short sequences of DNA (amplicons) increased in an exponential numbering and the short amplicons reaches 34 billion of sequence targeted DNA after 35 cycles of PCR (Mburu and Hanotte, 2005). The size of PCR final products is the total of the forward and reverse

primers adding to the size of the targeted region between the two primers (Erlich *et al*., 1991).



Map 3.3 DNA amplification mechanisms of the first four cycles of PCR. The first cycle generated two long ssDNA defined by primer’s starting location. The second cycle produced two short ssDNA defined by position of the two primers. In the third cycle, two short dsDNA were replicated exponentially followed by eight defined amplicons yielded in the fourth cycle.

3.3.1 PCR Stages

To start the PCR cycles an initial denaturation of dsDNA at 94^0C for three minutes was the first stage. The second stage of the processing was Denaturation of dsDNA at 94^0C for one minute followed by annealing of the two oligonucleotide (primers) at 45^0C and 50^0C for the static annealing temperatures, and for gradient 50-65^0C for one minute 35 cycles took place. An Extension of the nucleotides at 72^0C for two minutes was the next step of the PCR. A final extension of the nucleotides at 72^0C for seven minutes was to offer an ideal environment for a complete extension. The PCR assay was held at 4^0C for 15 minutes after the final PCR cycle**.**

3.3.2 PCR Components

3.3.2.1 DNA Templates

DNA is comprised of two polynucleotide strands intertwined in the form of a double helix conveying the building block data of all organisms. DNA molecules are comprised of the pentose sugar (deoxyribose), phosphate groups, and Nitrogenous heterocyclic bases of Adenine, Thymine, Cytosine, and Guanine (Zien, 2004).

The nitrogen bases comprised of Adenine (A) and Guanine (G) consist of double-rings composed of a five-atom ring attached to a six-atom ringed molecule derivative of purine. Thymine (T) and Cytosine (C) are six-atom ringed molecule derivative of pyrimidine. The double helix, non-symmetrical structure, is polarized because of different chemicals making up the two ends. The complementary strands are oriented in opposite directions; one strand runs in the 3' -5' prime direction, whereas the other in the 5' -3' prime direction. A single DNA probe is marked by its 5' prime containing phosphate groups at its beginning and 3' prime containing hydroxyl group as its end. Knowing the chemical structure of each end is crucial when the strand is being manipulated in the lab because some enzymes only interact with the 3' or the 5' terminus (Fitzgerald-Hayes and Reichsman, 2010). The strand pointing to the 5' terminus is termed “upstream” and the strand pointing to the 3' terminus is called the “downstream” DNA strand (Flowers *et al.*, 2004).

The dsDNA is held together by hydrogen bonds linking its complementary nitrogen base pairs: Adenine with Thymine or A-T and Guanine with Cytosine or G-C. The complementary base pairing is always formed between a pyrimidine and a purine base. A-T base pairs are linked by two hydrogen bonds, whereas G-C is linked with three hydrogen bonds to maintaining the molecules uniformly in the helix form. This orientation protects the chemical characteristics of the bases. The hydrogen bonds keep nitrogen bases inside the double helix while the deoxyribose sugar and phosphate groups shield the external. The DNA probe sequence is denoted from 5' – 3' prime (Wu *et al*., 2008). Heterogeneity of DNA strands is a by-product of differences between the stabilization forces of its distinctive A-T and G-C base pairs stacking and the hydrogenous bonding (Sinden, 1994).

The base-pairing and physical features of the double strands shield the genetic information from alteration caused by environmental chemical substances (Sinden 1994). Moreover, DNA template containing high G-C base pairs, or a steady secondary structure might replicate inefficiently generating nonspecific products (Lander *et al.*, 1995).

3.3.2.2 Primers (forward and reverse)

Primers are short single strands of oligonucleotides of a known sequence ranging between 15-30bp. Primers are designed to hybridise to either priming side of DNA template, and to define the site at which synthesizing complementary strands is to commence. The complementarity of the primers to the priming regions enhances the efficacy of PCR products by providing an extension side to synthesize complementary strands of the DNA template. Moreover, the free hydroxyl group at the primer 3'-end is linked to the phosphate groups at the extended nucleotide 5'-end by phosphodiester process, creating a stable covalent bond between the primer and the newly added nucleotide. A primer with complemented sequence to priming region evades production of nonspecific priming regions (Brown, 1989). Moreover, positioning three G or C bases within the final bases of the primer 3’-end facilitates the specificity annealing at

the primer 3'-end because G-C base pairs are linked by three hydrogen bonds (Borah, 2011).

Primers amplifying long strands function better when the PCR buffer salt intensity is low, whereas primers replicating short strands do better in high salt intensity where long strands would be more difficult to denature (Henegariu *et al*., 1997).

It is recommended that interior matching sections be avoided when developing PCR primers because compatible sections generate hairpin characters, which inhibits the formation of perfect annealing of the primer to the target sequence by impeding the PCR amplification (Mitsuhashi, 1996).

A primer that anneals to the DNA upstream strand is called the forward primer whereas the primer that hybridizes to the downstream strand is the reverse primer.

3.3.2.3 PCR Buffer

Buffer solution offers a perfect environment in terms of pH level for the PCR reagents to function at optimum degree and stabilise the DNA polymerase. The buffer is composed of 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% (W/V) gelatine, with a final10X intensity to preserve the pH level in the PCR mixture. Unstable pH ionises the bases causing disruption of the hydrogen bonds, which results in separation of the two strands of the double helix. The divalent cations existing in the buffer are crucial to shield the negativity of the triphosphate and to permit the hydroxyl oxygen of the 3'-end to link to the phosphate groups of the 5'-end of the incoming nucleotide (Das and Dash, 2015).

Magnesium ions intensity stretched between 0.5 mM to 5 mM is widely incorporated into the PCR ingredients where 1.0 µl intense of the divalent Mg^{+2} is frequently utilized. In fact, the ions' intensity might vary when different thermostable DNA polymerases integrate; altering intensity due to the existence of EDTA, and Ca^{2+} ions that might isolate or compete with the Mg^{+2} ions (Pelt-Verkuil *et al.*, 2008).

3.3.2.4 Deoxynucleotide triphosphate (dNTPs)

The quantity of Deoxynucleotide Triphosphate (dNTPs), the main aspect in extending the nucleotides, is added in equimolar amounts. The four-deoxynucleotide triphosphates of dATP, dTTP, dCTP, and dGTP are the core building blocks of the DNA strands. They are proportioned to the nitrogen base pairs (A-T) and (G-C) ratio when incorporated into the PCR ingredient. The dNTPs are acidic in nature and require neutralisation with alkaline before reserving for long term. Moreover, dNTPs are stable when they are conserved in marginally alkaline liquid solutions at -20^{0}C (Pelt-Verkuil *et al.*, 2008).

An equimolar intense of dNTPs ranging 20-200 mM and Mg^{2+} intensity ranging 0.5- 5.0 mM enriches the efficacy of the PCR products (Brown, 1998). However, increasing dNTPs intensity in PCR mixture more than 400 uM inhibits the amplification of the nucleotides, and lowering the dNTPs intensity to less than 50 uM lowers the PCR product's quantity (Henegariu *et al.*, 1997). Henegariu *et al.*, (1997) reported that thawing and freezing have influenced the efficacy of dNTPs to not function properly, and the multiplex PCRs products entirely were invisible after three to five cycles. To remedy this snag, small aliquots of dNTPs were made and kept frozen at -20^{0}C and centrifuged prior to use.

3.3.2.5 Taq polymerase

Taq polymerase is an enzyme repellent to denaturation by high temperature, extracted from *Thermus aquaticus* bacteria. The enzyme thermostability features suit the PCR high heating when it is raised to 94^{0}C in order to unwind the DNA templates. The enzyme binds into the primers during the amplification process, supplying the nucleotides initiating synthesis a complementary strand of the targeted regions of DNA template (Brown, 2010).

Notably, Taq DNA polymerase lacks the 3' to 5' prime exonuclease proofreading activity; however, throughout the polymerisation Taq DNA polymerase proofreads the synthesised DNA strands in the direction of 5' to 3' prime (Erlich *et al*., 1991). The absence of the 3' to 5' prime correction activity in Taq polymerase might cause the

appearance of non-specificity in the PCR final product. Despite of having correction activity, thermostable DNA polymerases might hybridise incompatible nucleotides into the extended DNA strands (Pelt-Verkuil *et al.*, 2008).

Unfortunately, Taq polymerase might generate nonspecific products because of its high activity in the temperatures used for the primers annealing in which the polymerase proceeds to synthesise the new strand despite relatively incompatible hybridisation of the primer at the non-targeted region. In fact, incompatible primer hybridising to the DNA template for a short time can initiate synthesising a DNA probe (Brown, 1998).

Remarkably, Taq polymerase has great efficacy in replicating short strands up to 5kb, but it might start to stumble after incompatible base-pairing installations and slow down or pause in further elongation. In order to replicate fragments up to 50 kb a mixture of 11:1 ratio of Taq polymerase and Pfu polymerase owning 3' to 5' prime exonuclease correction is recommended (Mulhardt, 2007).

3.3.2.6 Melting Temperature (T_m) and Annealing Temperature (T_a)

Melting Temperature is the state at which half of the presented DNA molecule is denatured into ssDNA and the remainder is in random coil or double stranded state. In order for the forward and reverse primers to replicate the DNA template, the T_a of the PCR has to be 10^0C below the T_m of primers (Santalucia, 2007). The number of G-C and A-T bases, as well as primer length determines melting temperature. The melting temperature (T_m) and hybridization rates influence the molecule annealing process. Knowing the T_m of oligonucleotide and intensity of the stock solution validates the efficacy of the oligonucleotide signing as a primer (Brown, 1998). The primers' melting temperatures rely on the content of (G-C) percentage and stacking interrelation of the bases.

It is known that (G-C) percentage, melting temperature, and annealing temperature are strongly interrelated where structure and length of the primers governs the T_a degree in the PCR process. For a better PCR result, it is recommended to be at least 5^0C below the T_m of the less stable primer in the pair.

Notably, many protocols do not outline the significant role of T_a in having a successful or disappointing PCR result. Alternatively, they propose a custom recipe for primers with high (G-C) or (A-T) content to be annealed at 55^0C and 60^0C respectively. However, for primers to reach optimum specificity the T_a should be 1 to 2 degrees below DNA primer annealing to DNA templates (Brown, 1989). Effectual and definite amplicons can be acquired by lowering the T_a as 10 to 12^0C, when DNA template is less than 30 ng (Henegariu *et al.*, 1997). It is known that reducing the T_a increases PCR production and amplification of nonspecific products. Conversely, elevating the T_a increases the specificity of the products at the cost of reducing the PCR yield.

Further, the monovalent and divalent salt and Mg^{2+} intensity in the PCR solution stimulates the annealing temperatures where the T_a is commonly set 5^0C below the primer's T_m. However, the optima T_a might exceed the primers' T_m by 5^0C to 10^0C (Viljoen *et al*., 2005). The annealing temperature is proportional to the intensity of the primers, percentage of (G-C) content, primer length, and intensity of salt in the PCR buffer solution (Tong, 2014)**.**

3.3.3 PCR static annealing temperature

A standard PCR was conducted for a static T_a of 45^0C and 50^0C to assess the eighteen primers (forward and reverse) pairs' efficiency in amplifying *D. alata*, *D. bulbifera*, and *D. cayenensis* specimens. Similarly, a static T_a of 50^0C was deployed using four primers: Ym30, Da1A01, Da3G04, and Dpr3B12 incorporated with M13 FAM. The objective was to amplify *Dioscorea* species and to analyse their fragment sizes.

3.3.4 PCR Gradient DNA increment annealing temperature

PCR Thermocycler with twelve columns by eight rows was employed for the DNA gradient. An increment temperature of 50 to 65^0C was proportioned into twelve partial of temperature degrees. Each individual column of the Thermocycler was operated according to ascending temperature degrees starting from the first column on the left

with the lowest temperature of 50^0C through to the high temperature of 65^0C with the rightmost column. The application objective was to achieve an optimum-T_a of the eighteen forward and reverse primers using DNA specimens from *D. alata*, *D. bulbifera,* and *D. cayenensis*.

It was noted that the high annealing temperature improves the specificity of the PCR products, but might reduce the yield of the PCR. The DNA template used for PCR in most cases was pooled of multiple DNA templates having very close quantification.

3.3.4.1 PCR Processing steps to deploy forward and reverse primers

Master Mix for 14 reactions of PCR samples was prepared as 151.5µL of SDW was loaded to a 2.0ml Eppendorf tube. Then, 28µL of 10x PCR Buffer (Vivantis A), and 8.4µL of 50 mM $MgCl_2$ were loaded into the tube. 28µL of 2uM (10x) forward and reverse primers were then added. Subsequent to adding 5.3µL 10 mM of dNTPs and 24µL of the DNA template, the tubes were loaded with 20µL of the Master Mix. After adding 2.8µL of Taq polymerase (5U/µL), two negative controls of 18µL of master mix and 2µL SDW were used. Lastly, the twelve samples and the two negative controls were loaded into the PCR machine Thermal-Cycler (MasterCycler/ BioRap) for amplification.

3.3.4.2 FluorChem HD2 (UV application)

The PCR gel was photographed using an Alpha-Innotech technology unit (FluorChem HD2) after gently placed on the exposed UV tray, and the photograph was taken and saved.

3.4 $MgCL_2$ ROLE IN PCR APPLICATION

Magnesium is an essential cofactor for numerous enzymes and, in particular, for deoxyribonuclease (DNA) during PCR amplification. It is known that high intensity of Mg^{2+} can increase the amplification yield, but decreases specificity. A concentration of

1.5mM $MgCl_2$ was recommended for optimal PCR. However, Mg^{2+} concentration incorporated with Taq DNA polymerase should be optimised for each group of primers; to achieve this objective, a comparison of multiple series of PCR containing a series of Mg^{2+} intensity starting from 0.5 increased to 5.0mM was carried out (Sambrook and Russell, 2001). A further specificity of amplicons might be achieved by increasing $MgCl_2$ intensity from 1.8 to 10.8mM, whereas nonspecific bands vanished when dNTPs intensity was at 200mM. However, intensifying the $MgCl_2$ up to 20mM might drive the PCR yield backward and become barely visible (Henegariu *et al.*, 1997). The PCR yield reached an adequate intensity at 10.8mM concentration.

Remarkably, magnesium intensity considerably stimulates the PCR products due to the ions' interaction with the DNA and the enzymes activity being altered. Moreover, the influence of Mg^{2+} intensity varies with different pairings and DNA templates. Subsequently, optimisation of Mg^{2+} concentration between 0.5 to 5.0 mM is widely incorporated into PCR ingredients when 1.0µl divalent is utilized (Brown, 1989).

Three different quantities of $MgCl_2$ ranging from 0.07µl, 0.6µl, and 1.0µl, 2.5µl for a reaction of 20µl were deployed in this project.

3.5 FRAGMENT ANALYSIS

To analyse PCR fragment length employing Capillary-electrophoresis and laser exposure system, the A 6-carboxy-fluorescence was integrated into the forward primer's 5'-end deployed in PCR (Schuelke, 2000). The capillary split fragments according to their ions' total charges into different sizes. Buffer type, concentration, pH, temperature, voltage applied, and type of the polymerase are factors influencing the capillary process. The fragment length was calculated according to the standard measurements with which the fragments co-migrated. The size of the fragments might vary with instrument variation, size of the capillary array, and the size standard (Life Technology, 2014). Amplicons samples with strong visibility were pooled after the PCR was conducted, and the products were sent for fragment analysis using GeneMapper software where the DNA fragments were co-migrated with 199.49bp low, and 300.52bp high size-standard

3.5.1 Microsatellites markers

Microsatellites are short oligonucleotides with lengths ranging from one to four base pairs of tandem motif sequences, deployed for linking analysis, investigating genetic arrays, and gene mapping. The markers are mostly positioned in the introns or the noncoding regions of the gene. Molecular markers are classified according to the technical concept **(**Hoshino *et al.*, 2012).

The scheme of incorporating a tailed primer which has no homology with the targeted template into the forward primer 5' terminus pairing with a reverse primer in a three primers application has dropped the cost of employing fluorescent labelling microsatellites in genotyping assays (Missiaggia and Grattapaglia, 2006).

Molecular genetic markers are efficient in identifying and distinguishing between cultivars with identical morphological traits and evolutionary relationships because molecular markers are unaffected by unstable environmental situations or plant life-cycle activities (Aggarwal *et al.*, 2008; Muthamia *et al.*, 2013).

The quantity of fluorescent dye M13 FAM incorporated into the forward primer was less than half of the reverse primer. During the first round of PCR, the labelled M13/forward primer integrated into the replicated PCR products. When T_a dropped and the forward primer was consumed, the action activated the universal M13 to precede the amplification as a forward-M13 primer and to integrate the fluorescent dye into the PCR products (Schuelke, 2000).

Theoretically, PCR is capable of replicating microsatellites from a single cell, but replicating from inadequate quantity of DNA template is frequently accompanied with high error rates where alleles with inaccurate sizes are replicated or an allele of heterozygous organisms is not amplified (Schlotterer and Pemberton, 1998).

For sharp and high yield of PCR products, primers are drafted out of the minimum space from the duplicated motif by positioning the markers nearby (within 10-20bp) because the motif might escalate the tendency of PCR stammering. Moreover, the primers must have sufficient length and (G-C) bases to overcome the T_a stringent, and to secure primers hybridisation to a single side of the targeted DNA. Designing primers with a

higher (A-T) content at the 3' prime secures primer hybridisation before DNA polymerase proceeds to elongate the nucleotides (Scribner and Pearce, 2000).

Arguably, designed microsatellites for one species might amplify several closely linked species. Markers' transferability efficacy across species has considerably endorsed the comprehensive use of microsatellites (Schlotterer and Pemberton, 1998). Additionally, the success level of microsatellites amplification across species has revealed the direct link to the evolutionary differences between the donor species of the loci and the recipient species wherein the heterologous loci are being implanted (Primmer *et al.*, 1996; Primmer *et al.*, 2005).

Markers comprising of repetitions of one to six base-pairs long DNA motifs have different forms of alleles with varying number of replicated motif units. The loci comprising microsatellites are greatly adaptable, making their analysis a useful tool in various genomic applications. Microsatellites have regularly highlighted a significant degree of genetic variation in species that have been classified as having low levels of genetic variance by other methods of detection (Korpelainen *et al.*, 2007).

Moreover, short motifs might generate an extra number of slipped strands mismatching throughout the PCR forming genotype slips (Buschiazzo and Gemmell, 2006). Yet, long and ideal SSR loci are known to display higher allelic divergence (Buschiazzo and Gemmell, 2006; Kelkar *et al.*, 2008; Zalapa *et al.*, 2012).

Polymerases enzymes might assemble non-template dNTPs to the ends of PCR products while extending the polynucleotides generating alleles with two different lengths, the genuine length and longer length of the same alleles. Therefore, having two sizes for a single allele mistranslated the fragment sizes because the two sizes were representing the same allele. Accordingly, more than one band or peak might be observed on electrophoresis gel and electropherograms diagrams (Stone, 2000).

Microsatellites with dinucleotide motifs might form more than one peak on electropherograms. Figures 4.75, 4.76, 4.78, 4.79, and 4.80 illustrate the double bands and multiple peaks on the electropherograms. Conversely, the microsatellites of two alleles containing 30% percentage of heterozygosity in a species would have an identical size of alleles where markers exhibited a single band and one peak on the

electrophoresis gel and electropherograms after amplification. Commonly, species with single nucleotide polymorphisms (SNPs) will have low percentages of heterozygous alleles. Thus, most of these SNPs have identical alleles (Kluwe, 2013). Heterozygous markers with double alleles might vary in their length and nucleotide stacking.

Since a single primer was incorporated with fluorescence dye, one strand of each of the marker allele appeared on the electropherograms. Therefore, the revealed allele on the electropherograms might not be visualised as one peak, but a group of peaks within one main peak as the biggest, brightest, and more intense compared to the other smaller shadowing peaks (Life Technology, 2014).

The use of Bioinformatics tools eased microsatellite-loci segregation and transferring between closely linked species (Hoshino *et al*., 2012**).** Moreover, having a high allelic variation and co-dominancy augmented the microsatellites use (Vartia *et al.*, 2014). Because of their high mutation rate, microsatellites are considered the most efficient molecular markers with the advantage of simplicity and economical detection by PCR.

3.5.2 Microsatellites markers list

Four microsatellite markers were incorporated with M13-FAM tailed primer:

Ym30 (249-300bp) F: CACGACGTTGTAAAACGACGGTCCTCTTCTATCCCAACAA
R: CACGTATTAACTCCAT

Da1A01 (222-237bp) F: CACGACGTTGTAAAACGACTATAATCGGCCAGAGG
R: TGTTGGAAGCATAGAGAA

Da3G04 (321–329bp) F: CACGACGTTGTAAAACGACCACGGCTTGACCTATC
R: TTATTCAGGGCTGGTG

Dpr3B12 (152–168bp) F: CACGACGTTGTAAAACGACCATCAATCTTTCTCTGCTT
R: CCATCACACAATCCATC

3.5.3 PCR Processing steps: Forward + M13 Tailed and reverse primers

The 1000X dye-labelled M13-SSR, 5′-CACGACGTTGTAAAACGAC-3′ solution was diluted with the ratio of 1:9. 1µL of M13 stock solution was added to 9µL of SDW to produce a 100 X dye-labelled M13-SSR concentration. Master mixture for seven reactions of PCR with 100X dye-labelled M13 and two negative samples were prepared

by loading 87.2µL of SDW into a 2.0ml Eppendorf tube, and then 14µL of 10x PCR Buffer A (Vivants /without $MgCl_2$) was added. Then, 4.2µL of 50mM $MgCl_2$, and 1.4µL of 2uM (10x) forward and reverse primers were added. Subsequently, 1.26µL of 100x dye-labelled M13, and 2.63µL 10mM of dNTPs were added. By adding 1.4µL of Taq polymerase (5U/µL), and 10µL of the DNA template the mixture was ready for experimentation. Two negative controls were loaded with 18µL of the master mix and 2µL SDW added to complete the volume to 20µL.

4 Chapter 4: RESULTS

4.1 DNA ISOLATION

The 48 accessions collected from Southwestern Ethiopia (*D. alata* 8*, D. bulbifera* 12*, and D. cayenensis* 20), and from Southwestern Malaysia (*D. alata* 8) generated samples with varying DNA quantity based on the electrophoresis data. Some samples were excluded due to their poor DNA quality.

4.2 DNA QUANTIFICATION

4.2.1 Spectrophotometers (Nano-Drop)

The spectrophotometer data were analysed to acquire the sample's mean of the DNA quantities. Table 4.1 gives the mean of *Dioscorea alata* samples with a mean sample concentration of 33.1ng/µl, ranging from 5.7 ng/µl to 4.8 ng/µl. *Dioscorea bulbifera* species DNA quantity mean was 110.5 ng/µl where the high and the low samples were 329 ng/µl and 12.2 ng/µl respectively. While, *Dioscorea cayenensis* had an average concentration of 46 ng/µl, and ranged from 97 ng/µl to 7.1ng/µl. Table 4.2 shows the spectrophotometers results of *D. alata* from Malay Peninsula, which averaged 63.1ng/µl, and ranged from 149.2 ng/µl to 6.4ng/µl.

The average concentration of all the three *Dioscorea* species was 62.9mg/µl.

Spectrophotometers/DNA Quantification for *Dioscorea sp* . of yam Ethiopia Dec. 2013						
Code	Species	DNA. Qua.ng/ul				
BE 35 2nd	D. alata	18.2				
BE 35 1st	D. alata	55.7				
Sh 43 2nd	D. alata	51.7.				
Sh 60 2nd	D. alata	44.5				
Sh19 2nd	D. alata	30.9				
Sh30 2nd	D. alata	4.8				
Sh30 1st	D. alata	39.2				
Sh67 1st	D. alata	5.9				
Sh 56 1st	D. alata	46.7				
Sh24C 1st	D. bulbifera	103.9				
Sh24C 2nd	D. bulbifera	26.9				
Sh24E 1st	D. bulbifera	144.6				
Sh25c 1st	D. bulbifera	307.0				
Sh25C 2nd	D. bulbifera	107.5				
Sh75A 1st	D. bulbifera	329.0				
Sh75A 2nd	D. bulbifera	33.9				
Sh75D 1st	D. bulbifera	57.6				
Sh75D 2nd	D. bulbifera	12.2				
Sh80 1st	D. bulbifera	65.2				
Sh82D 1st	D. bulbifera	71.0				
Sh82D 2nd	D. bulbifera	37.1				
DB	D. bulbifera	140.0				
Be29 2nd	D. cayenensis	97.0				
Be29 2nd	D. cayenensis	41.9				
Be38 1st	D. cayenensis	48.7				
Be40 1st	D. cayenensis	28.7				
Be40 2nd	D. cayenensis	79.0				
Sh11 1st	D. cayenensis	65.4				
Sh54 1st	D. cayenensis	28.2				
Sh54 2nd	D. cayenensis	35.4	*Dioscorea*	*alata*	*bulbifera*	*cayenesis*
Sh54 2nd	D. cayenensis	35.4	Average	33.1	110.5	46
Be5 2nd	D. cayenensis	30.6	µg/µl			
Be3 1st	D. cayenensis	85.0				
Be14 1st	D. cayenensis	25.0	DNA high	55.7	329.0	97.0
Be4 1st	D. cayenensis	45.0	Quantity			
Sh18 2nd	D. cayenensis	213.0	DNA Low	4.8	12.2	7.1
Sh38	D. cayenensis	10.0	Quantity			
Sh61 1st	D. cayenensis	23.1				
Sh55 1st	D. cayenensis	7.1				
Sh48 1st	D. cayenensis	47.6				
Sh48 2nd	D. cayenensis	14.1				
BE7 1st	D. cayenensis	92.0				
Sh 3 2nd	D. cayenensis	45.0				

Table 4.1 Displaying spectrophotometer reading of DNA quantification *D. alata, D. bulbifera,* and *D. cayenensis* species of Ethiopia with average of high and low concentration in DNA Qua. ng/ul.

Spectrophotometers for DNA quantification of *D. alata* of Malay peninsula								
M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	
DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	DNA Qua. ng/ul	
146.0	71.8	98.6	92.5	112.2	149.2	136.5	127.4	H=149.2 ng/ul
68.7	61.6	97.2	71.9	98.9	136.8	100.5	91.8	L=6.4 ng/ul
57.4	61.4	39.0	31.8	83.0	98.9	99.6	52.4	
22.3	46.5	29.0	25.6	67.9	91.5	67.3	35.4	
9.1	45.3	13.5	20.0	66.2	89.5	59.9	33.3	
6.4	55.0	11.4	15.3	61.8	86.2	50.9	20.5	
51.6	56.9	48.1	42.8	81.7	108.7	85.8	60.2	Average

Table 4.2 Displaying spectrophotometer reading of DNA quantification of *D. alata* species of Malay Peninsula, and the average of group of specimens, the high and low of intensity in DNA Qua. ng/ul**.**

4.2.2 Electrophoresis of DNA quantification

The *D. alata, D. bulbifera,* and *D. cayenensis* sample quantities were estimated based on the electrophoresis gel run under the UV light and quantified according to the DNA ladder (Thermo-Scientific GeneRuler 100 X).

Table 4.3 shows that the *D. alata* of Ethiopia sample average was 123.6ng/µl, with a high of 275 ng/µl and a low of 55 ng/µl in DNA quantity. The average quantity of *D. bulbifera* was 246.25 ng/µl, with a high of 350 ng/µl and low of 155 ng/µl in DNA quantity. *D. cayenensis* displayed an average of 108.86 ng/µl, and displayed 240 ng/µl and 30 ng/µl of a high and low in DNA quantity respectfully.

The electrophoresis data from table 4.4 of Malaysian *D. alata* revealed an average of 74.1 ng/µl, and a high of 200 ng/µl with a low of 5 ng/µl in DNA quantity.

Notably, there was a significant difference in the quantification data of the spectrophotometer and electrophoresis measurements. Therefore, the next step was to find the mean of the two quantifications records as exhibited by table 4.5. The DNA quantification images on agarose gel displayed by figures 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6.

Electrophoresis for DNA Quantification of Dioscorea sp. of Yam Ethiopia							
		Electrophoresis reading					
Sample ID	Species	DNA Qua .(ng/ul)					
Sh19	D. alata	55					
Sh 56	D. alata	100					
Sh 67	D. alata	130					
Sh 43	D. alata	58					
Sh 30	D. alata	275					
Sh 24C	D. bulbifera	220					
Sh 24E	D. bulbifera	220					
Sh 25C	D. bulbifera	335					
Sh 75A	D. bulbifera	350					
Sh 82D	D. bulbifera	155					
Sh 75D	D. bulbifera	200					
Sh 80	D. bulbifera	190					
Sh 25C	D. bulbifera	300					
Be35	D. cayenensis	180					
Sh 60	D. cayenensis	190					
Be 29	D. cayenensis	150					
Sh 54	D. cayenensis	105					
Be 5	D. cayenensis	240					
Sh 55	D. cayenensis	55					
Sh 48	D. cayenensis	150					
Be 38	D. cayenensis	250		D.alata	D.bulbifera	D.cayenesis	
Be 40	D. cayenensis	85	Average of DNA	123.6	246.3	108.9	
Sh 11	D. cayenensis	145	Qua. ng/ul				
Be 29	D. cayenensis	150	High Quantity	275	350	240	
Be 38	D. cayenensis	180	Low quantity	55	155	30	
Be3 1st	D. cayenensis	85	Qua. ng/ul				
Be14 1st	D. cayenensis	25					
Be4 1st	D. cayenensis	50					
Sh18 2nd	D. cayenensis	40					
Sh61 1st	D. cayenensis	45					
Sh55 1st	D. cayenensis	40					
Sh48 1st	D. cayenensis	50					
BE7 1st	D. cayenensis	100					
Sh 3 2nd	D. cayenensis	50					
Sh61 1st	D. cayenensis	30					

Table 4.3 exhibited Electrophoresis gel reading of DNA quantification of *D. alata, D. bulbifera,* and *D. cayenensis* species of Ethiopia with the average, high and low intensity in DNA Qua. ng/ul**.**

Electrophoresis for DNA Quantification *D. alata* sp. Yam Malay peninsula , J								
M1/20ul	M2/20ul	M3/20ul	M4/20ul	M5/20ul	M6/20ul	M7/20ul	M8/20ul	
DNA Qua. ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	DNA Quant ng/ul	
200	120	200	110	85	95	75	20	H=200 ng/
185	110	200	100	50	85	35	10	
180	115.0	120	90	45	80	20	10	L=5 ng/ul
150	115.0	120	85	10	60	10	5	
110	115.0	110	55	5	50	5	5	
85	115.0	110	50	5	45	5	5	
75	115.0	100	35	5	40	5	5	
140.7	115.0	137.1	75.0	29.3	65.0	22.1	8.6	average

Table 4.4 exhibited Electrophoresis gel reading DNA quantification of *D. alata* of Malay Peninsula, and the average, high and low intensity in DNA Qua. ng/ul**.**

Spectrophotometers & Electrophoresis of DNA Quantification Dioscorea sp. Yam						
		Spectrophometers	**Electrophoresis**			
sample	Species	DNA Qua. ng/ul	DNA Qua. ng/ul			
Sh19	D. alata	30.29	55			
Sh 56	D. alata	12.01	100			
Sh 67	D. alata	5.88	130			
Sh 43	D. alata	51	58			
Sh 30	D. alata	46	275			
Sh 24C	D. bulbifera	41	220			
Sh 24E	D. bulbifera	209	220			
Sh 25C	D. bulbifera	307	335			
Sh 75A	D. bulbifera	329	350			
Sh 82D	D. bulbifera	71	155			
Sh 75D	D. bulbifera	57	200			
Sh 80	D. bulbifera	115	190			
Sh 25C	D. bulbifera	307	300			
Be35	D. cayenensis	65	180			
Sh 60	D. cayenensis	18	190			
Be 29	D. cayenensis	152	150			
Sh 54	D. cayenensis	48	105			
Be 5	D. cayenensis	90.59	240			
Sh 55	D. cayenensis	7.12	55			
Sh 48	D. cayenensis	50	150			
Be 38	D. cayenensis	70	250			
Be 40	D. cayenensis	79	85			
Sh 11	D. cayenensis	87	145			
Be 29	D. cayenensis	152	150			
Be 38	D. cayenensis	70	180	Spectrophotometer High Qua		350 ng/ul
Be3 1st	D. cayenensis	85	85	Electrophoresis High Qua		329 ng/ul
Be14 1st	D. cayenensis	25	25	Spectrophotometer Low Qua		25 ng/ul
Be4 1st	D. cayenensis	45	50	Electrophoresis Low Qua		7 ng/ul
Sh18 2nd	D. cayenensis	21	40			
Sh61 1st	D. cayenensi	23	45			
Sh55 1st	D. cayenensis	7	40			
Sh48 1st	D. cayenensis	45	50			
BE7 1st	D. cayenensis	92	100			
Sh 3 2nd	D. cayenensis	45	50			
Sh61 1st	D. cayenensi	1.22	30			

Table 4.5 Spectrophotometers and electrophoresis gel reading DNA quantification of *D. alata, D. bulbifera,* and *D. cayenensis* species of Ethiopia with the average, high and low intensity in DNA Qua. ng/ul**.**

Figure 4.1 DNA quantification for *D. alata*, *D. bulbifera,* and *D. cayenensis* species viewed on electrophoresis 1% agarose gel prepared with 1X TAE buffer. The results were migrated with Fermentas GeneRuler 1K ladder.

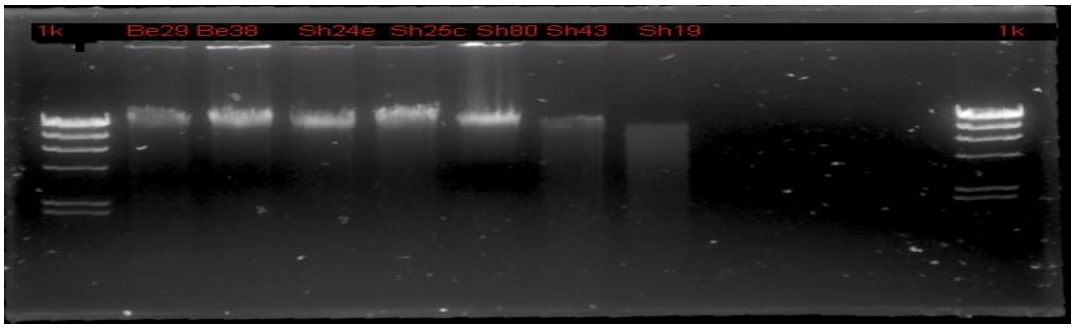


Figure 4.2 DNA quantification for *D. alata, D. bulbifera,* and *D. cayenensis* using electrophoresis gel 1% agarose. 1X TAE buffer, Fermentas GeneRuler 1K ladder lambda used as a standard size.

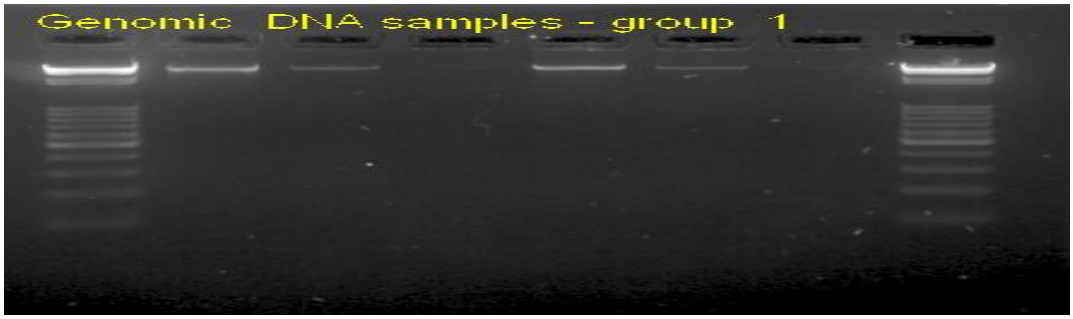


Figure 4.3 DNA quantification for *D. alata, D. bulbifera,* and *D. cayenensis* displayed on electrophoresis 1% agarose gel prepared with 1X TAE buffer, the samples quantified according to Fermentas GeneRuler 1K ladder.

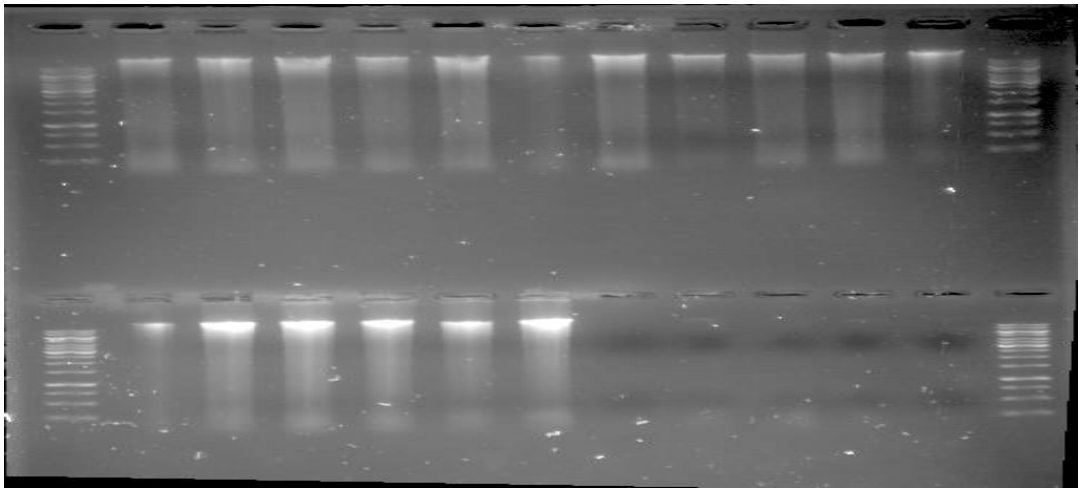


Figure 4.4 DNA quantification for *D. alata* (Malaysian) using electrophoresis 1% agarose gel readied by 1X TAE buffer and Fermentas GeneRuler 1K ladder to quantify the samples.

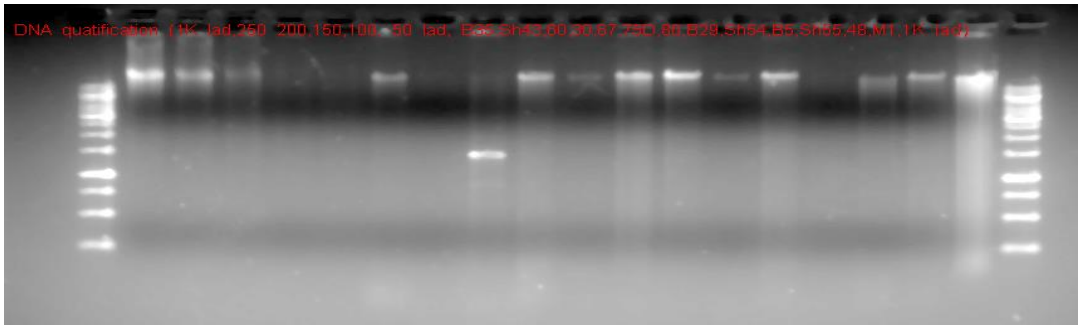


Figure 4.5 DNA quantification for *D. alata, D. bulbifera,* and *D. cayenensis* using electrophoresis 2% agarose gel, 1X TAE buffer, and Fermentas GeneRuler 1K ladder, 250, 200, 150,100, 50 ng/µl lambda ladders. A contamination of artefact was observed on the gel.

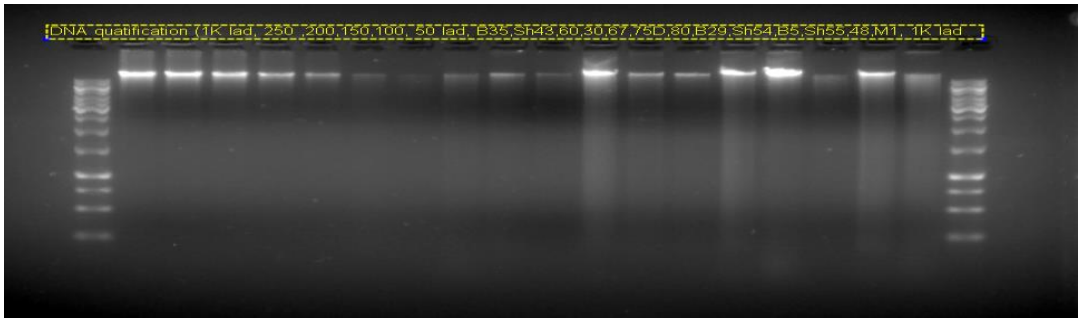


Figure 4.6 DNA quantification for *D. alata, D. bulbifera*, and *D. cayenensis* species using electrophoresis 2% agarose gel and 1X TAE buffer,. Fermentas GeneRuler 1K ladder, 250, 200, 150,100, 50 ng/µl lambda ladders used in determining the DNA quantity.

4.3 PCR APPLICATION

The PCR results of the eighteen primers amplifying *D. alata, D. bulbifera,* and *D. cayenensis* were examined by electrophoresis-gel under UV-light, and the markers categorised into amplified and non-amplified and being transferable or not across the three species.

4.3.1 Primers List

18 primers developed by Tostain et al. (2006), Number of alleles, Tm, %GC.				
Primer & allele size	**Primer Sequence (5' → 3')**	**No of alleles**	**Tm ^{0}C**	**%GC**
Ym13	F:TTCCCTAATTGTTCCTCTTGTTG	**7**	**67.3**	**39.1**
200-350	R:GTCCTCGTTTCCCTGT		**65.3**	**56.2**
Ym30	F:GGTCCTCTTCTATCCCAACAA	**14**	**67.6**	**47.6**
236-350	R:CACGTATTAACTCCATCTATCCAA		**66.4**	**37.5**
Da1A01	F:TATAATCGGCCAGAGG	**11**	**61.9**	**50**
200-300	R:TGTTGGAAGCATAGAGAA		**63.4**	**38.9**
Da3G04	F:CACGGCTTGACCTATC	**12**	**63.4**	**56.2**
175-350	R:TTATTCAGGGCTGGTG		**63.2**	**50**
Dab2C12	F:AGGCATCTTGGGAAA	**2**	**62.8**	**46.7**
120-195	R:CGAACGATCCAATAAAA		**60.1**	**35.3**
Dab2D06	F:TGTAAGATGCCCACATT	**7**	**63.8**	**41.2**
185-280	R:TCTCAGGCTTCAGGG		**64.4**	**60**
Dab2D08	F:ACAAGAGAACCGACATAGT	**6**	**65.3**	**42.1**
315-355	R:GATTTGCTTTGAGTCCTT		**63**	**38.9**
Dpr3B12	F:CATCAATCTTTCTCTGCTT	**11**	**62.9**	**36.8**
152-298	R:CCATCACACAATCCATC		**62.2**	**47.1**
Dpr3D06	F:ATAGGAAGGCAATCAGG	**4**	**63.1**	**47.1**
130-300	R:ACCCATCGTCTTACCC		**64.8**	**56.2**
Dpr3F04	F:AGACTCTTGCTCATGT	**2**	**62.5**	**43.8**
160-170	R:GCCTTGTTACTTTATTC		**57.9**	**35.3**
Dpr3F10	F:TCAAAGGAATGTTGGG	**2**	**61.6**	**43.8**
270-320	R:ACGCACATAGGGATTG		**63.6**	**50**
Dpr3F12	F:TCCCCATAGAAACAAAGT	**4**	**63.5**	**38.9**
190-320	R:TCAAGCAAGAGAAGGTG		**63.8**	**47.1**
Da1D08	F:GATGCTATGAACACAACTAA	**2**	**63**	**35**
180-250	R: TTTGACAGTGAGAATGGA		**63.7**	**38.9**
Da1F08	F: AATGCTTCGTAATCCAAC	**4**	**63**	**38.9**
175-190	R: CTATAAGGAATTGGTGCC		**62.1**	**44.4**
Dab2C05	F: CCCATGCTTGTAGTTGT	**6**	**64.3**	**47.1**
175-240	R: TGCTCACCTCTTTACTTG		**63.9**	**44.4**
Dab2E09	F: AACATATAAAGAGAGATCA	**1**	**58.6**	**26.3**
210	R: ATAACCCTTAACTCCA		**59**	**37.5**
Dab2E07	F: TTGAACCTTGACTTTGGT	**4**	**64.8**	**38.9**
110-200	R: GAGTTCCTGTCCTTGGT		**65.3**	**52.9**
Da1C12	F: GCCTTTGTGCGTATCT	**5**	**64.3**	**50**
120-200	R: AATCGGCTACACTCATCT		**65.2**	**44.4**
Tm (melting temperature)				

Table 4.6 Eighteen markers sequencing, allele number and size, T_m (melting temperature), GC percentages, used in this project, developed by Tostain *et al.* (2006).

4.3.2 PCR Static annealing temperature at 45^0C and 50^0C

SSR markers Da1D08 failed amplifying *D. alata* at 45 and 50^0C. Table 4.7 and figures 4.7, 4.8, 4.9, and 4.10 depict the results of the 18 markers at static T_a at 45^0C and 50^0C.

PCR for *Dioscorea alata* static annealing temperature (45 and 50 Celsius				
Dioscorea alata			Temperature	
Num	Marker & Allele si	5' to 3'-end	*45 °C*	*50 °C*
1	**Ym 13**	F:TTCCCTAATTGTTCCTCTTGTTG	+	+
	200-350bp	R:GTCCTCGTTTCCCTGT		
2	**Ym 30**	F:GGTCCTCTTCTATCCCAACAA	+	+
	236-350bp	R:CACGTATTAACTCCATCTATCCAA		
3	**Da1A01**	F:TATAATCGGCCAGAGG	+	+
	200-300bp	R:TGTTGGAAGCATAGAGAA		
4	**Da3G04**	F:CACGGCTTGACCTATC	+	+
	175-350bp	R:TTATTCAGGGCTGGTG		
5	**Dab2C12**	F:AGGCATCTTGGGAAA	+	+
	120-195bp	R:CGAACGATCCAATAAAA		
6	**Dab2D06**	F:TGTAAGATGCCCACATT	+	+
	185-280bp	R:TCTCAGGCTTCAGGG		
7	**Dab2D08**	F:ACAAGAGAACCGACATAGT	+	+
	315-355bp	R:GATTTGCTTTGAGTCCTT		
8	**Dpr3B12**	F:CATCAATCTTTCTCTGCTT	+	+
	152-298bp	R:CCATCACACAATCCATC		
9	**Dpr3D06**	F:ATAGGAAGGCAATCAGG	+	+
	130-300bp	R:ACCCATCGTCTTACCC		
10	**Dpr3F04**	F:AGACTCTTGCTCATGT	+	+
	160-170bp	R:GCCTTGTTACTTTATTC		
11	**Dpr3 F10**	F:TCAAAGGAATGTTGGG	+	+
	270-320bp	R:ACGCACATAGGGATTG		
12	**Dpr3F12**	F:TCCCCATAGAAACAAAGT	+	+
	190-320bp	R:TCAAGCAAGAGAAGGTG		
13	**Da1D08**	F:GATGCTATGAACACAACTAA	-	-
	180-250bp	R:TTTGACAGTGAGAATGGA		
14	**Da1F08**	F:AATGCTTCGTAATCCAAC	+	+
	175-190bp	R:CTATAAGGAATTGGTGCC		
15	**Dab2C05**	F:CCCATGCTTGTAGTTGT	+	+
	175-240bp	R:TGCTCACCTCTTTACTTG		
16	**Dab2E09**	F:AACATATAAAGAGAGATCA	+	+
	210bp	R:ATAACCCTTAACTCCA		
17	**Dab2E07**	F:TTGAACCTTGACTTTGGT	+	+
	110-200bp	R:GAGTTCCTGTCCTTGGT		
18	**Da1C12**	F:GCCTTTGTGCGTATCT	+	+
	120-200bp	R:AATCGGCTACACTCATCT		
(-) No amplification			**(+) amplification**	

Table 4.7 PCR static annealing at 45^0C and 50^0C for *D. alata* species deploying eighteen primers developed by Tostain *et al.* (2006).

Figure 4.7 PCR static T_a at 45^0C of *D. alata* species incorporated with Da3G04-320bp, Dab2C12-330bp, Dab2D06-200bp, Dab2D08-240bp, Dpr3D06-300bp, Dpr3F04-170bp, Dpr3F12-245bp & Dab2F07-210bp primers to amplify the accessions. Electrophoresis 1% agarose gel displayed the result.

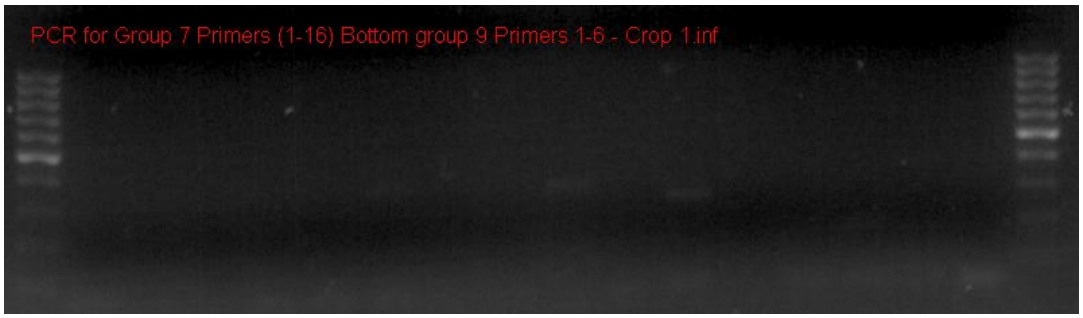


Figure 4.8 PCR static annealing temperature at 50 ^{0}C for *D. alata* results of Dab2D06-280bp**,** Dpr3D06-300bp, Dpr3F10-270bp & Da1F08-180bp primers positive amplification visualised 1% agarose gel.

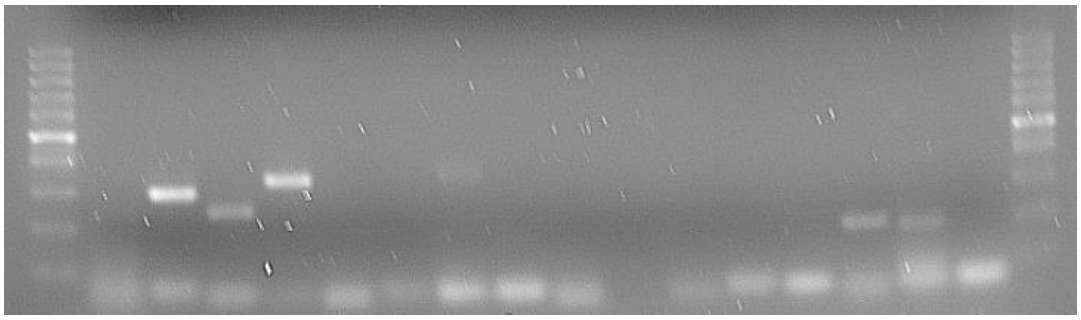


Figure 4.9 PCR, static T_a 50.^{0}C for *D. alata* species amplified deploying Ym30- 290bp, Da1A01-250bp, Da3G04-340bp, Dab2D08-345bp, Da1F08-185bp & Dab2C05-180bp primers. The products observed on 1% agarose gel.

Figure 4.10 PCR static annealing temperature at 45^0C and 50^0Cfor *D. alata* & *D. bulbifera* species employing Da1A01-240bp & Da3G04-320bp primers observed on 1% agarose gel.

Similarly, the SSR markers of Dpr3B12, Da1D08, and Dab2E09 failed to amplifying *D. bulbifera* species in PCR at 45^0C and 50^0C T_a. Table 4.8 and figures 4.10, 4.11, 4.12, 4.13, 4.14, 4.16, and 4.17 exhibited the PCR results of the markers incorporated with *D. bulbifera* species.

PCR for *Dioscorea bulbifera static* annealing temperature 45 and 50 C				
	Dioscorea bulbifera		Temperature	
Numbe	Marker & Alle	5' to 3'-end	*45 °C*	*50 °C*
1	**Ym 13**	F:TTCCCTAATTGTTCCTCTTGTTG	+	+
	200-350bp	R:GTCCTCGTTTCCCTGT		
2	**Ym 30**	F:GGTCCTCTTCTATCCCAACAA	+	+
	236-350bp	R:CACGTATTAACTCCATCTATCCAA		
3	**Da1A01**	F:TATAATCGGCCAGAGG	+	+
	200-300bp	R:TGTTGGAAGCATAGAGAA		
4	**Da3G04**	F:CACGGCTTGACCTATC	+	+
	175-350bp	R:TTATTCAGGGCTGGTG		
5	**Dab2C12**	F:AGGCATCTTGGGAAA	+	+
	120-195bp	R:CGAACGATCCAATAAAA		
6	**Dab2D06**	F:TGTAAGATGCCCACATT	+	+
	185-280bp	R:TCTCAGGCTTCAGGG		
7	**Dab2D08**	F:ACAAGAGAACCGACATAGT	+	+
	315-355bp	R:GATTTGCTTTGAGTCCTT		
8	**Dpr3B12**	F:CATCAATCTTTCTCTGCTT	-	-
	152-298bp	R:CCATCACACAATCCATC		
9	**Dpr3D06**	F:ATAGGAAGGCAATCAGG	+	+
	130-300bp	R:ACCCATCGTCTTACCC		
10	**Dpr3F04**	F:AGACTCTTGCTCATGT	+	+
	160-170bp	R:GCCTTGTTACTTTATTC		
11	**Dpr3 F10**	F:TCAAAGGAATGTTGGG	+	+
	270-320bp	R:ACGCACATAGGGATTG		
12	**Dpr3F12**	F:TCCCCATAGAAACAAAGT	+	+
	190-320bp	R:TCAAGCAAGAGAAGGTG		
13	**Da1D08**	F:GATGCTATGAACACAACTAA	-	-
	180-250bp	R:TTTGACAGTGAGAATGGA		
14	**Da1F08**	F:AATGCTTCGTAATCCAAC	+	+
	175-190bp	R:CTATAAGGAATTGGTGCC		
15	**Dab2C05**	F:CCCATGCTTGTAGTTGT	+	+
	175-240bp	R:TGCTCACCTCTTTACTTG		
16	**Dab2E09**	F:AACATATAAAGAGAGATCA	-	-
	210bp	R:ATAACCCTTAACTCCA		
17	**Dab2E07**	F:TTGAACCTTGACTTTGGT	+	+
	110-200bp	R:GAGTTCCTGTCCTTGGT		
18	**Da1C12**	F:GCCTTTGTGCGTATCT	+	+
	120-200bp	R:AATCGGCTACACTCATCT		
(-) No amplification			**(+) amplification**	

Table 4.8 PCR static annealing temperatures at 45^0C & 50^0C results for *D. bulbifera* species deploying eighteen primers developed by Tostain *et al.* (2006).

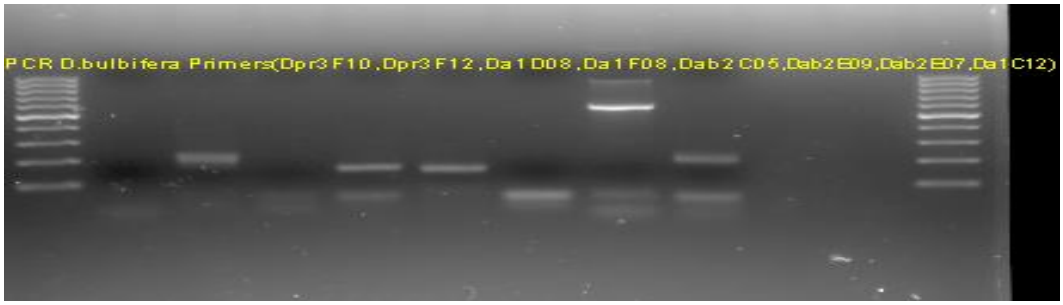


Figure 4.11 PCR static Ta 50^{0}C for *D. bulbifera* species positive Dpr3F12-210bp, Da1F08-190bp, Dab2C05-185bp, Dab2E07-110bp & Da1C12-190bp primers were detected on electrophoresis 1% agarose gel.

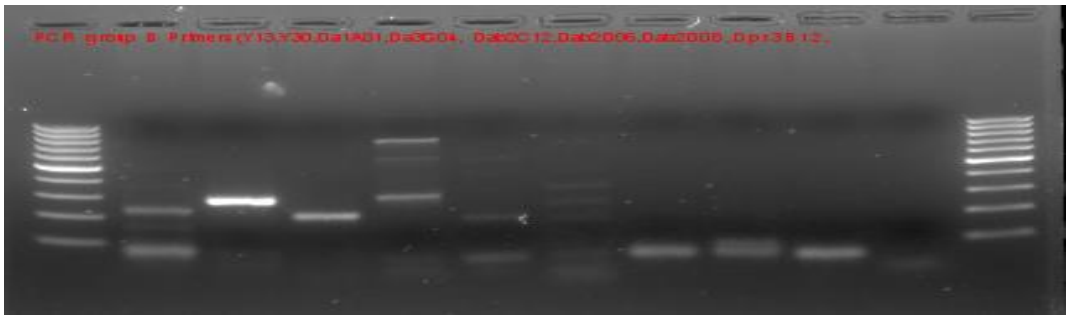


Figure 4.12 PCR static Ta 50^{0}C for *D. bulbifera* species, primers Ym13-240bp, Ym30-270bp, Da1A01-200bp, Da3G04-280bp, Da1C12 -190bp & Dab2D06-185bp results exhibited on electrophoresis 1% agarose gel.

Figure 4.13 PCR static Ta 50^{0}C for *D. bulbifera* species' positive Dpr3F04-160bp primers amplification was visualised on electrophoresis 1% agarose gel.

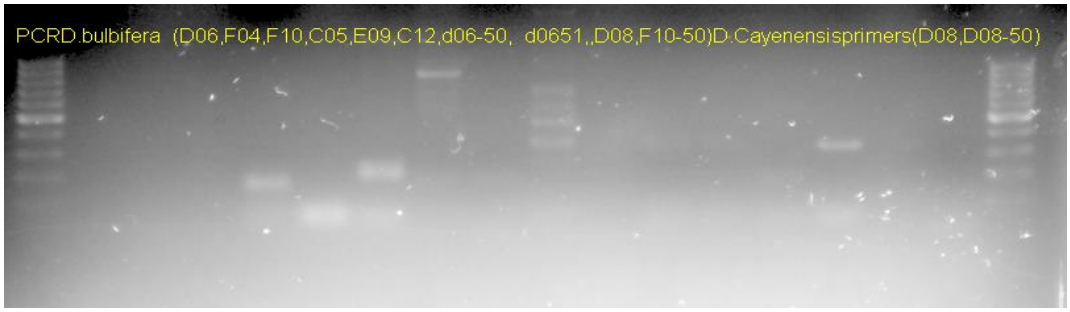


Figure 4.14 PCR static Ta at 45^{0}C & 50^{0}C. *D. bulbifera & cayenensis species'* positive results of Dab2C05-200bp, Dabc12-220bp, Dab2D06-210bp, Dab2D06-210, Dab2D06-210, Dab2D08-350bp, Dpr3F10-320bp, Dpr3F10-320bp, Da1D08-300bp & Da1D08-300bp markers pictured using electrophoresis 1% agarose gel.

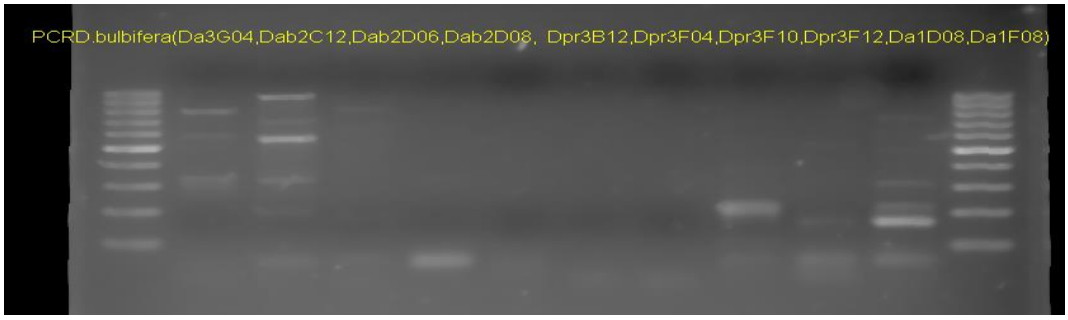


Figure 4.15 PCR, static Ta 45^{0}C for *D. bulbifera species* using Da3G04-320bp, Dab2C12-200bp, Dpr3F12-220bp & Da1F08-190bp primers. Heterozygosity was observed on electrophoresis 1% agarose gel**.**

Figure 4.16 PCR static Ta 50^{0}C for *D. bulbifera species'* positive Ym13-230bp, Ym30-280bp, Da1A01-240bp, Dpr3E07-190bp, Da3G04- 320bp & Dab2D08- 320bp primers amplification were observed on electrophoresis 1% agarose gel.

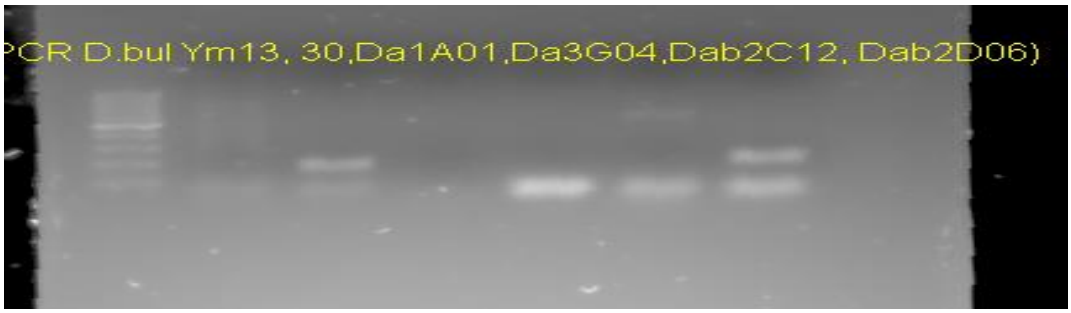


Figure 4.17 PCR static T_a 50^{0}C for *D. bulbifera species'* positive Ym13-290bp, Ym30-240, Dab2C12 -120bp & Dab2D06-220bp primers amplifications was observed on electrophoresis 1% agarose gel.

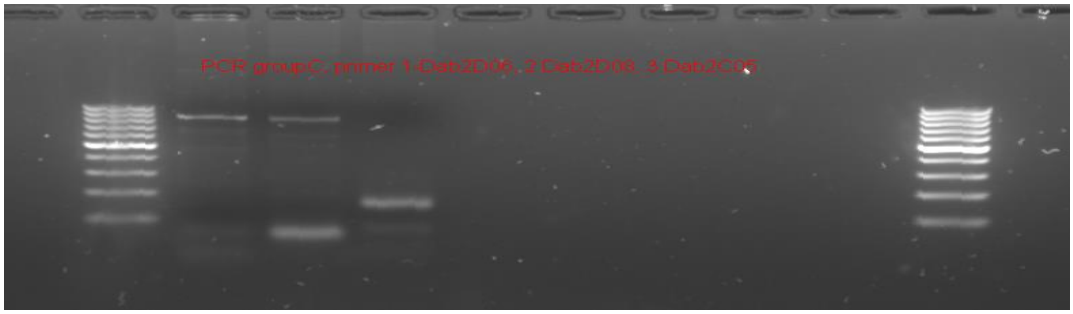


Figure 4.18 PCR static Ta 50^0C for *D. cayenensis* species' positive Dab2D06-220bp, Dab2D08-330bp &Dab2C05-175bp primers amplifications were observed on electrophoresis 1% agarose gel.

Moreover, Table 4.9 and Figures 4.19, 4.20, 4.21, and 4.22 indicate that all the eighteen primers successfully amplified *D. cayenensis* species at 45^0C and 50^0C T_a.

PCR for *Dioscorea cayenensis* static annealing temperature 45 and 50 C				
Dioscorea cayenensis			Temperature	
Numbe	rker & Allele s	5' to 3'-end	*45°C*	*50 °C*
1	**Ym 13**	F:TTCCCTAATTGTTCCTCTTGTTG	+	+
	200-350bp	R:GTCCTCGTTTCCCTGT		
2	**Ym 30**	F:GGTCCTCTTCTATCCCAACAA	+	+
	236-350bp	R:CACGTATTAACTCCATCTATCCAA		
3	**Da1A01**	F:TATAATCGGCCAGAGG	+	+
	200-300bp	R:TGTTGGAAGCATAGAGAA		
4	**Da3G04**	F:CACGGCTTGACCTATC	+	+
	175-350bp	R:TTATTCAGGGCTGGTG		
5	**Dab2C12**	F:AGGCATCTTGGGAAA	+	+
	120-195bp	R:CGAACGATCCAATAAAA		
6	**Dab2D06**	F:TGTAAGATGCCCACATT	+	+
	185-280bp	R:TCTCAGGCTTCAGGG		
7	**Dab2D08**	F:ACAAGAGAACCGACATAGT	+	+
	315-355bp	R:GATTTGCTTTGAGTCCTT		
8	**Dpr3B12**	F:CATCAATCTTTCTCTGCTT	+	+
	152-298bp	R:CCATCACACAATCCATC		
9	**Dpr3D06**	F:ATAGGAAGGCAATCAGG	+	+
	130-300bp	R:ACCCATCGTCTTACCC		
10	**Dpr3F04**	F:AGACTCTTGCTCATGT	+	+
	160-170bp	R:GCCTTGTTACTTTATTC		
11	**Dpr3 F10**	F:TCAAAGGAATGTTGGG	+	+
	270-320bp	R:ACGCACATAGGGATTG		
12	**Dpr3F12**	F:TCCCCATAGAAACAAAGT	+	+
	190-320bp	R:TCAAGCAAGAGAAGGTG		
13	**Da1D08**	F:GATGCTATGAACACAACTAA	+	+
	180-250bp	R:TTTGACAGTGAGAATGGA		
14	**Da1F08**	F:AATGCTTCGTAATCCAAC	+	+
	175-190bp	R:CTATAAGGAATTGGTGCC		
15	**Dab2C05**	F:CCCATGCTTGTAGTTGT	+	+
	175-240bp	R:TGCTCACCTCTTTACTTG		
16	**Dab2E09**	F:AACATATAAAGAGAGATCA	+	+
	210bp	R:ATAACCCTTAACTCCA		
17	**Dab2E07**	F:TTGAACCTTGACTTTGGT	+	+
	110-200bp	R:GAGTTCCTGTCCTTGGT		
18	**Da1C12**	F:GCCTTTGTGCGTATCT	+	+
	120-200bp	R:AATCGGCTACACTCATCT		

Table 4.9 PCR static annealing temperatures at 45^0C and 50^0C data for *D. cayenensis* species using eighteen primers developed by Tostain *et al.* (2006).

PCR results for static annealing 45 , 50 Celsius and 50 to 65 gradient products of *Dioscc*														
locus 5' to 3'-end	species	**45**	**50**	**50.4**	**51.3**	**52.7**	**54.7**	**56.6**	**58.4**	**60.3**	**62.3**	**63.7**	**64.6**	65
Ym 13	D. alata	+	+	+	+	+	+	+	+	+				
F:TTCCCTAATTGTTCCTCTTGTTG	D. bulbifera	+	+	+	+	+	+	+	+	+	+	+	+	+
R:GTCCTCGTTTCCCTGT	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Ym 30	D. alata	+	+	+	+	+	+	+	+	+	+	+		
F:GGTCCTCTTCTATCCCAACAA	D. bulbifera	+	+	+	+	+	+	+	+	+	+	+	+	
R:CACGTATTAACTCCATCTATCCAA	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Da1A01	D. alata	+	+	+	+	+								
F:TATAATCGGCCAGAGG	D. bulbifera	+	+	+	+	+	+	+	+	+	+			
R:TGTTGGAAGCATAGAGAA	D. cayenensis	+	+	+	+	+	+	+	+	+				
Da3G04	D. alata	+	+	+	+	+	+	+	+					
F:CACGGCTTGACCTATC	D. bulbifera	+	+											
R:TTATTCAGGGCTGGTG	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Dab2C12	D. alata	+	+											
F:AGGCATCTTGGGAAA	D. bulbifera	+	+											
R:CGAACGATCCAATAAAA	D. cayenensis	+	+											
Dab2D06	D. alata	+	+	+	+	+	+	+	+					
F:TGTAAGATGCCCACATT	D. bulbifera	+	+	+	+	+	+	+	+	+				
R:TCTCAGGCTTCAGGG	D. cayenensis	+	+											
Dab2D08	D. alata	+	+											
F:ACAAGAGAACCGACATAGT	D. bulbifera	+	+	+	+	+	+	+	+	+				
R:GATTTGCTTTGAGTCCTT	D. cayenensis	+	+	+	+	+	+							
Dpr3B12	D. alata	+	+	+	+	+	+	+						
F:CATCAATCTTTCTCTGCTT	D. bulbifera	-	-	-										
R:CCATCACACAATCCATC	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Dpr3D06	D. alata	+	+	+	+	+	+	+	+					
F:ATAGGAAGGCAATCAGG	D. bulbifera	+	+	+	+									
R:ACCCATCGTCTTACCC	D. cayenensis	+	+	+	+	+	+	+	+					
Dpr3F04	D. alata	+	+											
F:AGACTCTTGCTCATGT	D. bulbifera	+	+											
R:GCCTTGTTACTTTATTC	D. cayenensis	+	+											
Dpr3 F10	D. alata	+	+											
F:TCAAAGGAATGTTGGG	D. bulbifera	+	+											
R:ACGCACATAGGGATTG	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Dpr3F12	D. alata	+	+	+	+	+	+	+						
F:TCCCCATAGAAACAAAGT	D. bulbifera	+	+	+	+	+	+							
R:TCAAGCAAGAGAAGGTG	D. cayenensis	+	+											
Da1D08	D. alata	-	-	-										
F:GATGCTATGAACACAACTAA	D. bulbifera	-	-	-										
R:TTTGACAGTGAGAATGGA	D. cayenensis	+	+											
Da1F08	D. alata	+	+	+	+	+	+	+	+					
F:AATGCTTCGTAATCCAAC	D. bulbifera	+	+	+										
R:CTATAAGGAATTGGTGCC	D. cayenensis	+	+	+	+	+	+							
Dab2C05	D. alata	+	+											
F:CCCATGCTTGTAGTTGT	D. bulbifera	+	+											
R:TGCTCACCTCTTTACTTG	D. cayenensis	+	+	+	+	+	+							
Dab2E09	D. alata	+	+	+	+	+								
F:AACATATAAAGAGAGATCA	D. bulbifera	-	-	-										
R:ATAACCCTTAACTCCA	D. cayenensis	+	+	+	+	+	+	+	+	+	+	+	+	+
Dab2E07	D. alata	+	+	+	+	+	+	+	+	+				
F:TTGAACCTTGACTTTGGT	D. bulbifera	+	+											
R:GAGTTCCTGTCCTTGGT	D. cayenensis	+	+	+	+	+	+	+	+					
Da1C12	D. alata	+	+	+	+	+	+	+	+	+				
F:GCCTTTGTGCGTATCT	D. bulbifera	+	+											
R:AATCGGCTACACTCATCT	D. cayenensis	+	+	+										
(-) No amplification	**(+) amplification**													

Table 4.10 Gradients PCR for optimum annealing temperatures from 50^0C to 65^0C results for *D. alata, D. bulbifera,* and *D. cayenensis* species utilizing 18 primers produced by Tostain *et al.*, (2006).

Figure 4.19 PCR static Ta 50^0C for *D. cayenensis* species' positive Ym13-170bp,-240bp-350bp, Ym30-270bp, Da1F08-175bp & Dab2E07-200bp primers amplifications and more than one band for Ym13 were observed on 1% agarose gel.

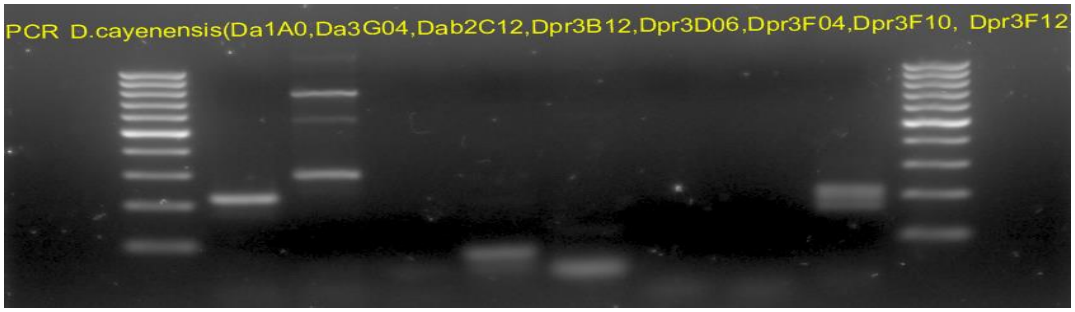


Figure 4.20 PCR static Ta 50^0C for *D. cayenensis* species' positive Da1A01-220bp, Da3G04-280bp, Dpr3B12-180bp, Dpr3D06-130pb, Dpr3F12-200bp primers amplifications were observed on 1% agarose gel

Figure 4.21 PCR static T_a 50^0C for *D. cayenensis* species Ym13-240bp, Ym30-250bp, Da3G04-280bp, Dab2D06-245bp, Dpr3B12-220bp, Dab2D06-210bp, Dab2C05-230bp, Dab2E09-210bp & Dab2E07-190bp primers in the bottom were visualised on 1% agarose gel.

Figure 4.22 PCR static T_a 50^0C for *D. cayenensis* species result employed Dpr3F12, 220bp and for *D. alata* deployed Dab2D08, 345bp primers. The affirmative results were observed on 1% agarose gel.

4.3.3 Gradient PCR (increment annealing temperature 50-65^{0}C)

The gradient PCRs were used to optimise the eighteen primers optimal T_a of the *D. alata, D. bulbifera,* and *D. cayenensis* specimens as Table 4.10 depicts.

4.3.3.1 Dioscorea alata

Table 4.11 depicts the optimisation data of the PCR increment T_a 50-65^{0}C where Da1D08 primers failed to amplify *D. alata* species. Dab2C12, Dab2D08, Dpr3F04, Dpr3F10, and Dab2C05 primers recorded an optima T_a of 50^0C, while Da1A01 and Dab2C05 primers' optimum temperatures was 52.7^0C. Moreover, Dpr3B12 and Dpr3F12 primers' optimum Ta was 56.7^0C, whilst Da3G04, Dab2D06, Dpr3D06, and Da1F08 primers were optimised at 58.4^0C T_a. Optimum T_a of 60.3^0C was reached with Ym13, Dab2E07, and Da1C12 primers, while Ym30 primer pairs had the highest optimum T_a of 63.7^0C.

Figures 4.23, 4.24, 4.25, 4.26, 4.27, 4.28, 4.29, 4.30, 4.31, 4.32, and 4.33 depict the amplification results and the optimum annealing temperatures of *Dioscorea alata* specimens.

Gradient PCR for *Dioscorea alata* increment annealing temperature 50- 65 Celsius													
Marker &	Primer sequence	Temperature											
allele size	5' to 3'-end	*50 ℃*	*50.4 ℃*	*51.3 ℃*	*52.7 ℃*	*54.7 ℃*	*56.6 ℃*	*58.4 ℃*	*60.3 ℃*	*62.3 ℃*	*63.7 ℃*	*64.6 ℃*	*65 ℃*
Ym 13	F:TTCCCTAATTGTTCCTCTTGTTG	+	+	+	+	+	+	+	+				
200-350	R:GTCCTCGTTTCCCTGT												
Ym 30	F:GGTCCTCTTCTATCCCAACAA	+	+	+	+	+	+	+	+	+	+		
236-350	R:CACGTATTAACTCCATCTATCCAA												
Da1A01	F:TATAATCGGCCAGAGG	+	+	+	+								
200-300	R:TGTTGGAAGCATAGAGAA												
Da3G04	F:CACGGCTTGACCTATC	+	+	+	+	+	+	+					
175-350	R:TTATTCAGGGCTGGTG												
Dab2C12	F:AGGCATCTTGGGAAA	+											
120-195	R:CGAACGATCCAATAAAA												
Dab2D06	F:TGTAAGATGCCCACATT	+	+	+	+	+	+	+					
185-280	R:TCTCAGGCTTCAGGG												
Dab2D08	F:ACAAGAGAACCGACATAGT	+											
315-355	R:GATTTGCTTTGAGTCCTT												
Dpr3B12	F:CATCAATCTTTCTCTGCTT	+	+	+	+	+	+						
152-298	R:CCATCACACAATCCATC												
Dpr3D06	F:ATAGGAAGGCAATCAGG	+	+	+	+	+	+	+					
130-300	R:ACCCATCGTCTTACCC												
Dpr3F04	F:AGACTCTTGCTCATGT	+											
160-170	R:GCCTTGTTACTTTATTC												
Dpr3 F10	F:TCAAAGGAATGTTGGG	+											
270-320	R:ACGCACATAGGGATTG												
Dpr3F12	F:TCCCCATAGAAACAAAGT	+	+	+	+	+	+						
190-320	R:TCAAGCAAGAGAAGGTG												
Da1D08	F:GATGCTATGAACACAACTAA	-											
180-250	R:TTTGACAGTGAGAATGGA												
Da1F08	F:AATGCTTCGTAATCCAAC	+	+	+	+	+	+	+					
175-190	R:CTATAAGGAATTGGTGCC												
Dab2C05	F:CCCATGCTTGTAGTTGT	+											
175-240	R:TGCTCACCTCTTTACTTG												
Dab2E09	F:AACATATAAAGAGAGATCA	+	+	+	+								
210	R:ATAACCCTTAACTCCA												
Dab2E07	F:TTGAACCTTGACTTTGGT	+	+	+	+	+	+	+	+				
110-200	R:GAGTTCCTGTCCTTGGT												
Da1C12	F:GCCTTTGTGCGTATCT	+	+	+	+	+	+	+	+				
120-200	R:AATCGGCTACACTCATCT												
(-) No amplification			**(+) amplification**										

Table 4.11 Gradients PCR for optimum annealing temperatures from 50^0C to 65^0C results for *D. alata* species incorporating eighteen primers developed by Tostain *et al.*, (2006).

Figure 4.23 Gradients PCR 35 cycles. Increment Ta, optimum of 50 ^{0}C to 60.3 ^{0}C for *D. alata* species deploying Ym13-275bp primers visualised on 1% agarose gel. Heterozygosity as double banding was observed.

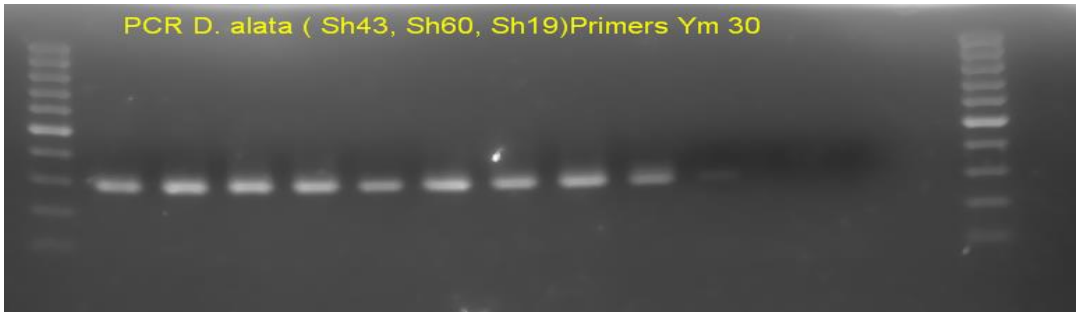


Figure 4.24 Gradient PCR increment Ta from 50 ^{0}C to 63.7 ^{0}C optima for *D. alata* species deploying Ym30- 290bp primers positive result pictured using electrophoresis 1% agarose gel.

Figure 4.25 Gradients PCR increment Ta with optimum at 50 ^{0}C- 52.7^{0}C for *D. alata* species incorporating Da1A01-250bp primers, the two first wells were punctured. The positive result screened on electrophoresis 1% agarose gel.

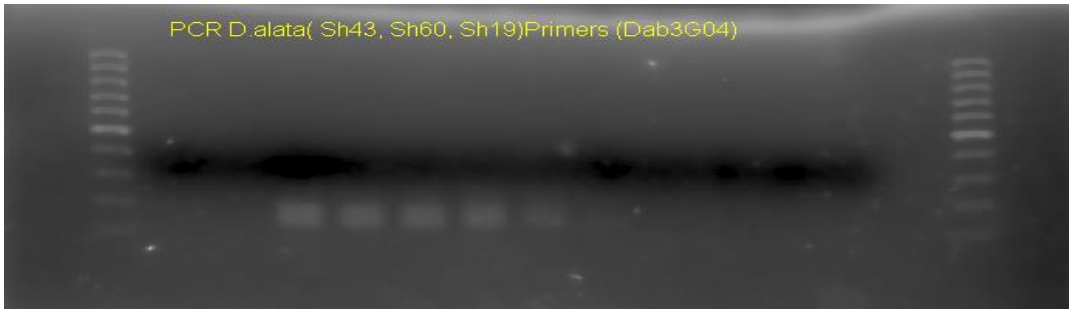


Figure 4.26 Gradient PCR increment Ta, 50-58.4^{0}C optima for *D. alata* species using Da3G04- 190bp primers' positive results were observed on 1% agarose gel. Two wells were damaged. More than one band was showing and the allele sizes were 160- 175bp.

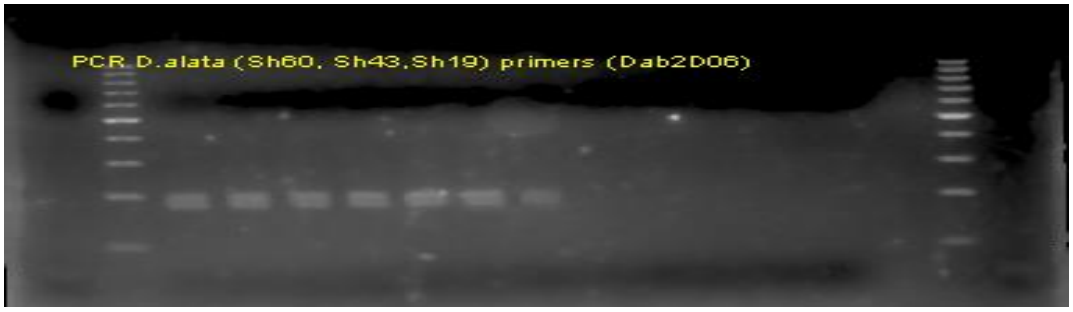


Figure 4.27 Gradient PCR, Ta 50-58.4^{0}C optima for *D. alata* species employed Dab2D06-200bp primers. Heterozygosity was observed from the double banding on 1% agarose gel.

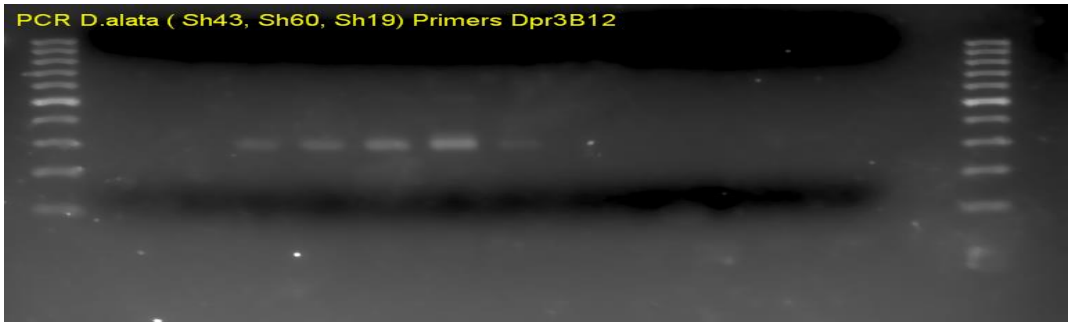


Figure 4.28 Gradient PCR, Ta 50-56.7^{0}C optima for *D. alata* species deployed Dpr3B12-290bp primers results were observed on 1% agarose gel. Three wells were damaged.

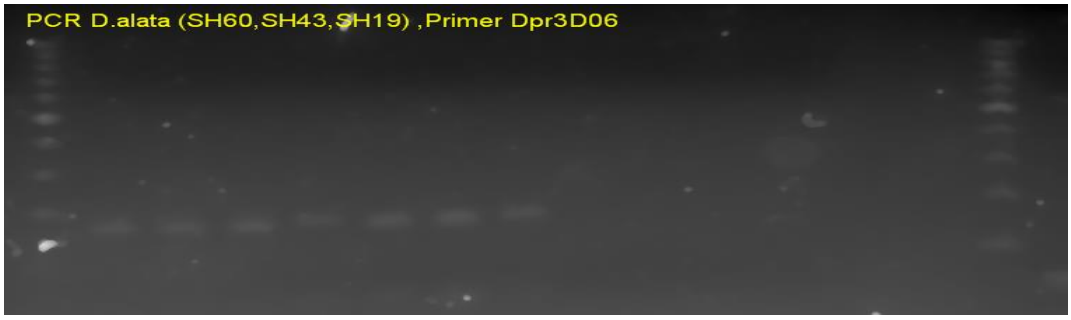


Figure 4.29 Gradient PCR, Ta at 50-58.4^{0}C optima for *D. alata* species, integrating Dpr3D06-150bp primers. The results visualised on 1% agarose gel.

Figure 4.30 Gradient PCR, Ta 50-56.4^{0}C optima for *D. alata* species employing Dpr3F12-220bp primers. The result observed on 1% agarose gel.

Figure 4.31 Gradient PCR, Ta 50-58.4^{0}C optima for *D. alata* species employing Da1F08-190bp primers.

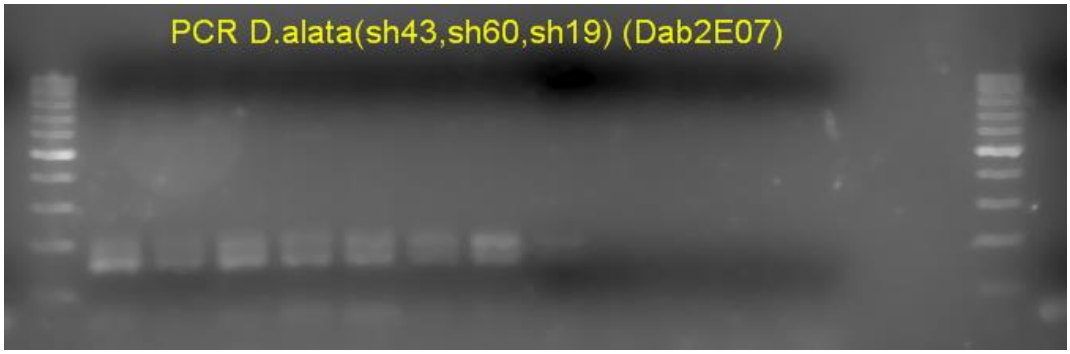


Figure 4.32 Gradient PCR, Ta 50-60.3^{0}C optima for *D. alata* species using Dab2E07, 165-190bp primers. The result showed heterozygosity on the observed 1% agarose gel.

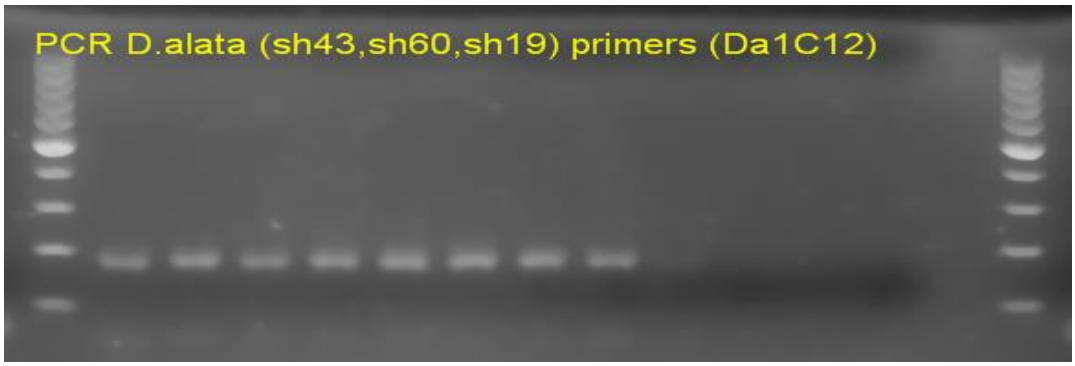


Figure 4.33 Gradient PCR, Ta 50-60.3^{0}C optima for *D. alata* species using Da1C12-190bp primers. The result observed on 1% agarose gel.

4.3.3.2 Dioscorea bulbifera

Primers Dpr3B12, Da1D08 & Dab2E09 failed to amplify *D. bulbifera* species as table 4.12 shows. Notably, Da3G04, Dab2C12, Dpr3F04, Dpr3F10, Dab2C05, Dab2E07, and Da1C12 primers have shared optima T_a of 50^{0}C. The Da1F08 primers recorded an optimum annealing at 50.4^{0}C, whereas Dpr3D06 marker registered 51.3^{0}C optima T_a. Moreover, Dpr3F12 reached an optimum T_a 54.7^{0}C. Markers Dab2D06 and Dab2D08 were optimised at 60.3^{0}C T_a. The two markers Da1A01 and Ym30 amplified *D. bulbifera* at optima T_a of 62.3^{0}C and 64.6^{0}C respectively.

Interestingly, replacing a pooled DNA template of *D. bulbifera* with a single specimen of (Sh75A) in PCR, the Ym30 markers optima T_a dropped from 65.0^{0}C to 60.3^{0}C.

Figures 4.34, 4.35, 4.36, 4.37, and 4.38 demonstrate the results of *D. alata* species.

Gradient PCR for *D. bulbifera* increment annealing temperature 50 - 65 Celsius													
Marker &	Primer sequence	Temperature											
allele size	5' to 3'-end	50 °C	50.4 °C	51.3 °C	52.7 °C	54.7 °C	56.6 °C	58.4 °C	60.3 °C	62.3 °C	63.7 °C	64.6 °C	65 °C
Ym 13	F:TTCCCTAATTGTTCCTCTTGTTG	+	+	+	+	+	+	+	+	+	+	+	+
200-350	R:GTCCTCGTTTCCCTGT												
Ym 30	F:GGTCCTCTTCTATCCCAACAA	+	+	+	+	+	+	+	+	+	+	+	
236-350	R:CACGTATTAACTCCATCTATCCAA												
Da1A01	F:TATAATCGGCCAGAGG	+	+	+	+	+	+	+	+	+			
200-300	R:TGTTGGAAGCATAGAGAA												
Da3G04	F:CACGGCTTGACCTATC	+											
175-350	R:TTATTCAGGGCTGGTG												
Dab2C12	F:AGGCATCTTGGGAAA	+											
120-195	R:CGAACGATCCAATAAAA												
Dab2D06	F:TGTAAGATGCCCACATT	+	+	+	+	+	+	+	+				
185-280	R:TCTCAGGCTTCAGGG												
Dab2D08	F:ACAAGAGAACCGACATAGT	+	+	+	+	+	+	+	+				
315-355	R:GATTTGCTTTGAGTCCTT												
Dpr3B12	F:CATCAATCTTTCTCTGCTT	-											
152-298	R:CCATCACACAATCCATC												
Dpr3D06	F:ATAGGAAGGCAATCAGG	+	+	+									
130-300	R:ACCCATCGTCTTACCC												
Dpr3F04	F:AGACTCTTGCTCATGT	+											
160-170	R:GCCTTGTTACTTTATTC												
Dpr3 F10	F:TCAAAGGAATGTTGGG	+											
270-320	R:ACGCACATAGGGATTG												
Dpr3F12	F:TCCCCATAGAAACAAAGT	+	+	+	+	+							
190-320	R:TCAAGCAAGAGAAGGTG												
Da1D08	F:GATGCTATGAACACAACTAA	-											
180-250	R:TTTGACAGTGAGAATGGA												
Da1F08	F:AATGCTTCGTAATCCAAC	+	+										
175-190	R:CTATAAGGAATTGGTGCC												
Dab2C05	F:CCCATGCTTGTAGTTGT	+											
175-240	R:TGCTCACCTCTTTACTTG												
Dab2E09	F:AACATATAAAGAGAGATCA	-											
210	R:ATAACCCTTAACTCCA												
Dab2E07	F:TTGAACCTTGACTTTGGT	+											
110-200	R:GAGTTCCTGTCCTTGGT												
Da1C12	F:GCCTTTGTGCGTATCT	+											
120-200	R:AATCGGCTACACTCATCT												
(-) No amplification		**(+) amplification**											

Table 4.12 Gradients PCR for optimum annealing temperatures from 50^0C to 65^0C marks for *D. bulbifera* species using eighteen primers developed by Tostain *et al.*, (2006).

Figure 4.34 Gradient PCR increment Ta, 50- 62.3^{0}C optima for *D. bulbifera species* using Da1A01-300bp primers. The affirmative results were viewed on 1% agarose gel.

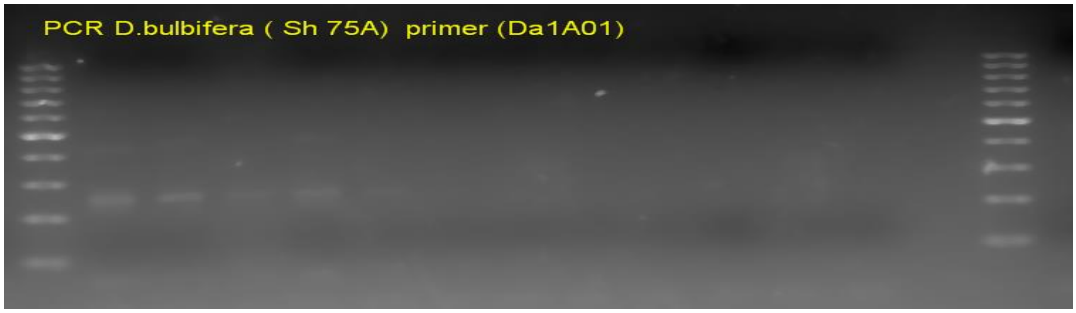


Figure 4.35 Gradient PCR increment Ta, 50- 52.7^{0}C optima for *D. bulbifera* species using Da1A01-250bp primers. The affirmative results were viewed on 1% agarose gel.

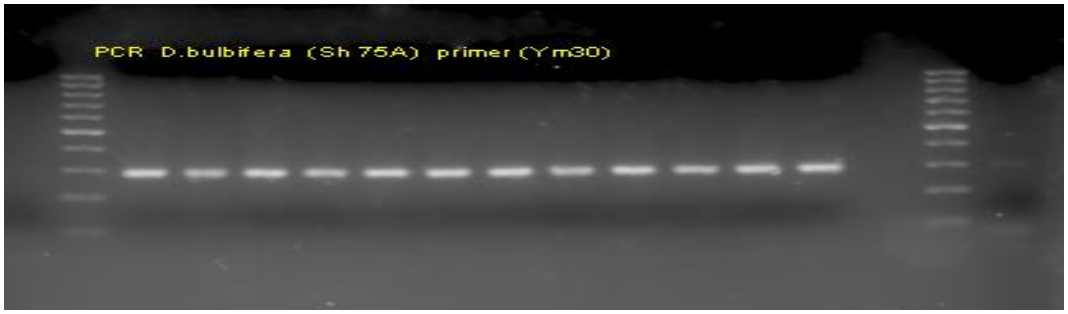


Figure 4.36 Gradient PCR increment Ta, 50-65^{0}C optima for *D. bulbifera* species utilising Ym30-300bp primers. The affirmative results was exhibited on 1% agarose gel. Using high intensity of $MgCl_2$ 2.5 ul where PCR reached the highest optima Ta and the amplicons are bright and clear.

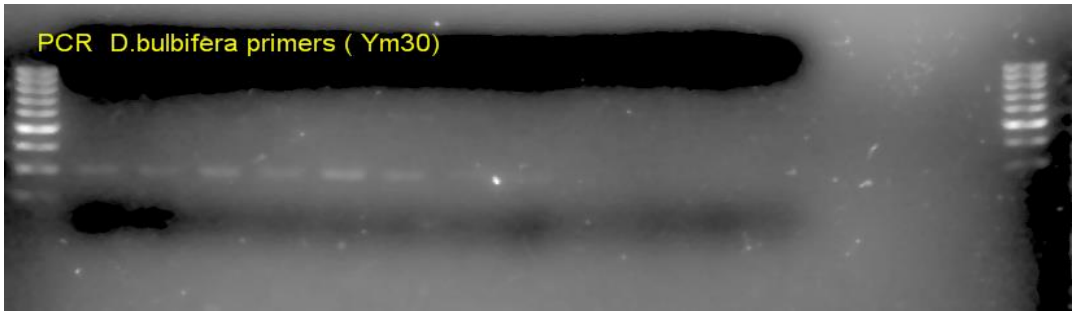


Figure 4.37 Gradient PCR increment Ta, 50-60.3^{0}C optima for *D. bulbifera* species utilising Ym30-300bp primers. The affirmative results was exhibited on 1% agarose gel. Using intensity of $MgCl_2$ 0.6 ul where PCR reached optima Ta at 60.3^{0}C and the amplicons are less intense.

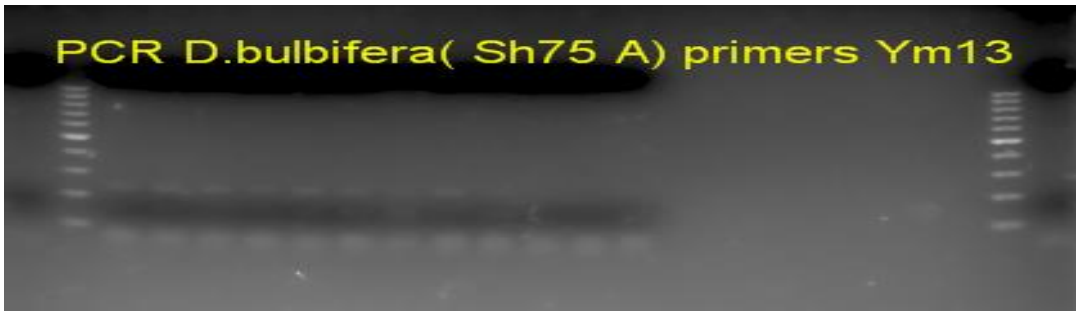


Figure 4.38 Gradient PCR increment Ta, 50-65^{0}C optima for *D. bulbifera* species employing Ym13-250bp primers. The positive results were observed on 1% agarose gel.

4.3.3.3 Dioscorea cayenensis

Dioscorea cayenensis was the most successful in amplification by the eighteen primers as is illustrated by table 4.13 and figures 4.39, 4.40, 4.41, 4.42, 4.43, 4.44, 4.45, and 4.46.

The optima T_a was significant where 65^{0}C was recorded by Ym13, Ym30, Da3G04, Dpr3B12, Dpr3F10, and Dab2E09 primers. Notably, Dab2C12, Dab2D06, Dpr3F04, and Da1D08 markers shared the optima T_a of 50^{0}C. Furthermore, Da1C12 primers recorded an optima T_a of 50.4^{0}C, while Dab2D08, Da1F08 & Dab2C05 primers registered an optimum T_a of 54.7^{0}C. The 58.4^{0}C optima T_a was registered by Dpr3D06 & Dab2E07 primers. Notably, Da1A01 was the only marker to have an optima T_a of 60.3^{0}C.

Gradient PCR for *D. cayenensis* increment annealing temperature 50-65 Celsius													
Marker	Primer sequence	Temperature											
allele size	5' to 3'-end	50 °C	50.4 °C	51.3° C	52.7 °C	54.7 °C	56.6 °C	58.4 °C	60.3 °C	62.3 °C	63.7 °C	64.6 °C	65° C
Ym 13	F:TTCCCTAATTGTTCCTCTTGTTG	+	+	+	+	+	+	+	+	+	+	+	+
200-350	R:GTCCTCGTTTCCCTGT												
Ym 30	F:GGTCCTCTTCTATCCCAACAA	+	+	+	+	+	+	+	+	+	+	+	+
236-350	R:CACGTATTAACTCCATCTATCCAA												
Da1A01	F:TATAATCGGCCAGAGG	+	+	+	+	+	+	+	+				
200-300	R:TGTTGGAAGCATAGAGAA												
Da3G04	F:CACGGCTTGACCTATC	+	+	+	+	+	+	+	+	+	+	+	+
175-350	R:TTATTCAGGGCTGGTG												
Dab2C12	F:AGGCATCTTGGGAAA	+											
120-195	R:CGAACGATCCAATAAAA												
Dab2D06	F:TGTAAGATGCCCACATT	+											
185-280	R:TCTCAGGCTTCAGGG												
Dab2D08	F:ACAAGAGAACCGACATAGT	+	+	+	+	+							
315-355	R:GATTTGCTTTGAGTCCTT												
Dpr3B12	F:CATCAATCTTTCTCTGCTT	+	+	+	+	+	+	+	+	+	+	+	+
152-298	R:CCATCACACAATCCATC												
Dpr3D06	F:ATAGGAAGGCAATCAGG	+	+	+	+	+	+	+					
130-300	R:ACCCATCGTCTTACCC												
Dpr3F04	F:AGACTCTTGCTCATGT	+											
160-170	R:GCCTTGTTACTTTATTC												
Dpr3 F10	F:TCAAAGGAATGTTGGG	+	+	+	+	+	+	+	+	+	+	+	+
270-320	R:ACGCACATAGGGATTG												
Dpr3F12	F:TCCCCATAGAAACAAAGT	+											
190-320	R:TCAAGCAAGAGAAGGTG												
Da1D08	F:GATGCTATGAACACAACTAA	+											
180-250	R:TTTGACAGTGAGAATGGA												
Da1F08	F:AATGCTTCGTAATCCAAC	+	+	+	+	+							
175-190	R:CTATAAGGAATTGGTGCC												
Dab2C05	F:CCCATGCTTGTAGTTGT	+	+	+	+	+							
175-240	R:TGCTCACCTCTTTACTTG												
Dab2E09	F:AACATATAAAGAGAGATCA	+	+	+	+	+	+	+	+	+	+	+	+
210	R:ATAACCCTTAACTCCA												
Dab2E07	F:TTGAACCTTGACTTTGGT	+	+	+	+	+	+	+					
110-200	R:GAGTTCCTGTCCTTGGT												
Da1C12	F:GCCTTTGTGCGTATCT	+	+										
120-200	R:AATCGGCTACACTCATCT												

Table 4.13 Gradients PCR for optimum annealing temperatures from 50^0C to 65^0C results for *D. cayenensis* species utilizing 18 primers created by Tostain *et al.*, (2006).

Figure 4.39 Gradient PCR of optimum Ta 50- 65^{0}C for *D. cayenensis* species deployed Ym30-290bp primers. The affirmative results were observed on 1% agarose gel.

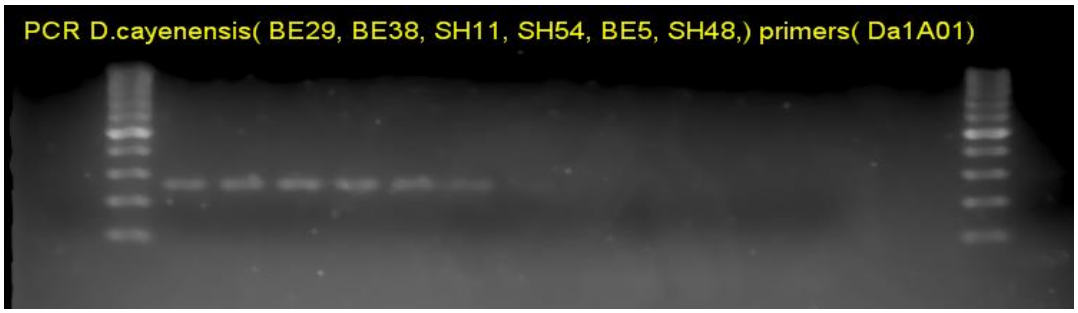


Figure 4.40 exhibited Gradient PCR, optimum Ta, 50- 60.3^{0}C for *D. cayenensis* species using Da1A01-280bp primers. The positive results was observed on 1% agarose gel.

Figure 4.41 showed Gradient PCR, optimum Ta, 50- 58.4^{0}C for *D. cayenensis* species using Dab2D06-275bp primers. The positive result was observed on 1% agarose gel.

Figure 4.42 showed Gradient PCR, optimum Ta, 50- 54.7^{0}C for *D. cayenensis* species using Dab2D08-345bp primers. The positive result was observed on 1% agarose gel.

Figure 4.43 exhibited Gradient PCR, optimum Ta, 50- 58.4^{0}C for *D. cayenensis* species using Dpr3D06-160bp primers.

Figure 4.44 showed Gradient PCR, optimum Ta 50- 54.7^{0}C for *D. cayenensis* species using Dab2C05-240bp primers. The positive result was observed on 1% agarose gel.

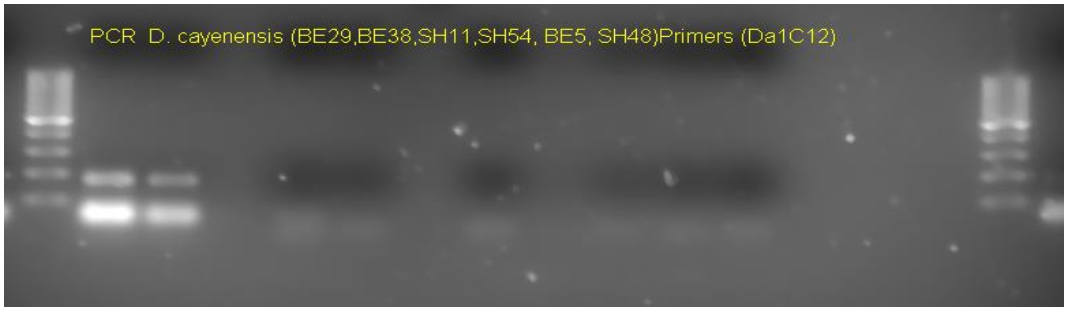


Figure 4.45 exhibited Gradient PCR, optimum Ta 50- 50.4^{0}C for *D. cayenensis* species using Da1C12-190bp primers. The positive result was observed on 1% agarose gel.

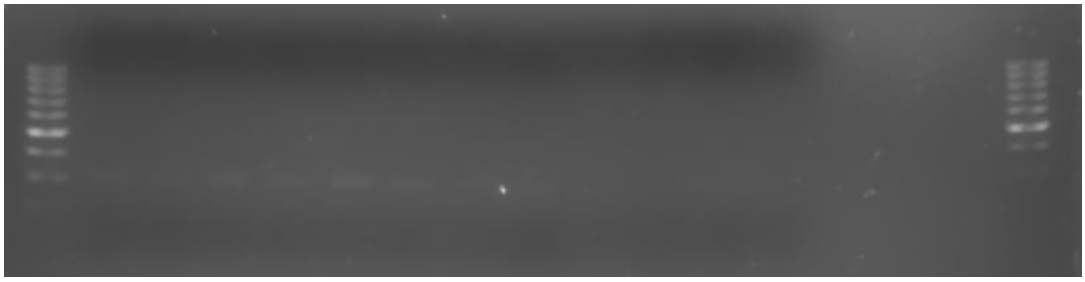


Figure 4.46 showed Gradient PCR, optimum Ta 50- 54.7^{0}C for *D. cayenensis* species using Da1F08-290bp primers. The positive results were observed on 1% agarose gel.

4.3.4 Optimum Annealing Temperature

Table 4.14 displays the optimal annealing temperature where the optimum T_a increased the specificity of the PCR products for primers engaged. A notable variation of optima T_a was observed between *D. alata, D. bulbifera,* and *D. cayenensis* species. Markers Dpr3B12, Da1D08, and Dab2E09 miscarried to amplify *D. bulbifera* while Da1D08 primer was unsuccessful in duplicating *D. alata* specimens. Notably, Dab2C12 and Dpr3F04 markers were the only two with an identical optima T_a for all of the three species.

PCR Optimum Annealing Temperature for *D. alata, D. bulbifera, & D. cayen*					
lumber	ker & Allele s	5' to 3'-end	*D. alata*	*D. bulbifera*	*D. cayenensis*
1	**Ym 13**	F:TTCCCTAATTGTTCCTCTTGTTG	60.3	65	65
	200-350bp	R:GTCCTCGTTTCCCTGT			
2	**Ym 30**	F:GGTCCTCTTCTATCCCAACAA	63.7	64.6	65
	236-350bp	R:CACGTATTAACTCCATCTATCCAA			
3	**Da1A01**	F:TATAATCGGCCAGAGG	52.7	62.3	60.3
	200-300bp	R:TGTTGGAAGCATAGAGAA			
4	**Da3G04**	F:CACGGCTTGACCTATC	58.4	50	65
	175-350bp	R:TTATTCAGGGCTGGTG			
5	**Dab2C12**	F:AGGCATCTTGGGAAA	50	50	50
	120-195bp	R:CGAACGATCCAATAAAA			
6	**Dab2D06**	F:TGTAAGATGCCCACATT	58.4	60.3	50
	185-280bp	R:TCTCAGGCTTCAGGG			
7	**Dab2D08**	F:ACAAGAGAACCGACATAGT	50	60.3	54.7
	315-355bp	R:GATTTGCTTTGAGTCCTT			
8	**Dpr3B12**	F:CATCAATCTTTCTCTGCTT	56.7	-	65
	152-298bp	R:CCATCACACAATCCATC			
9	**Dpr3D06**	F:ATAGGAAGGCAATCAGG	58.4	51.3	58.4
	130-300bp	R:ACCCATCGTCTTACCC			
10	**Dpr3F04**	F:AGACTCTTGCTCATGT	50	50	50
	160-170bp	R:GCCTTGTTACTTTATTC			
11	**Dpr3 F10**	F:TCAAAGGAATGTTGGG	50	50	65
	270-320bp	R:ACGCACATAGGGATTG			
12	**Dpr3F12**	F:TCCCCATAGAAACAAAGT	56.7	54.7	50
	190-320bp	R:TCAAGCAAGAGAAGGTG			
13	**Da1D08**	F:GATGCTATGAACACAACTAA	-	-	50
	180-250bp	R:TTTGACAGTGAGAATGGA			
14	**Da1F08**	F:AATGCTTCGTAATCCAAC	58.4	50.4	54.7
	175-190bp	R:CTATAAGGAATTGGTGCC			
15	**Dab2C05**	F:CCCATGCTTGTAGTTGT	50	50	54.7
	175-240bp	R:TGCTCACCTCTTTACTTG			
16	**Dab2E09**	F:AACATATAAAGAGAGATCA	52.7	-	65
	210bp	R:ATAACCCTTAACTCCA			
17	**Dab2E07**	F:TTGAACCTTGACTTTGGT	60.3	50	58.4
	110-200bp	R:GAGTTCCTGTCCTTGGT			
18	**Da1C12**	F:GCCTTTGTGCGTATCT	60.3	50	50.4
	120-200bp	R:AATCGGCTACACTCATCT			
(-) No amplification					

Table 4.14 Gradients PCR for optimum annealing temperatures from 50^0C to 65^0C results for *D. alata, D. bulbifera,* and *D. cayenensis* species utilizing the 18 primers produced by Tostain *et al.*, (2006).

4.3.5 Markers transferability across *Dioscorea sp*.

The PCR results in table 4.15 and figures 4.47, 4.48, 4.49, 4.50, 4.51, 4.52, 4.53, and 4.54 depict the eighteen primers transferability across *D. alata, D. bulbifera,* and *D. cayenensis* species with percentage of transferring. Of note, markers Dpr3B12, Da1D08, and Dab2E09 PCR results indicate they miscarried in transferring across the three species.

PCR results at 50 to 65 Celsius, *and primers transferability across Dioscorea species*					
locus	5' to 3'-end	D. alata	D. bulbifera	D. cayenensis	transferability across species
Ym 13	F:CACGACGTTGTAAAAC	+	+	+	**100%**
200-350	R: GTCCTCGTTTCCCTGT				
Ym 30	F:CACGACGTTGTAAAAC	+	+	+	**100%**
236-350	R: CACGTATTAACTCCAT				
Da1A01	F: TATAATCGGCCAGAGG	+	+	+	**100%**
200-300	R: TGTTGGAAGCATAGAGAA				
Da3G04	F: CACGGCTTGACCTATC	+	+	+	**100%**
175-350	R: TTATTCAGGGCTGGTG				
Dab2C12	F: AGGCATCTTGGGAAA	+	+	+	**100%**
120-195	R: CGAACGATCCAATAAAA				
Dab2D06	F: TGTAAGATGCCCACATT	+	+	+	**100%**
185-280	R: TCTCAGGCTTCAGGG				
Dab2D08	F: ACAAGAGAACCGACATAGT	+	+	+	**100%**
315-355	R: GATTTGCTTTGAGTCCTT				
Dpr3B12	F: CATCAATCTTTCTCTGCTT	+	-	+	**66%**
152-298	R: CCATCACACAATCCATC				
Dpr3D06	F: ATAGGAAGGCAATCAGG	+	+	+	**100%**
130-300	R: ACCCATCGTCTTACCC				
Dpr3F04	F: AGACTCTTGCTCATGT	+	+	+	**100%**
160-170	R: GCCTTGTTACTTTATTC				
Dpr3 F10	F: TCAAAGGAATGTTGGG	+	+	+	**100%**
270-320	R: ACGCACATAGGGATTG				
Dpr3F12	F: TCCCCATAGAAACAAAGT	+	+	+	**100%**
190-320	R: TCAAGCAAGAGAAGGTG				
Da1D08	F: GATGCTATGAACACAACTAA	-	-	+	**33%**
180-250	R: TTTGACAGTGAGAATGGA				
Da1F08	F: AATGCTTCGTAATCCAAC	+	+	+	**100%**
175-190	R: CTATAAGGAATTGGTGCC				
Dab2C05	F: CCCATGCTTGTAGTTGT	+	+	+	**100%**
175-240	R: TGCTCACCTCTTTACTTG				
Dab2E09	F: AACATATAAAGAGAGATCA	+	-	+	**66%**
210	R: ATAACCCTTAACTCCA				
Dab2E07	F: TTGAACCTTGACTTTGGT	+	+	+	**100%**
110-200	R: GAGTTCCTGTCCTTGGT				
Da1C12	F: GCCTTTGTGCGTATCT	+	+	+	**100%**
120-200	R: AATCGGCTACACTCATCT				
(+) Amplification (-) No amplifica		**(%) Transferrability across *Dioscorea* *sp* .**			

Table 4.15 Gradients PCR amplification results for 50^0C to 65^0C, and the 18 primers transferability across *D. alata, D. bulbifera & D. cayenensis* species. (+) successful amplification and (-) unsuccessful in transferring.

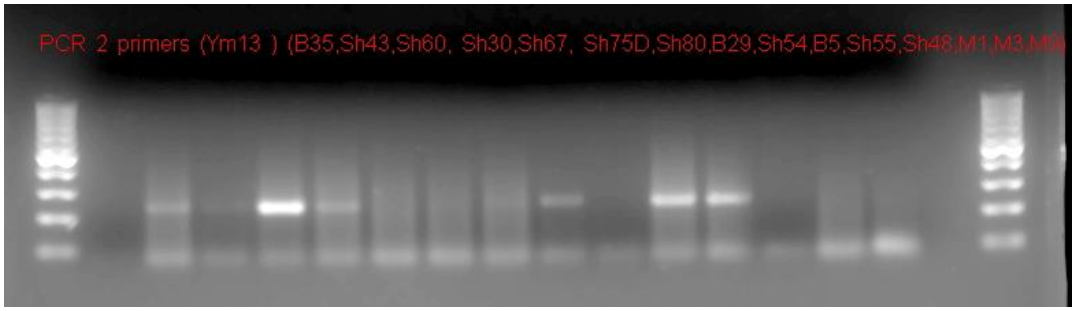


Figure 4.47 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Ym13-250bp primers. The positive results were observed on 1% agarose gel.

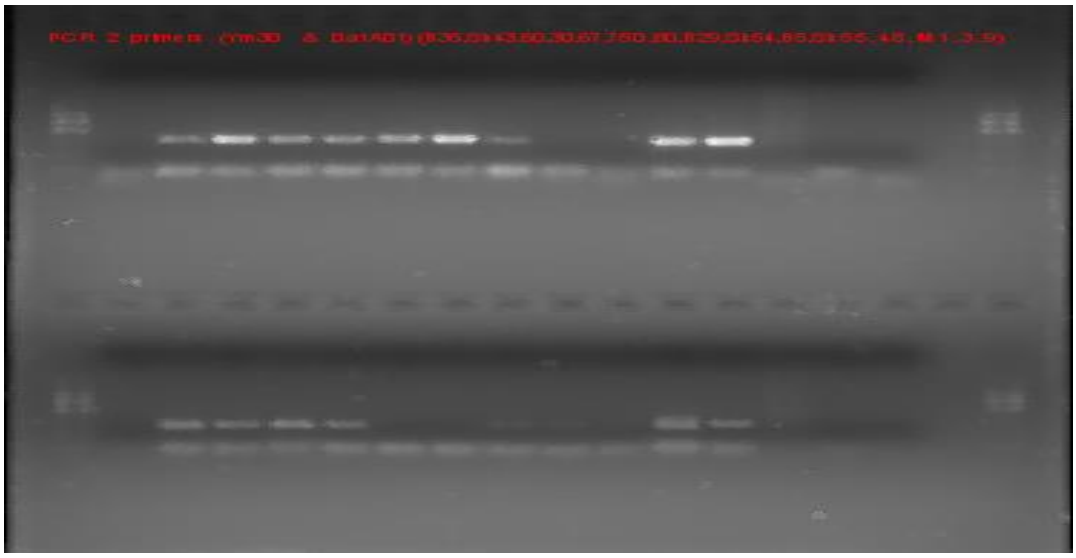


Figure 4.48 PCR, static Ta 50^{0}C for *D. alata*, *D. bulbifera,* and *D. cayenensis* species incorporating Ym30-300bp primers on the top gel, and Da1A01-260bp on the bottom gel. The positive results were observed on 1% agarose gel.

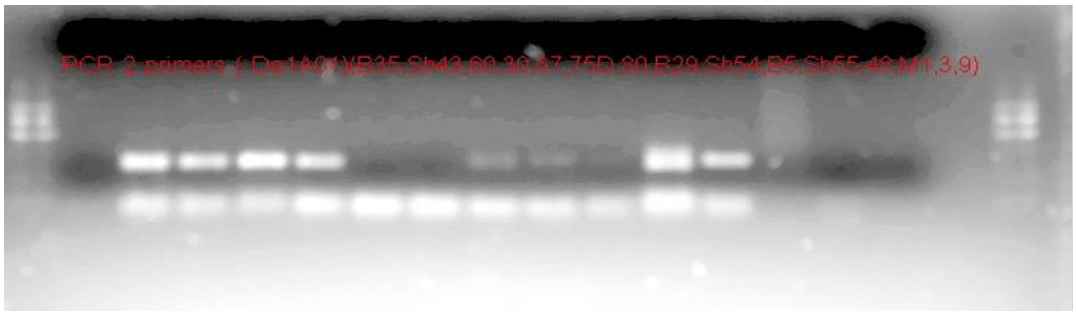


Figure 4.49 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da1A01-270bp primers. The positive results were observed on 1% agarose gel.

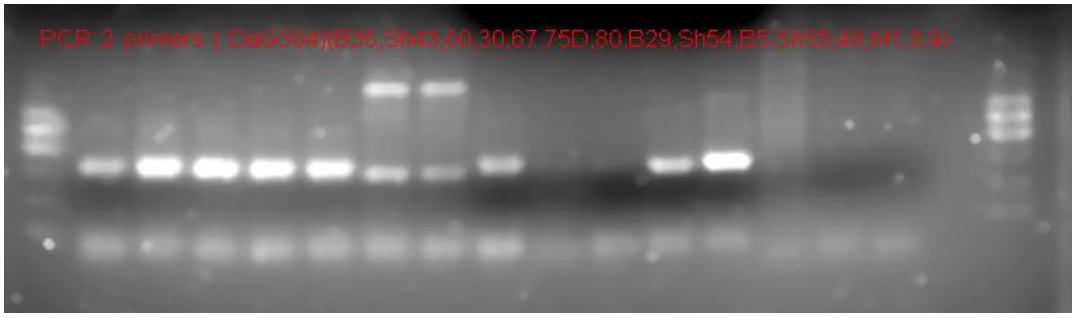


Figure 4.50 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da3G04-350bp primers. The positive results were observed on 1% agarose gel.

Figure 4.51 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Dab2D08-350bp primers. The positive results were observed on 1% agarose gel.

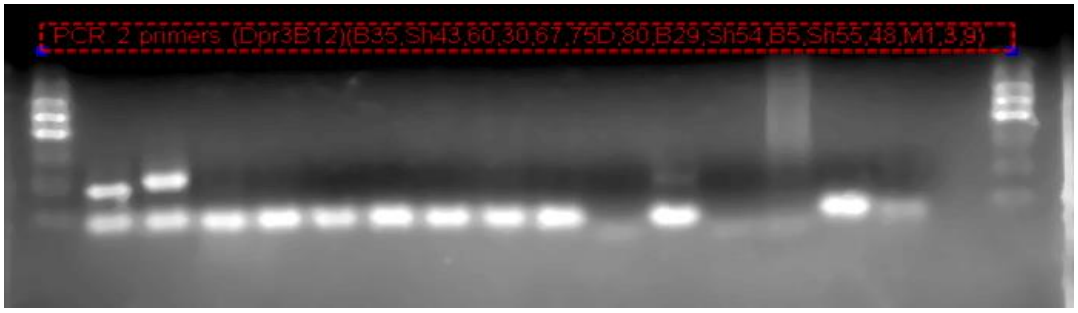


Figure 4.52 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species using Dpr3B12-200bp primers. The positive results were observed on 1% agarose gel.

Figure 4.53 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Dab2D08-320bp primers. The positive results were observed on 1% agarose gel.

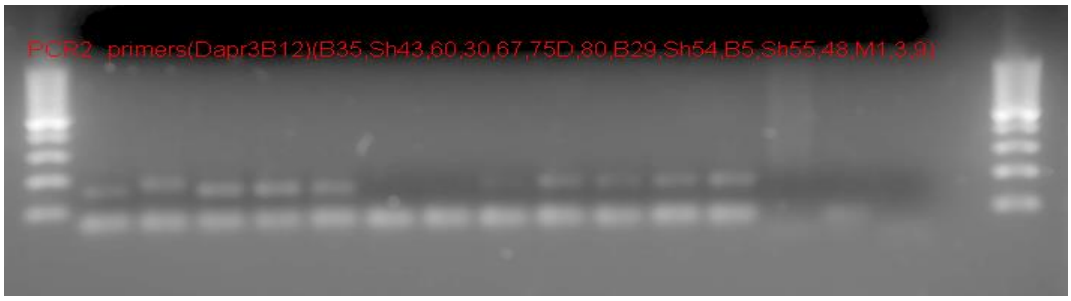


Figure 4.54 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Dpr3B12- 200bp primers. The positive results were observed on 1% agarose gel.

4.3.6 Forward and M13 Tailed Primers

Primers Ym30, Da1A01, Dab2D08, Da3G04, and Dpr3B12 incorporated with M13 tailed were tested prior to the incorporation of M13 FAM to assess their efficacy in amplifying *D. alata, D. bulbifera,* and *D. cayenensis* targeted DNA regions. The M13 tailed primer was integrated into the forward primer's 5'-end. The results were adequate for fragment analysis as figures 4.55, 4.56, 4.57, 4.58, 4.59, 4.60, 4.61, 4.62, 4.63, 4.64, and 4.65 illustrate.

Figure 4.55 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Ym13-250 bp with M13 FAM tailed primers. Because the result observed on gel of 2 % agarose was not clear, therefore the primers were excluded from fragment analysis.

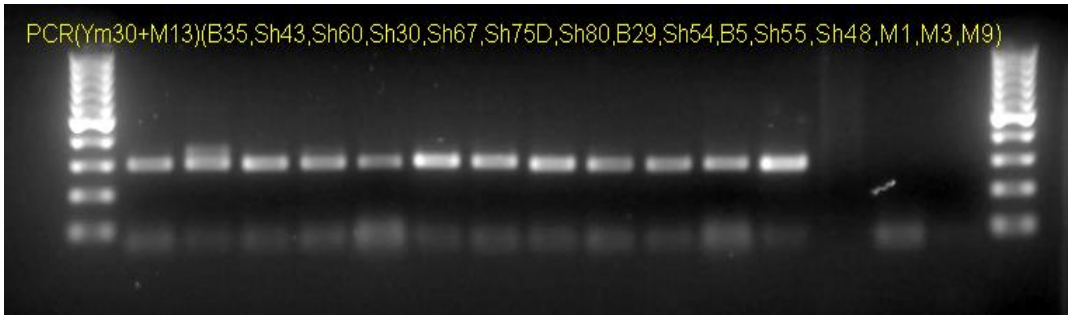


Figure 4.56 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Ym30, 290-310bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. More than 1.0 ul of $MgCl_2$ was incorporated in PCR**.**

Figure 4.57 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Ym30-290 bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. 0.6 ul of $MgCl_2$ was incorporated in PCR.

Figure 4.58 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da1A01-250 bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. More than 2.5 ul of $MgCl_2$ was incorporated in PCR.

Figure 4.59 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da1A01-250bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. 2.5 ul of $MgCl_2$was incorporated in PCR.

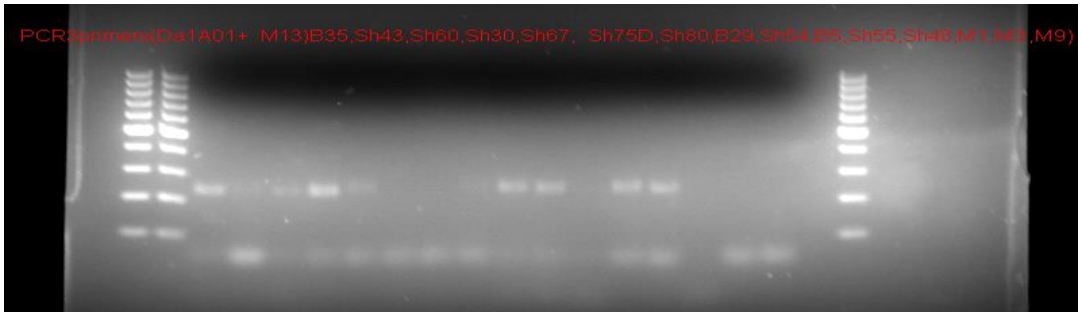


Figure 4.60 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da1A01-250bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. 0.6 ul of $MgCl_2$ was incorporated in PCR.

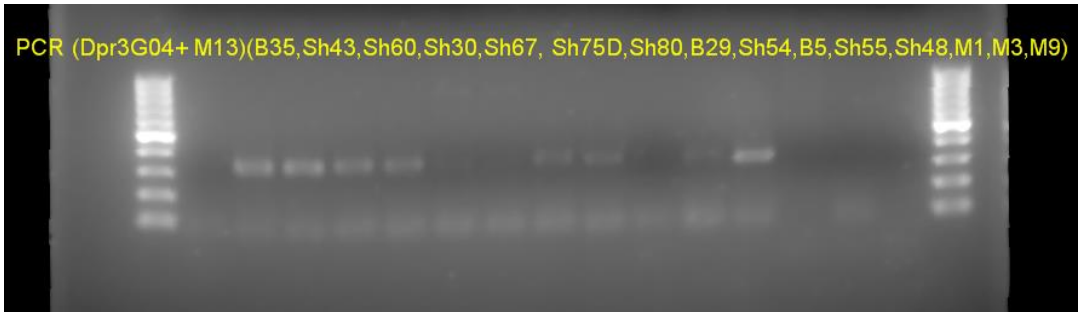


Figure 4.61 PCR, static Ta 50^{0}C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da3G04- 320 bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. More than 2.5 ul of $MgCl_2$ was incorporated in PCR.

Figure 4.62 PCR, static Ta 50^0C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da3G04-300 bp with M13 FAM tailed primers. The results was observed on gel of 2 % agarose. 0.6 ul of $MgCl_2$ was incorporated in PCR.

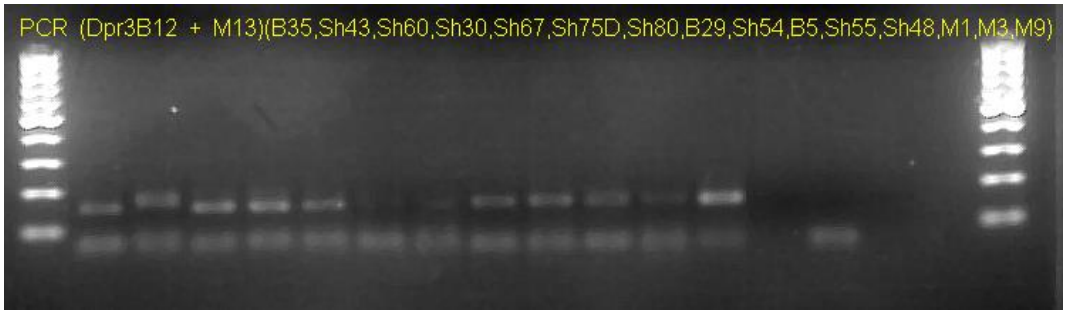


Figure 4.63 PCR, static Ta 50^0C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Dpr3B12-180bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. 2.5 ul of $MgCl_2$ was incorporated in PCR**.**

Figure 4.64 PCR static Ta 50^0C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporated Dpr3B12-175bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose. 0.6 ul of $MgCl_2$ was incorporated in PCR.

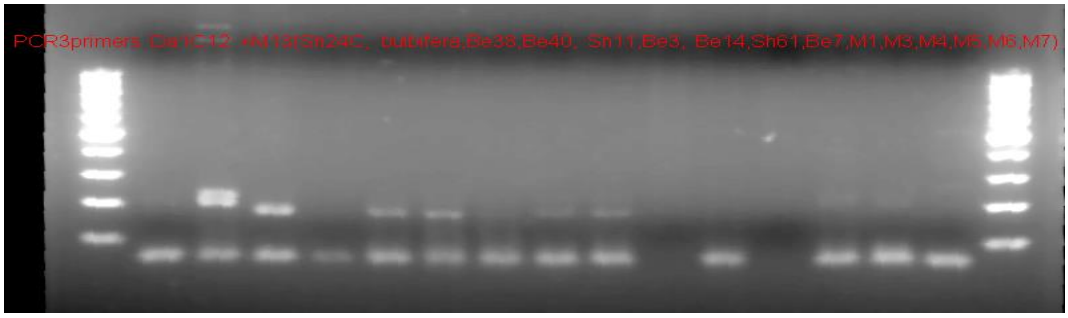


Figure 4.65 PCR, static Ta 50^0C for *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating Da1C12-190bp with M13 FAM tailed primers. The positive results were observed on gel of 2 % agarose.

4.4 Mg^{2+} QUANTITY AND M13 IMPACT ON PCR

Magnesium chloride concentrations altered the PCR yield, in particular when M13 tailed primers were incorporated into the forward primers' 5'-end. Increasing $MgCl_2$ quantity in the 20µl PCR solution from 0.07 to 0.6, and then into 1.0µl and 2.5 µl significantly influenced the product yield. Figure 4.66 of *D. bulbifera* employing Ym30 primes resulted in elevating the optimum T_a from 60.3^0C into 65^0C when the $MgCl_2$ 1.0µl quantity was interchanged with 0.6µl. Moreover, higher number and better resolution of bands were achieved when M13 and $MgCl_2$ 1.0 µl incorporated with Dpr3B12, Da1A01, and Da3G04 primers as figures 4.67, 4.68, 4.69, 4.70, 4.71, and 4.72 showed. The optima T_a of Dab2D06 a primer amplifying *D. alata* was reduced from 58.4^0C to 56.7^0C and in the meantime the allele's size were extended from 200bp into 250bp as figure 4.73 demonstrates. Similarly, incorporating a higher intensity of $MgCl_2$ within Da1A01 primers replicating *D. bulbifera* samples increased the optima T_a from 52.7^0C into 62.3^0C, and the bands sizes expanded from 250bp into 300bp as figure 4.74 depicts.

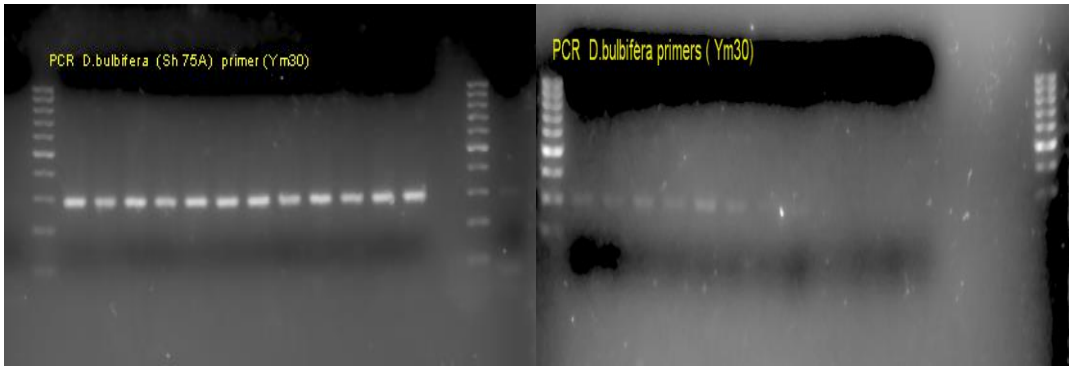


Figure 4.66

Gradient PCR, *D. bulbifera* incorporated with Ym30-290bp primers. 1.0 **µl** $MgCl_2$ incorporated in PCR, optima Ta 65^0C. The bands were more visible.

Gradient PCR, *D. bulbifera* species incorporated with Ym30-290bp primers. 0.6 **µl** $MgCl_2$ was used in the PCR optima Ta 60.3^0C. The bands were less visible.

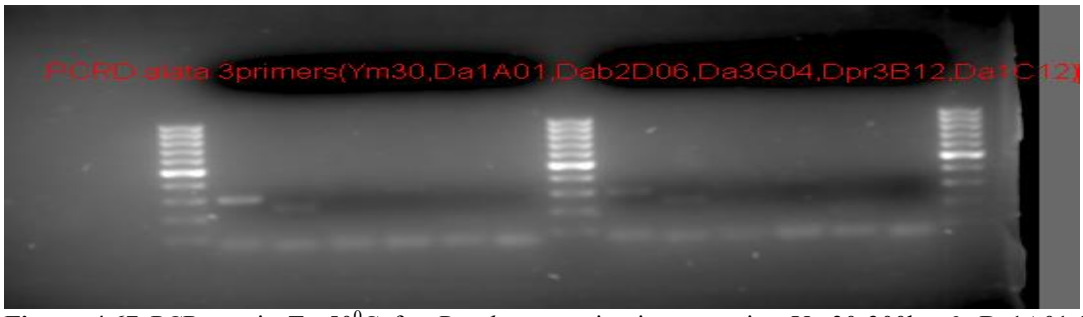


Figure 4.67 PCR, static Ta 50^0C for *D. alata*, species incorporating Ym30-300bp & Da1A01-260bp incorporated with M13 FAM markers. The positive results were observed on 2 % agarose gel. The differences of 1.0µl and 0.6ul MgCl2 quantity were incorporated in the PCR, more sharpness bands with high $MgCl_2$ intensity**.**

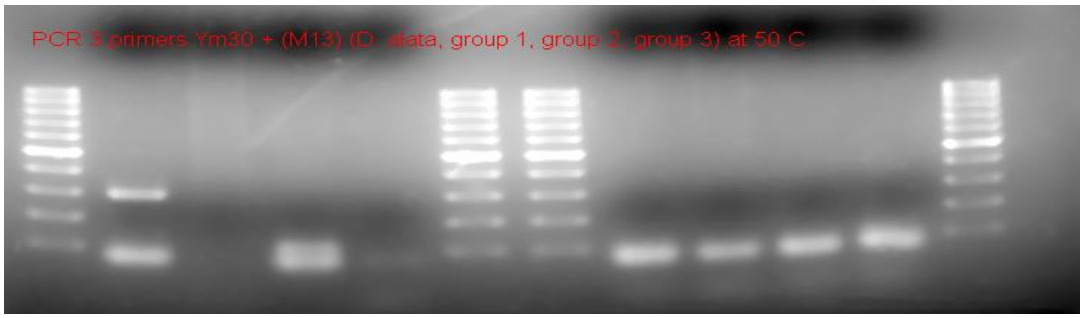


Figure 4.68 PCR, static Ta 50^0C for *D. alata*, species incorporated Ym30-290bp with M13 FAM primers. The positive results were observed on 2 % agarose gel. 1.0**µl** $MgCl_2$ had amplification comparing with 0.6ul no amplification in the PCR.

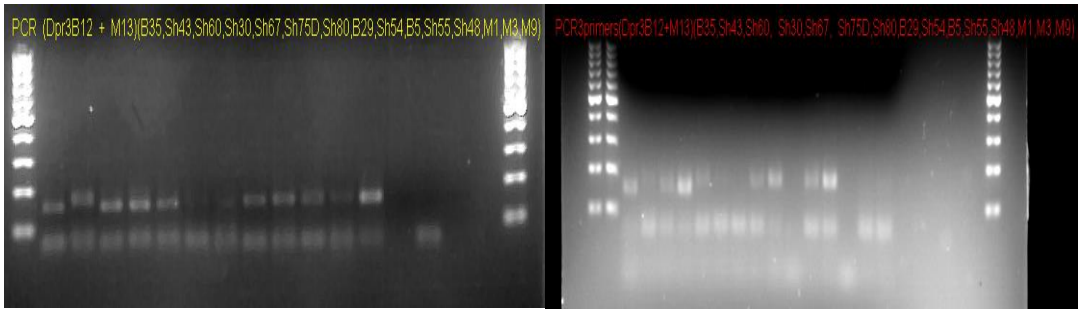


Figure 4.69

PCR Ta 50^{0}C for *D. alata*, *D. bulbifera*, and *D. cayenensis* species incorporated with Dpr3B12 and M13 FAM markers. 1.0**µl** $MgCl_2$ resulted in better and more bands amplified in PCR.

PCR Ta 50^{0}C for *D. alata*, *D. bulbifera*, and *D. cayenensis* species incorporated with Dpr3B12 and M13FAM markers. 0.6**µl** $MgCl_2$ resulted in less bands amplified in PCR.

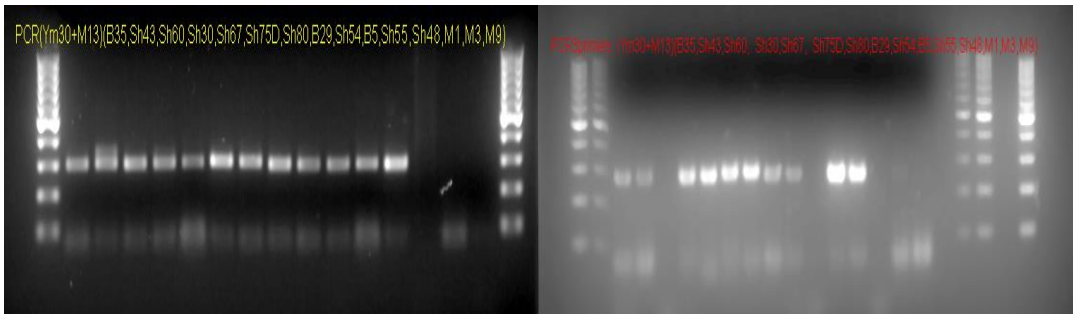


Figure 4.70

PCR, Ta 50^{0}C for *D. alata*, *D. bulbifera* & *D. cayenensis* species incorporating Ym30 with M13FAM primers was observed on 2 % agarose gel. 1.0**µl** $MgCl_2$ was incorporated in PCR resulted in brighter and more bands.

PCR, Ta 50^{0}C for *D. alata*, *D. bulbifera* & *D. cayenensis* species incorporating Ym30 with M13FAM primers was observed on 2 % agarose gel. 0.6**µl** $MgCl_2$ was incorporated in PCR generated less number of bands.

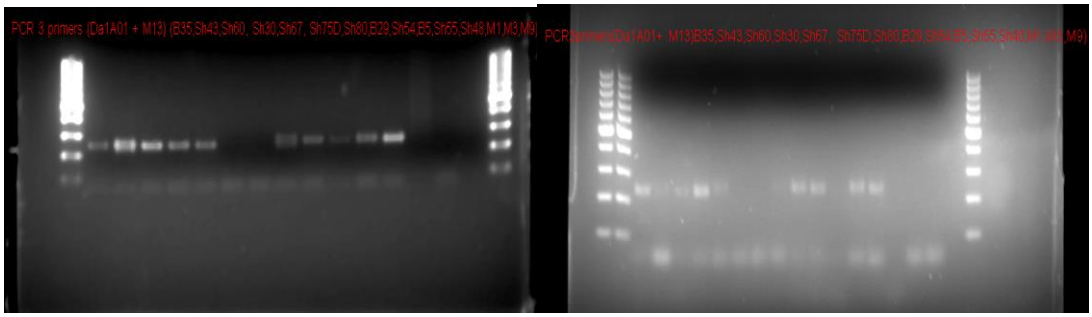


Figure 4.71

PCR Ta 50^{0}C for *D. alata*, *D. bulbifera* & *D. cayenensis* species incorporating Da1A01 with M13 FAM primers were observed on 2 % agarose gel. 1.0**µl** $MgCl_2$ incorporated in PCR brighter bands.

PCR Ta 50^{0}C for *D. alata*, *D. bulbifera*, and *D. cayenensis* species incorporating Da1A01 with M13FAM primers were observed on 2 % agarose gel. 0.6**µl** $MgCl_2$ incorporated in PCR less brightness.

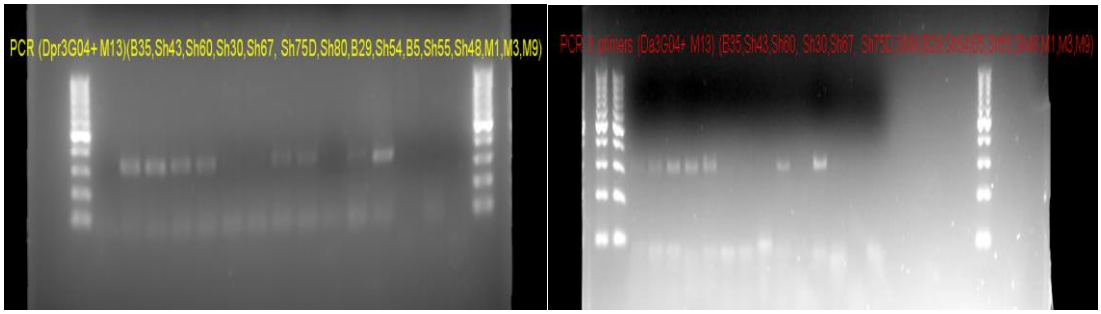


Figure 4.72

PCR Ta 50^{0}C for *D. alata*, *D. bulbifera*, and *D. cayenensis* species incorporating Dpr3G04 with M13 FAM primers were observed on 2 % agarose gel. 1.**0µl** $MgCl_2$ incorporated in PCR better amplifications.

PCR Ta 50^{0}C for *D. alata, D. bulbifera*, and *D. cayenensis* species incorporating Dpr3G04 with M13 FAM primers were observed on 2 % agarose gel. 0.6**µl** $MgCl_2$ incorporated in PCR less amplification**.**

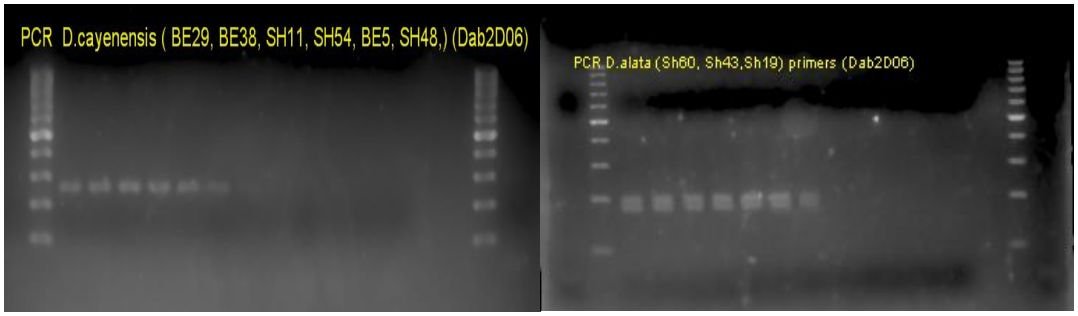


Figure 4.73

Gradient PCR *D. cayenensis* species incorporated with Dab2D06 primers. 1.0**µl** $MgCl_2$ quantity incorporated in PCR optima Ta 56.7^{0}C. The allele's size 250-275bp change was observed on 1 %

Gradient PCR for *D. alata* species incorporated with Dab2D06 primers. 0.6 **µl** $MgCl_2$ quantity incorporated in PCR optima Ta 58.4^{0}C. The allele's size 180-200bp and heterozygosity were observed on 1 % agarose gel.

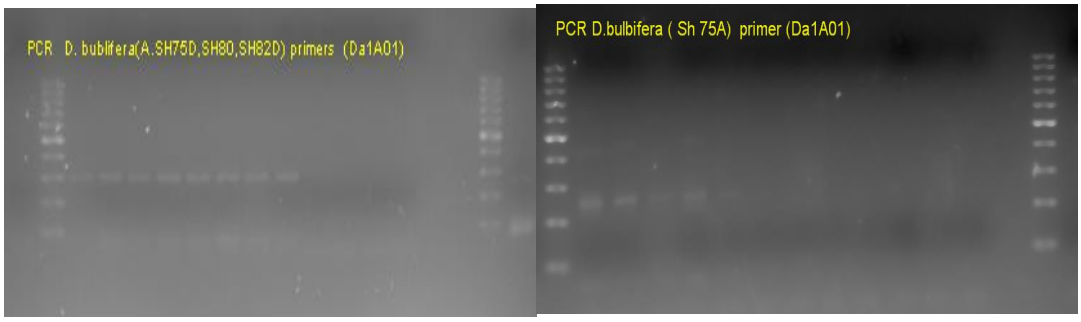


Figure 4.74

Gradient PCR increment Ta, 50- 62.3^{0}C optima for *D. bulbifera* species using Da1A01 primers. The affirmative results was viewed on gel of 1% agarose

Gradient PCR increment Ta, 50- 52.7^{0}C optima for *D. bulbifera* species using Da1A01 primers. The affirmative results was viewed on gel of 1% agarose

4.5 FRAGMENT ANALYSIS

Markers Ym30, Ga1A01, Da3G04, and Dapr3B12 were incorporated with M13 FAM in PCR to amplify *D. alata, D. bulbifera,* and *D. cayenensis* specimens. The PCR products were pooled when analysed by GeneMapper as illustrated by table 4.16

Fragment Analysis for Dioscorea sp. , June, 2014														
Primer + M13 Fam		*D. alata*					*D. bulbifera*		*D. cayenensis*					
	5' to 3' prime	Be35	Sh43	Sh60	Sh30	Sh 67	Sh75	Sh80	Be29	Sh54	Be5	Sh55	Sh48	fragmen
Ym30	F:CACGACGTTGTAAAACGACCACGACGTTGTAAAAC	287	291		249	249	248	249	249	290		290	290	
245-320 bp	R: CACGTATTAACTCCAT	291			291	290	299	299	290					16
Da1A01	F: CACGACGTTGTAAAACGACTATAATCGGCCAGAGG	237			237	222						232		
212–236 bp	R: TGTTGGAAGCATAGAGAA					237								
														5
Da3G04	F: CACGACGTTGTAAAACGACCACGGCTTGACCTATC		307	307	307	307				328		328		
318–341 bp	R: TTATTCAGGGCTGGTG		321	319	319	320								
														10
Dpr3B12	F:CACGACGTTGTAAAACGACCATCAATCTTTCTCTGCT	152		154	154	154			165	165		164	165	
129–141 bp	R: CCATCACACAATCCATC	154				163				167		167	167	
						167								14
											Total of sampl			45
		D. alata					*D. bulbifera*		*D. cayenensis*					
	Ym30 249-299 bp	249 - 291 bp					248 - 299 bp		249 - 290 bp					
	Da1A01 222-237 bp	222 - 237 bp							232 bp					
	Da3G04 321- 329 bp	307 - 321 bp							328 - 328 bp					
	Dpr3B12 152-168 bp	152 - 167 bp							164 - 167 bp					
D. alata ID(Be35, Sh43, Sh60, Sh30, Sh67) *D. bulbifera* ID(Sh75, Sh80) D. cayenesis ID (Be29, Sh54, Be5, Sh55, Sh48)														

Table 4.16 Fragment Analysis record deploying **GeneMapper Software** of *D. alata, D. bulbifera,* and *D. cayenensis* species incorporating forward incorporated with M13 FAM dye and reverse primers of Ym30, Da1A01, Da3G04, & Dpr3B12

4.5.1 *Dioscorea alata*

D. alata specimens amplified by Ym30 incorporated with M13 FAM markers generated seven fragments varying in size between 249 to 291 bp. Moreover, Da1A01 integrated with M13 FAM markers incorporated with *D. alata* yielded four different fragments

within the range of 222 to 237 bp long. The integration of Da3G04 incorporated with M13 FAM markers amplifying the targeted regions of *D. alata* produced eight fragments with different length ranging from 307 to 321bp. Integrating Dpr3B12 and M13 FAM microsatellites markers produced seven different fragments of varying lengths from 152 to 167bp. All the amplified fragments and their sizes are documented in table 4.16 and depicted in figures 4.75, 4.76, 4.77, 4.78, and 4.79.

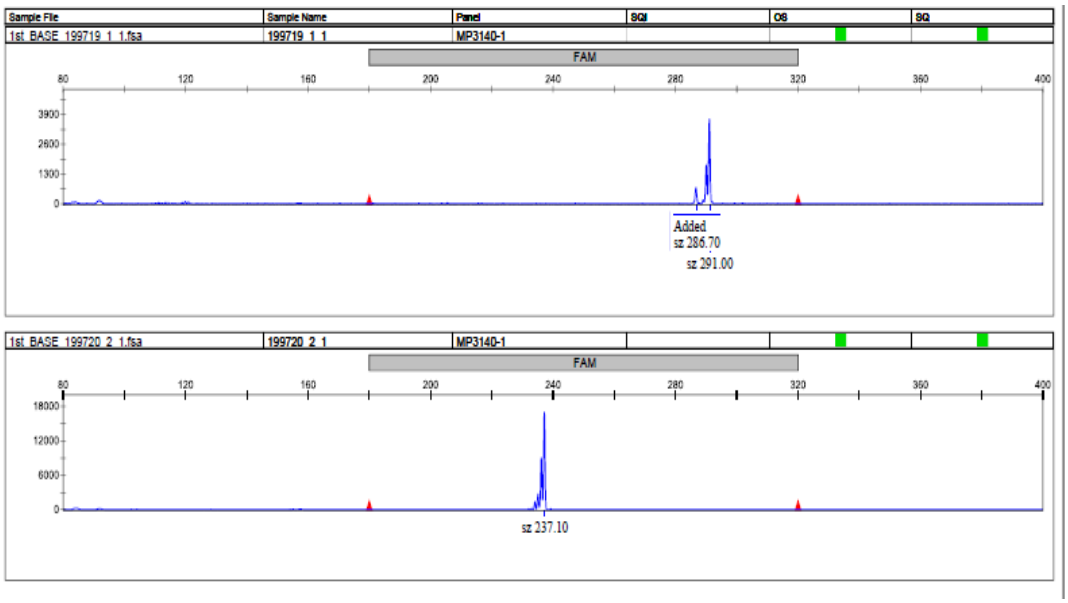


Figure 4.75 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. alata* species amplified by Ym30-287 & 291bp, Da1A01-237bp with M13 FAM markers.

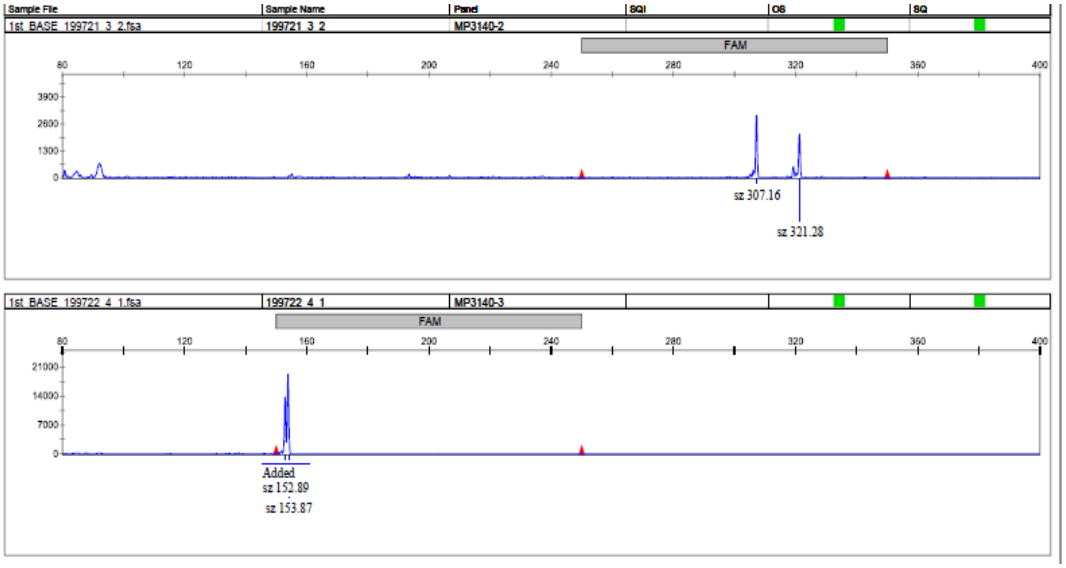


Figure 4.76 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. alata* species amplified by Da3G04-307 &322bp, Dpr3B12-152 &154bp incorporated with M13 FAM markers.

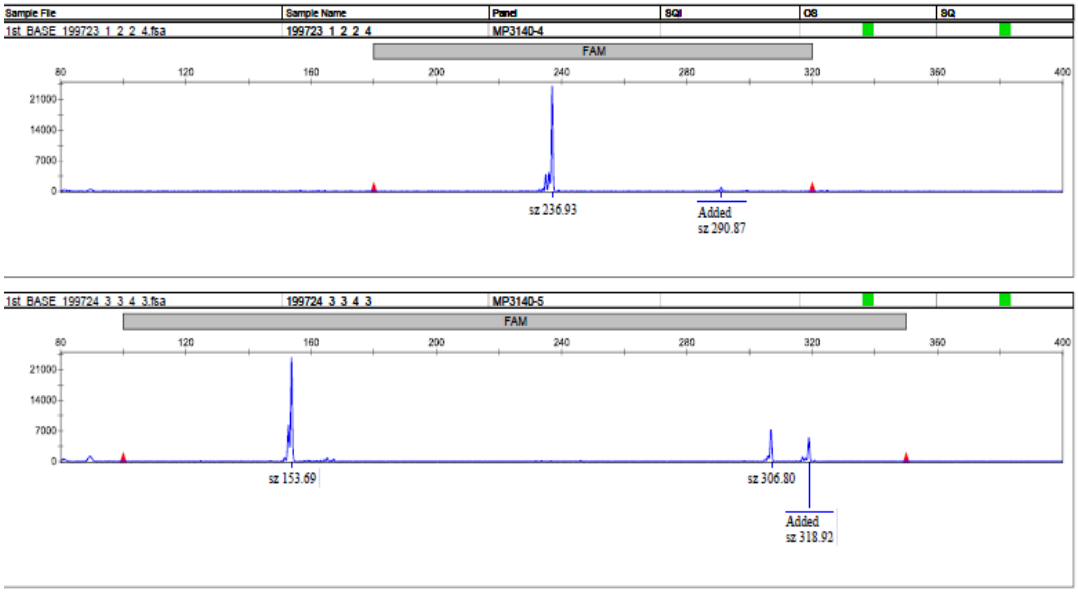


Figure 4.77 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. alata* species amplified by Da1A01-237bp, Ym30-290bp, Dpr3B12-154bp, and Da3G04-207 &319bp with M13 FAM markers.

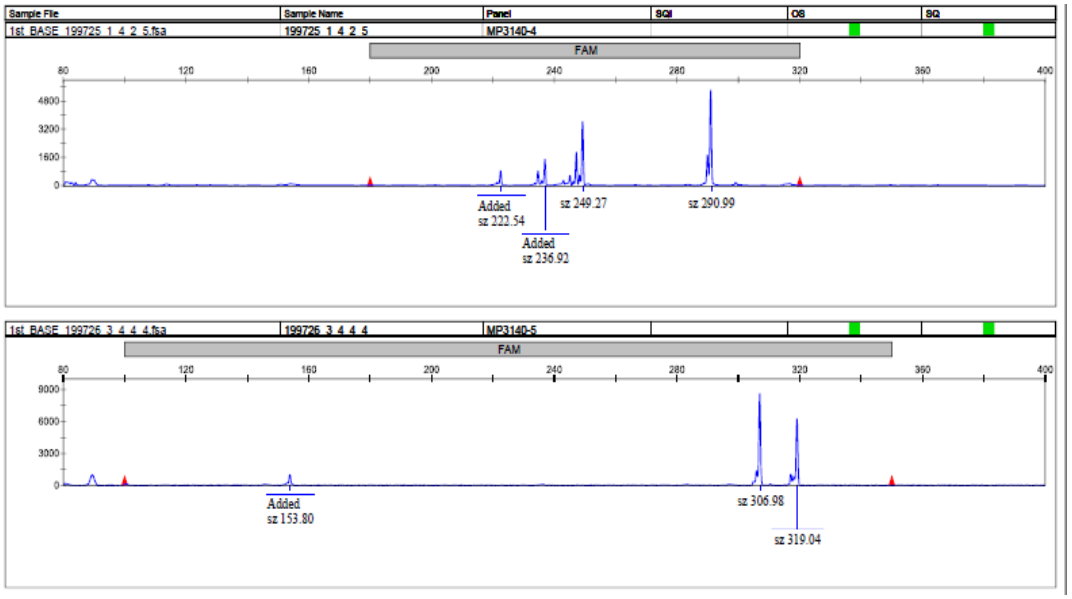


Figure 4.78 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. alata* species amplified by Da1A01-222 & 237bp, Ym30-249 & 291bp, DprB12-307 &319bp, and Da3G04-154bp with M13 FAM markers.

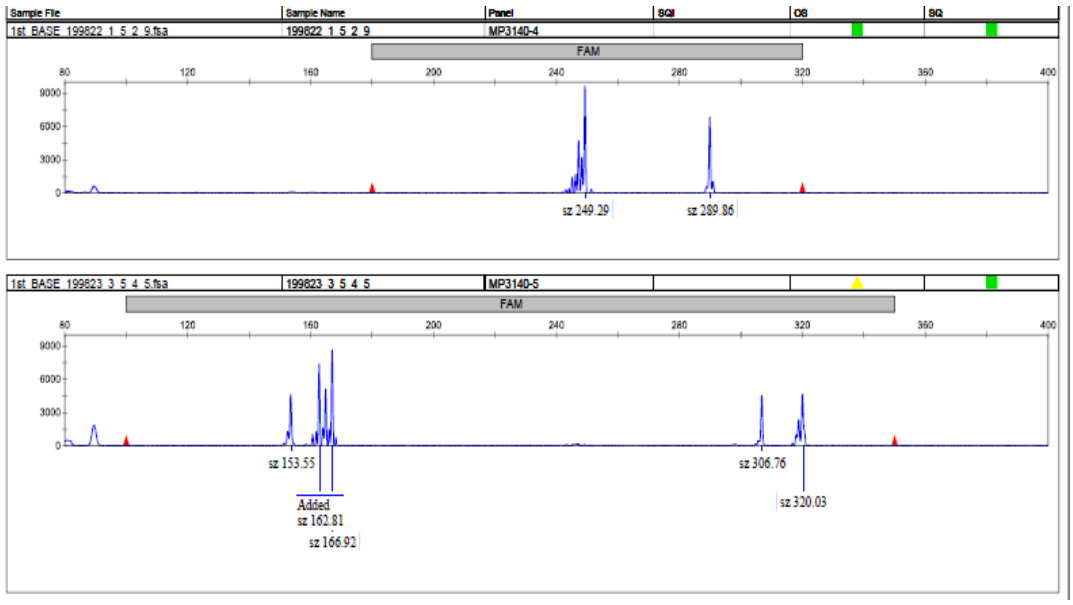


Figure 4.79 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. alata* species amplified by Ym30-249& 290bp, Dpr3B12-154, 163 &167bp, and Da3G04-307&321bp with M13 FAM markers.

4.5.2 *Dioscorea bulbifera*

The integration of Ym30 and M13 FAM markers amplifying *D. bulbifera* specimens created four fragments differing in length between 248 to 299 bp (Figure 4.80 and Table 4.16).

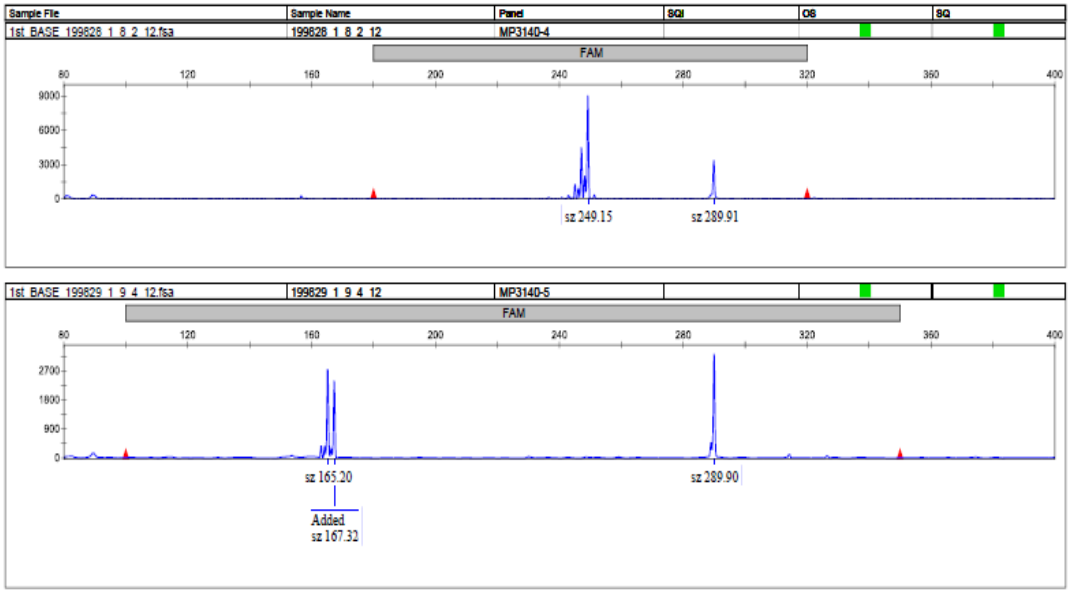


Figure 4.80 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. bulbifera species* and Ym30-249&290bp, Dpr3B12-165&167bp, and Ym30-290bp incorporated with M13 FAM markers**.**

4.5.3 *Dioscorea cayenensis*

Four fragments of 290bp and one of 249bp lengths were generated when Ym30 and M13 FAM amplified *D. cayenensis* species. Da1A01 and M13 FAM makers' production was one fragment with 232bp length. Da3G04 and M13 FAM markers yielded two fragments of size 328bp. Moreover, the incorporation of Dpr3B12 and M13 FAM produced seven fragments ranging between 164 to 167bp. Three fragments of 165bp and the other three with 167bp while one had 164bp. Figures 4.81, 4.82, 4.83, 4.84, and table 4.16 depict the fragment production and their length, and the fluorescent standard measurement.

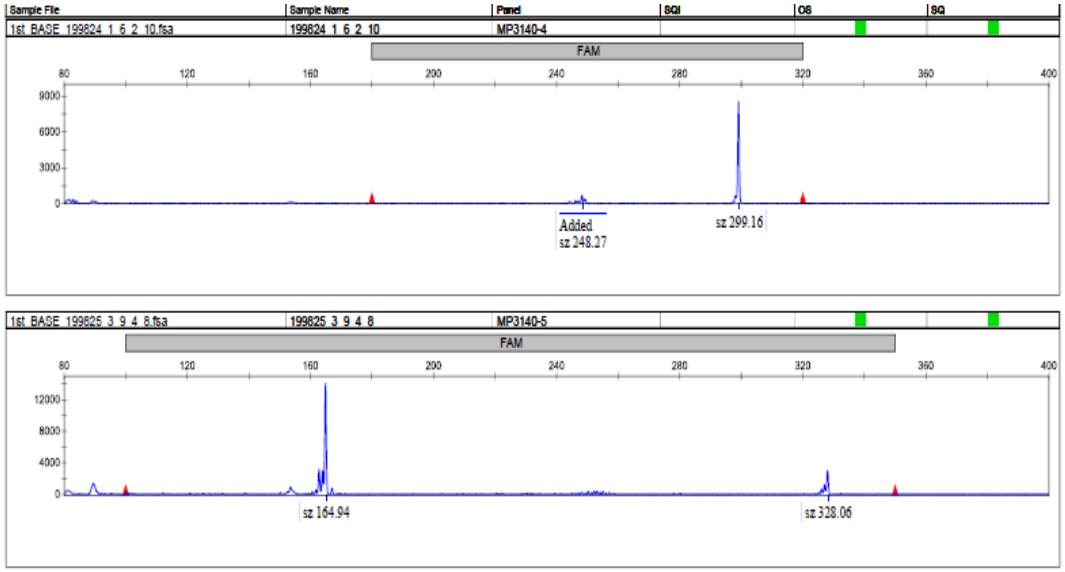


Figure 4.81 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. cayenensis* species amplified by Ym30-249 & 299bp, Dpr3B12-165bp, and Da3G04-328bp with M13 FAM markers.

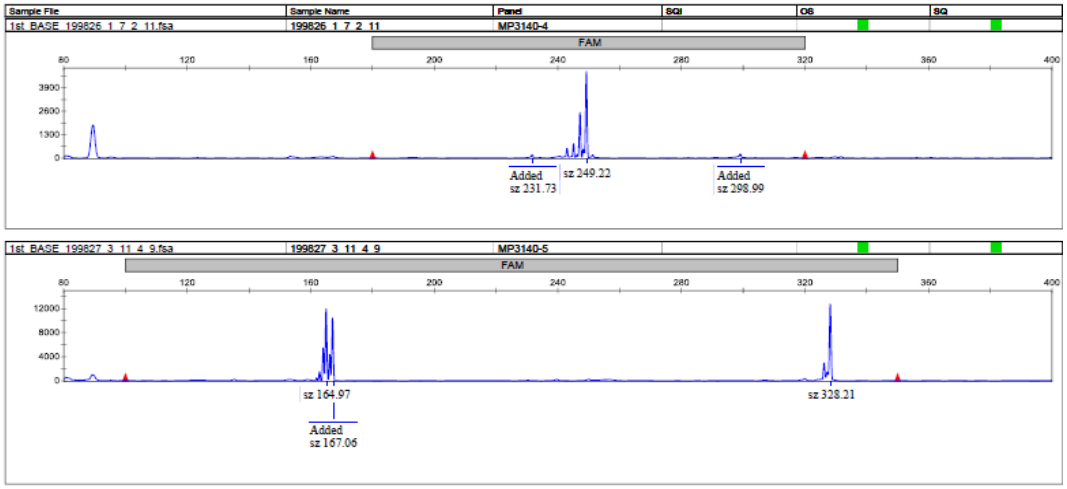


Figure 4.82 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. cayenensis* species amplified by Da1A01, Ym30, Dpr3B12, and Da3G04 with M13 FAM markers.

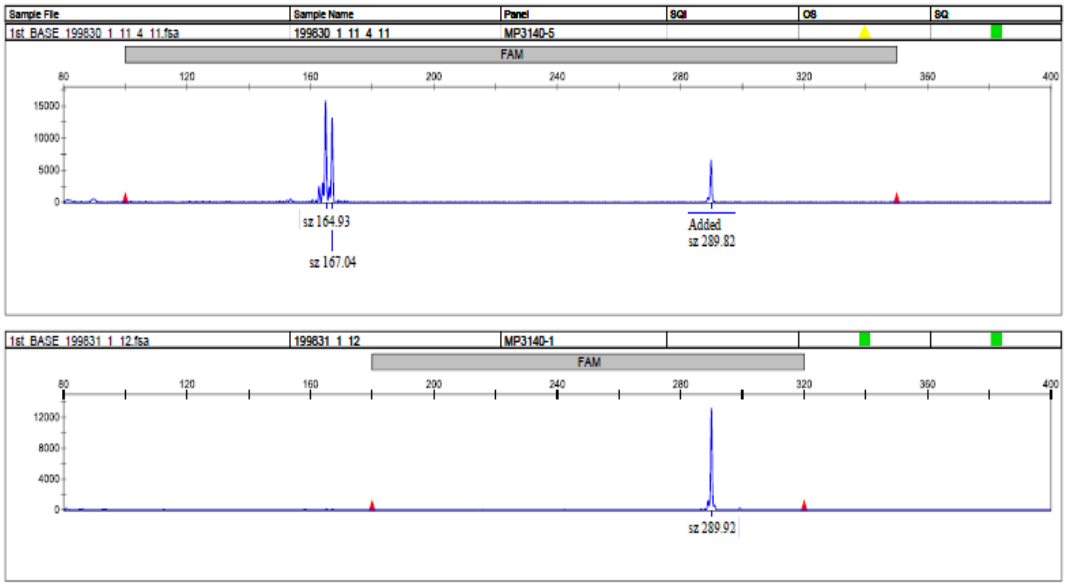


Figure 4.83 Electropherograms displaying fragment analysis- fluorescently labelled fragment of *D. cayenensis* species amplified by Dpr3B12-165& 167bp, Ym30-290 &290bp with M13 FAM markers.

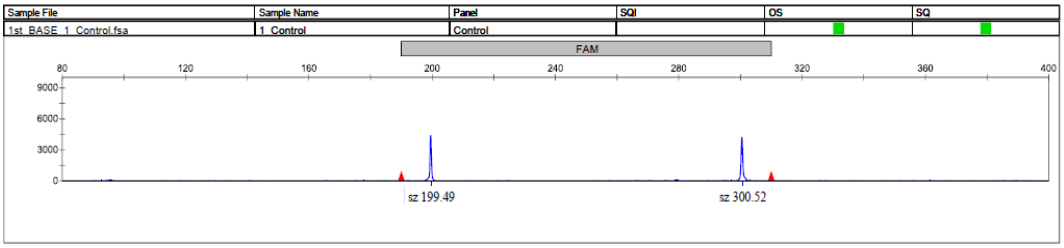


Figure 4.84 Electropherograms displaying fragment analysis- fluorescent standard size rule low 199bp, and the high of 300bp, used to analyse the fragments measurement.

5 Chapter 5: DISCUSSION

DNA was extracted from the 48 accessions of *D. alata, D. bulbifera,* and *D. cayenensis* species acquired from tropical regions of Southwestern Ethiopia (Maji district: Sheko and Bench villages), and Southwest Malay Peninsula (Selangor state: Semenyih and Kajang districts) using Sigma GenElute Kit, and i-genoimic Plant DNA Extraction Mini Kit. The isolated DNA was quantified deploying spectrophotometer and electrophoresis. Analysing electrophoresis data of DNA images on the gel governed the adequate of DNA templates for experimentations (Barbas *et al.*, 2007). Electrophoresis might be a better method in determining the quality and intactness of the DNA because DNA fluorescence was compared to a known quantity of lambda DNA mirroring the method used by Garner and Revzin (1981).

A second spectrophotometer quantification reading for the Ethiopian samples was performed because of the transit of the DNA from UK to Malaysia. Moreover, when variation in the results was observed, the mean of the two records was considered for accuracy in quantification data displayed in table 4.2. In spite of subjecting more than one sample to be quantified, low concentration and smearing on the gel were perceived in the Malaysian accessions due to poor preservation prior to DNA extraction as table 4.3 and 4.5 exhibited. Table 4.4 revealed that some of the Ethiopian samples had registered high quantity DNA concentration, possibly credited to adequate preservation and a perfect environment in the Future-Crop glasshouses at Sutton Bonington Campus where the newly expanded fresh leaves were frozen at below 80^0C degrees preceding the extraction.

Characterising yams require a precision in the process due to overlapping between the *Dioscorea* species traits. Therefore, the mean of the spectrophotometer and electrophoresis records was considered for estimation of the DNA quantity as table 4.6 and figures 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6 evidence the records.

The low quantity record was attributed to poor storage conditions accelerating biochemical alteration in the yam tubers causing degradation of the DNA molecules (Ikediobi and Oli, 1983). In addition, the phenolic compounds and the highly glutinous

polysaccharides in the tropical tuber crop leaves made extracting a decent quality of the DNA challenging.

Based on the spectrophotometers and electrophoresis records variation in DNA quantitative data, the existence of interspecific and intraspecific diversity between the species' individuals was inferred. Notably, since spectrophotometer UV can captivate tryptophan and tyrosine in which the absorption was subjected to the amount of wavelength absorbed by their chromophores (Schmid, 2001), an accurate spectrophotometer data can discriminate species amino acid structures.

A comparison of the three species average data and the difference between the highest and lowest in DNA concentration as indicated in tables 4.2, 4.3, 4.4, 4.5, and 4.6 showed that *D. alata* species were the poorest in a DNA concentration while *D. bulbifera* was the richest. However, the sample deviation from the mean *D. alata* samples were less deviated, and *D. bulbifera* was more deviated showing more consistency in the former.

After the DNA samples were quantified, the master mix was calculated to precede the PCR to stand on the eighteen primers efficacy in amplifying *D. alata, D. bulbifera,* and *D. cayenensis* species.

PCR static T_a at 45^0C assessed the eighteen primers where the PCR 35 cycles processed at a steady annealing temperature. PCR static T_a at 50^0C was used to reassess the primers efficiency in amplifying the *Dioscorea* species. Hence, concluding from table 4.7 and figures 4.8, 4.9, 4.10, and 4.11, in which Dpr3F10 and Da1D08 primers failed to amplify *D. alata* template, despite the pairs having a reasonable close T_m; the T_m delta of Dpr3F10 and Da1D08 primers were 0.07^0C and 1.83^0C respectively. Similarly, table 4.8 and figures 4.10, 4.11, 4.12, 4.13, 4.14, 4.16, and 4.17 depict *D. bulbifera* replications by PCR. The miscarriage of Dpr3B12, Da1D08, and Dab2E09 markers in amplifying *D. bulbifera* species, and Da1D08 markers in *D. alata* was attributed to the primer's failure in hybridising into complementary bases on the *Dioscorea sp.* several factors can be considered. DNA templates resulted in the absence of free hydroxyl groups to initiate a starting point elongating the nucleotides. Furthermore, multiple thawing and refreezing dNTPs might have impaired their functionality. Taq polymerase might have failed to break the Alpha to Beta phosphates connecting-bond on the dNTP 5'-end to acquire

energy required for phosphodiester bonding , thus the phosphate on the dNTP 5'-end was not linked to hydroxyl group on the primer 3'-end. In addition to that, the absence of a perfect denaturation, and Taq polymerase efficiency in carrying building blocks of dNTPs into the strand might have contributed to the miscarriage.

Amplification of *D. cayenensis* templates by the eighteen primers was outstanding as the results presented in table 4.9 and figures 4.19, 4.20, 4.21, and 4.22 depict.

Despite the T_a for the gradient PCRs being sufficiently below the markers melting temperature, some primers initiated replicating at 50^0C, but not at later stages. This variation in *Dioscorea* sp. amplification illustrated by table 4.10 was attributed to individual primers efficacy at suitable temperature at which annealing and elongation carried on. The failure was credited to the specificity of the PCR components, where only a specified temperature provided a perfect environment for the PCR ingredients to function and yield products, and any alteration in the temperature altering the PCR solution conductivity charges and the nature of the bases. As a result, the hydrogen atoms might have impounded and not formed hydrogen bonds, thus primers failed to hybridise the template at a higher or a lower temperature.

Nucleotides sequences differed between species, particularly in the noncoding section where the microsatellite loci were positioned (Scott and Williams, 1998). Thus, variation in the composition of nucleotides was existing as samples of the same species carried different genetic materials and having more than one allele or genetic locus representing characters of the species. Moreover, the two primers 3'-ends annealed to the target regions on the template manifesting the variation in the amplicons composition.

Analysing the eighteen primers PCR products integrated revealed diversification in the amplicons sizes. As figures 4.25, 4.34, and 4.40 presented diversification in the amplicons measurements resulted of Da1A01 marker amplifying *D. alata, D. bulbifera,* and *D. cayenensis* at 250-260bp, 290-300bp, and 260-270bp lengths respectively. Similarly, Dab2D06 produced bands 190-200bp long when integrated with *D. alata* and *D. bulbifera,* but produced bands of 340-355bp long when integrated with *D. cayenensis*. The variation in the length was attributed to the target region's distinctive

(A-T) and (C-G) bases stacking located between the two primers hybridisation points, and to the allele's variance being amplified.

Interestingly, the variation in optima T_a, and amplicons length might not be a conclusive evidence to validate the existence of intraspecific and interspecific diversification in *Dioscorea* species, but they can be a usable key to discriminate the species members.

In fact, the duration of the denaturation, annealing, extension stages and numbers of the PCR cycling were programmed according to the application's objective. Quantity of the material to start the PCR was crucial because the PCR cycling numbers were proportional to the amount of the products generated. However, PCR efficacy declines after exponential phase, and reaches a plateau phase where the primer ratio to the targeted DNA drops. This might lead the amplified products to hybridise to each other instead of the primers. Since Taq polymerase is not fully thermostable, some molecules will become inactive throughout some of the PCR cycles generating nonspecific products. In addition to that, primer and dNTP quantity might be used up prior to reaching the 40^{th} cycle (Brown, 1998). Re-association of DNA strands can be exploited to intercalate a complementary nucleotide of any foreign source to form new double-stranded DNA molecules (Karp, 2010).

Fortunately, the high transferability of the markers across species kept the linking venue between *D. alata, D. bulbifera,* and *D. cayenensis* wide open in which related species collectively shared some identical regions in their nucleotides stacking sequences. Moreover, this similarity in the sequences can be exploited in transferring desirable genes between the *Dioscorea sp.* efficiently aiding yam breeding programs and potentially developing a high quality yam.

To have a solid foundation upon which to characterise *Dioscorea* species, an M13 FAM marker was introduced into the forward primer's 5'-end paired with a normal reverse primer in a three-way scheme. Table 4.16 depicts the results of incorporating the M13 FAM marker into the forward primer's 5'-end paired with a normal reverse primer in a three-way scheme. The distinction in the DNA fragment sizes of Ym30 and M13 FAM microsatellites with *D. alata* species produced amplicons with varying sizes; exhibiting

a 42bp difference between the shortest fragment of 249bp and the longest fragment of 291bp.

It was concluded from using *D. alata* sample (Be35) amplified by Ym30 varied in the length of fragments where 287bp and 291bp were. These results inferred that each individual of the same species has its unique characters.

Similarly, specimen (Sh67) of *D. alata* amplified by Ym30 incorporated with M13 FAM markers generated 249bp and 290bp lengths fragments, but produced 222bp and 237bp lengths when amplified by Da1A01 incorporated with M13 FAM. In addition to that, 307bp and 320bp long fragments were yielded when amplified by Da3G04 markers. Then it produced three different lengths of 154bp, 163bp, and 167bp fragments when amplified by the corporation of Dpr3B12 with M13 FAM markers. Figures 4.78 and 4.79 documented the electropherograms results. The PCR products of various lengths assured that different alleles of the species were being amplified.

In the meantime, Ym30 and M13 FAM marker's ability to amplified *D. alata, D. bulbifera,* and *D. cayenensis* species reinforced the reality of linkage between the *Dioscorea* species where in spite of producing variation in the fragment lengths, the three species were being amplified by the primers. In spite of that, it indicates the existence of sharing similar DNA sequence in some part of their stands.

The differences in the fragment sizes were attributed to the Taq polymerase when adding nucleotides to the 3'-end. The added nucleotide, which might not have been extended in full without the long extension at the end of the PCR stages where a fraction of the amplicons obtained the extra nucleotide. Thus, peak of patterns was formed resulting in misinterpretation of the allele by GeneMapper software (Life Technology, 2014).

It is known that a mistranslation of the allele size might have caused by selecting low T_a where nonspecific products generated and nonspecific priming hybridised to the wrong regions in early and late cycles (Brown, 1998). Moreover, DNA template comprising high (GC), or a steady secondary structure might have replicated inefficiently generating nonspecific products (Lander *et al*., 1995).

In fact, the variation in the lengths of *D. alata, D. bulbifera,* and *D. cayenensis* samples emphasised the existence of more than one allele characterising the specific genes and

the reality of intraspecific and interspecific diversity in the *Dioscorea* species. DNA polymerase might face challenges in duplicating sections of a genome that comprises microsatellites with repeated sequences. Arguably, microsatellites have caused variation in the DNA length through generation (Karp, 2010).

Impressively, $MgCl_2$ played a crucial role in stimulating the PCR products because of its ion interaction with the DNA replication and the enzyme whose activities altered with $MgCl_2$ intensity. However, growing Mg^{+2} ions intensity might impede the PCR amplification causing imperfect separation of the amplified double-stranded products, which accelerate the strength of mispriming and the threat of nonspecific PCR products being replicated (Pelt-Verkuil *et al.*, 2008). Therefore, $MgCl_2$ was tailored to customise the PCR need. Brown (1998) stated intensifying Mg^{2+} concentration from 0.5 to 5.0 mM enriched the efficacy of PCR products. The $MgCl_2$ impact varied with different pairing-primers and DNA templates.

Subsequently, specific PCR products, a higher optimum T_a, and resolution of the bands were obtained by intensifying $MgCl_2$ from 0.07, 0.6, 1.0 µl and into the PCR solution. The PCR yield was proportioned to $MgCl_2$ concentration until an optimised level was reached, elevating the intensity impeding the PCR yield.

It is known that Mg^{2+} assists the Taq polymerase in removing Beta and Gama: the two negatively charged phosphate groups of the nucleotides to build phosphodiester bonds in nucleotides elongation. In addition to that, $MgCl_2$ binds to the primers and stabilises dsDNA, thus increasing the melting temperature of the dsDNA by preventing a full separation of the strands. Increasing the intensity of the $MgCl_2$ to a higher level changed the productivity of the PCR components, and PCR specificity on the advantage of polymerase activity. Figures 4.66, 4.67, 4.68, 4.69, 4.70, 4.71, 4.72, and 4.73 displayed the difference in the outcomes when $MgCl_2$ altered and influenced the PCR yield.

The precision of DNA fragment sizes analysed by GeneMapper software undoubtedly highlighted the existence of diversity between *D. alata, D. bulbifera,* and *D. cayenensis* species. Although they shared characters linking them to each other, the individual species possessed unique phenotypic and genotypic features assisted researchers to alienate the species from each other. This variation occurred because external and an

internal combination of factors deformed the cultivars' characters, and this genotypic alteration evolved over generations enhancing this cultivar to survive and potentially yield a better quality tuber.

Bousalem *et al.*, (2006) stated that Dab2C05 microsatellite marker, deployed to segregate the American yam of Dioscorea species, was efficient at the T_a of 50^0C. However, it was reported that Dab2C05 was active in amplifying *D. alata* species at T_a 45, and 50^0C, and *D. bulbifera* at 50^0C; whereas it amplified *D. cayenensis* at 50-54.7^0C in which figures 4.9, 4.14, 4.19, 4.21, and 4.44 documents the statement.

Tostain *et al.* (2006) reported the absence of amplification for Da1F08 markers with *Dioscorea alata* and *Dioscorea bulbifera*. However, the amplification of the two species by Da1F08 markers was confirmed as presented in figures 4.8, 4.11, 4.15, 4.20, and 4.31. Tostain did not have amplification with Da3G04 and either *Dioscorea alata* or *Dioscorea bulbifera*. However, Da3G04 was successfully amplified in this project at 45^0C, 50^0C, and 58.4^0C as documented by figures 4.7, 4.9, 4.10, 4.12, 4.15, and 4.26. Furthermore, regarding the lack of amplification for Dpr3F10 markers, figures 4.8 and 4.14 depict the replication of *D. alata,* and *D. bulbifera at* 45-50^0C.

Siqueira *et al.*, (2013) reported that Da1D08 and Da1A01 primers amplified the *Dioscorea cayenensis* at T_a 53^0C and 50^0C respectively, while it was confirmed the T_a in this project were 50^0C and 60.3^0C for Da1D08 and Da1A01 respectively.

6 Chapter 6: SUMMARY AND CONCLUSION

The forty-eight accession of *D. alata* (8)*, D. bulbifera (*12), and *D. cayenensis* (20) from Ethiopia, beside another *D. alata* (8) from Malaysia were scientifically labelled and frozen at minus 80 Celsius. DNA was extracted deploying i-genomic Plant DNA Extraction Mini Kit, and Sigma Plant Miniprep Kit.

The isolated DNA was quantified using spectrophotometers and electrophoresis. Variation was detected in the DNA quantitative results of the spectrophotometers and electrophoresis, thus the mean of the two records was taken into consideration for calculating the master mix for PCR assay. Because of poor quantity, some specimens were excluded from the list.

In fact, the Eighteen primers were assessed by PCR static annealing temperature at 45^0C for 35 cycles for efficiency in amplifying the *Dioscorea* species. Likewise, the eighteen markers were re-evaluated deploying PCR static T_a at 50^0C for 35 cycles. The PCR products were screened employing the electrophoresis protocol and the band sizes were co-migrated with standard-size Lambda DNA ladder.

A thermocycler with 12 columns and 8 rows was deployed for the gradient PCR. Increment temperature was appointed for the 12 columns in the thermocycler starting the first column at 50^0C and ascending with temperature to the 12 column ending with 65^0C. The eighteen primers were incorporated into a gradient PCR to acquire the optimal T_a utilising *D. alata, D. bulbifera,* and *D. cayenensis* DNA templates. The PCR products were assessed deploying electrophoresis application in finding the optimum T_a and bands lengths. Notably, a significant variation in optimal T_a was observed between the three species, which was partly attributed to the diversification in the nucleotides stacking possessed by each species' DNA strands.

Furthermore, markers' exhibited varied levels of efficacy in transferring across *D. alata, D. bulbifera,* and *D. cayenensis* species. Remarkably, $MgCl_2$ contributed to the amplification of the DNA templates when sufficient quantity was added to the PCR protocol. The $MgCl_2$ stimulation was witnessed as the optimal T_a of primers altered when it was intensified. In addition, the size, numbers of amplicons, and products resolution alteration were observed.

Ym30, Ga1A01, Da3G04, and Dapr3B12 microsatellite markers were incorporated with M13 FAM in PCR using *D. alata, D. bulbifera,* and *D. cayenensis* specimens. The fluorescence products were evaluated employing electrophoresis 2% agarose gel, and TBE buffer, and the positive PCR results were sent for fragment analysis. The samples were pooled prior to co-migrating with standard-size measurement. The fluorescence fragments exhibited ascertain variation in the size within the species' specimens and between the three species of *Dioscorea*. In fact, this step was an ideal step to determine linking or distancing the species of *D. alata*, *D. bulbifera* and *D. cayenensis*. Conversely, the fragment analysis revealed the similarity partaking in the nucleotides stacking sequence when an appointed marker efficaciously amplified the three species. The alteration in the allele sizes of the species' DNA was caused by the ennoblement and the way farmers domesticated the cultivar of yams.

Given the implications of farming success of the best variety of this tuber cultivar in a country heavily dependent on a resilient crop, the costs are justified to establish growing dependency on such a low-maintenance food source.

This study is the beginning of research that will benefit yam farmers around the globe, and in particular, Ethiopia and Malaysia because it will open an avenue to developing an accurate genotype with grounds for future adaptation to pest and disease resistance. The success in transferring markers across species will aid yam growers to select desired genotype and transferred characters to produce a better quality yield. It will also assist in decision-making that is crucial to preserving the varieties of *Dioscorea* species before this cultivar is eradicated and overtaken by other crops.

Efficiently producing high-quality yam is a key to helping secure food supply for many nations combating climate change and resulting droughts due to its starch storage, nutritional value, and ability to survive long periods with little moisture. Specifically, yam coating and fiber tissue protect the edible part of the tuber for a longer period if well stored, and help the cultivar to cope with climate change compared to other tuber crops (Onwueme, 1978; Lebot, 2009). All of which, can make the yam an ideal crop for tough climates with the increasingly drier weather. The yam of each region earnestly

requires a tissue culture technique for advanced study and future adoption as the realities of food scarcity grow.

In fact, yam could be cultivated within coffee and maize fields as is done in the Bench and Sheko districts of Ethiopia. Farmers are eager to implement methods to improve their cultivars' nutritional value and high-quality production that secures better revenue. Ethiopian and Malaysian *Dioscorea* species have not been intensively studied to investigate ploipdy levels, DNA content estimation, and to decipher the nature of the Ethiopian *D. cayenensis* complex in relation to other yam species in other parts of Africa. This study can significantly contribute to remedying the lack of knowledge on the diversity, and classification of Ethiopian and Malaysian *D. cayenensis* complex. It can also create a foundation for future research to advance the yam's potential.

For future projects, the following variables can be explored:

Various ranges of $MgCl_2$ concentrations and volumes starting from low as 0.05mM and increased by 0.05 mM to high concentration of 20mM to justify the $MgCl_2$ role in the PCR application.

Using primers with various concentrations and volumes. Various concentrations and volumes of templates to be used.

Incorporating M13 tailed primers with the forward and with the reverse primers.

Using forward and reverse primers separately in PCR assays, and then reunites the two separated primers in one PCR application.

7 APPENDIX

7.1 ABBREVIATIONS LIST

ANOVA: Analysis of Variance

bp : base pair

^0C : Celsius

cal BP : calibrated year before the present

D. : Dioscorea

dH_2O :distilled water

DNA : deoxynucleotide

dNTP : deoxynucleotide triphosphate

dsDNA: double stranded deoxynucleotide

FAO : Food and Agriculture Organization

g : gram

Kb : kilo base

$MgCl_2$: Magnesium Chloride

ml : millilitre

mM : mill molar

ng : nanogram

PCR : Polymerase Chain Reaction

RAPD : Random Amplified Polymorphic DNA

RFLP : Restriction Fragment Length Polymorphism

sp. : species

SPN : single nucleotide polymorphism

ssDNA: single strand deoxynucleotide

SSR : Simple sequence repeats

T_a : Annealing temperature

T_m : Melting temperature

Taq : Thermus aquaticus

TAE : Tris-Acetic-EDTA

TBE : Tris-Borate-EDTA

ug : microgram

ul : microliter

UV : Ultraviolet

7.2 BIBLIOGRAPHY

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