Nuclear DNA Content and Genetic Structure of Yams (Dioscorea Species, Dioscoereaceae) Cultivated in Southwestern Ethiopia

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Abstract:

Yams (Dioscorea species, Dioscoreaceae) are grown either for their starchy tubers or medicinal properties and are important crops in the tropics and sub-tropics. Ethiopia is regarded as an isolated centre of yam production in Africa. Many wild and domesticated varieties have been lost over time due to various constraints of production. A clear understanding of their diversity (especially that of *D. cayenensis* complex domesticated from native wild yam) is needed for conservation and improvement. The study was conducted on three species of Dioscorea that were collected from Bench and Sheko districts in SW Ethiopia, namely, *D. alata, D. bulbifera* and the *D. cayenensis* complex. The main objective of this study was to investigate the genetic structure of cultivated and managed yams from SW Ethiopia. Chloroplast single nucleotide polymorphisms (SNPs), nuclear DNA content and morphological observations were used to assess the diversity present. DNA content was measured by including Lycopersicum esculentum as an internal standard, staining the samples with propidium iodide (PI) using a Becton Dickinson LSR II flow cytometer. Inter and intraspecific DNA content variation was tested using GenStat statistical software (v14). Alignment and UPGMA tree construction were constructed using CLC Genomics Workbench (v4.8). Morphological characterization was carried out using plants grown in glasshouses using IPGRI/IITA descriptors. Explants were also grown on Murashige and Skoog medium (MS) and Woody Plant medium (WP) to test for suitable growth medium. Overall, the mean 2C DNA contents of *D. alata*, and *D. bulbifera* were 1.153 ± 0.02 and 2.374 ± 0.02, respectively. These values are comparable to reported 2C values of *D. alata* and *D. bulbifera* from other parts of Africa. 2C value of the *D. cayenensis* complex was 1.275 ± 0.02 and comparable to *D. praehensilis, D. abyssinica* and *D. cayenensis-rotundata* complex reported earlier. Successful amplification in D. alata using primers ndhH-Exon,, ycf1-rrn5 and rrn4,5-trnN is reported. Five chlorotypes were defined based on SNPs from the sequence of products from the three primers, with addition of West African *D. praehensilis* and *D. abyssinica* sequences. The occurrence of an individual from *D. praehensilis* with the same chlorotype as the *D. cayenensis* complex, and comparable DNA amounts might indicate that they could be same the taxonomic entity with different gene expression in different environments. Intraspecific morphological variation was observed in all three species studied. In addition to MS, WP was found to be suitable to grow yams in culture.

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ABSTRACT

Yams (*Dioscorea* species, Dioscoreaceae) are grown either for their starchy tubers or medicinal properties and are important crops in the tropics and sub-tropics. Ethiopia is regarded as an isolated centre of yam production in Africa. Many wild and domesticated varieties have been lost over time due to various constraints of production. A clear understanding of their diversity (especially that of *D. cayenensis* complex domesticated from native wild yam) is needed for conservation and improvement. The study was conducted on three species of *Dioscorea* that were collected from Bench and Sheko districts in SW Ethiopia, namely, *D. alata*, *D. bulbifera* and the *D. cayenensis* complex. The main objective of this study was to investigate the genetic structure of cultivated and managed yams from SW Ethiopia. Chloroplast single nucleotide polymorphisms (SNPs), nuclear DNA content and morphological observations were used to assess the diversity present. DNA content was measured by including *Lycopersicum esculentum* as an internal standard, staining the samples with propidium iodide (PI) using a Becton Dickinson LSR II flow cytometer. Inter and intraspecific DNA content variation was tested using GenStat statistical software (v14). Alignment and UPGMA tree construction were constructed using CLC Genomics Workbench (v4.8). Morphological characterization was carried out using plants grown in glasshouses using IPGRI/IITA descriptors. Explants were also grown on Murashige and Skoog medium (MS) and Woody Plant medium (WP) to test for suitable growth medium.

Overall, the mean 2C DNA contents of *D. alata*, and *D. bulbifera* were 1.153 ± 0.02 and 2.374 ± 0.02, respectively. These values are comparable to reported 2C values of *D. alata* and *D. bulbifera* from other parts of Africa. 2C value of the *D. cayenensis* complex was 1.275 ± 0.02 and comparable to *D. praehensilis*, *D. abyssinica* and *D. cayenensis-rotundata* complex reported earlier. Successful amplification in *D. alata* using primers *ndhH-Exon,, ycf1-rrn5* and *rrn4,5-trnN* is reported. Five chlorotypes were defined based on SNPs from the sequence of products from the three primers, with addition of West African *D. praehensilis* and *D. abyssinica* sequences. The occurrence of an individual from *D. praehensilis* with the same chlorotype as the *D. cayenensis* complex, and comparable DNA amounts might indicate that they could be same the taxonomic entity with different gene expression in different environments. Intraspecific morphological variation was observed in all three species studied. In addition to MS, WP was found to be suitable to grow yams in culture.

1. INTRODUCTION

1.1 TAXONOMY

Yams are dioecious plants which belong to the genus *Dioscorea* Plum. ex L. and the family Dioscoreaceae in the order Dioscoreales. Yams were classified under the monocotyledons even if some features such as the presence of a second non emergent cotyledon and reticulate veining of the leaves are typical of certain dicotyledonous plants (Degras, 1993). Dioscoreales **was** placed within the monocots in phylogenetic tree of angiosperms, but distantly related to grasses (APG III, 2009; Figure 1).

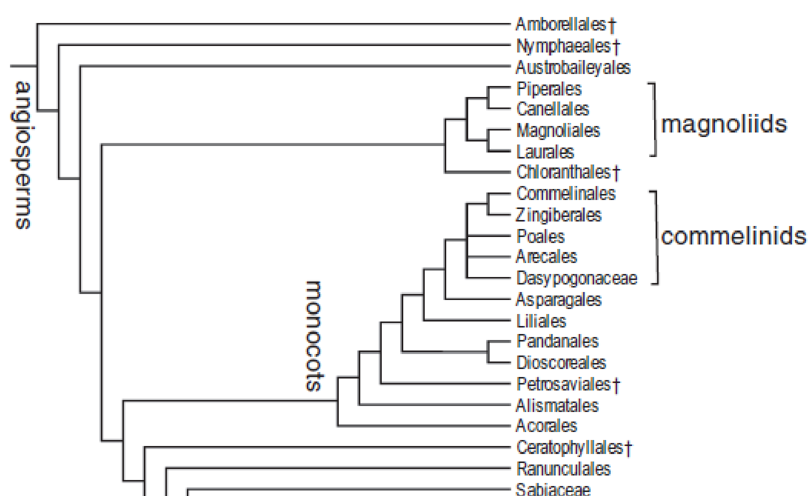

Figure 1. Relation of Dioscoreales (including the family Dioscoreaceae nom. cons.) to APG III orders and families (Source APG III, 2009).

Tacca and *Dioscorea* are the genera from Dioscoreaceae that is found in tropical Africa. Members of the *Dioscorea* genus are twining or climbing herbs, stems annual or rarely perennial and tubers perennial or renewed annually. Stems are with or without spines. The plants are dioecious. The flowers are small and unisexual and pollinated by insects, with an extremely irregular production of male and female flowers reported for members of the *D.*

cayenensis Lam. complex. The male inflorescences are spicate, racemose or rarely cymose, axillary or forming panicles at the ends of leafless branches. Male flowers have campanulate to spreading perianth and six stamens, either all fertile or three reduced to staminoides. The female inflorescences are spicate and axillary with perianth similar to the males. The capsules are triangular or deeply three-lobed dehiscing into three valves, and with 1-2 seeds in each locule. Seeds are mostly winged and rarely wingless (Meige and Demisew, 1997).

There are 640 *Dioscorea* species (Govaerts *et al.*, 2011) out of which four are major cultigens: *D. cayenensis* complex, *D. alata* L., *D. esculenta* (Lour.) Burkill and *D. polystachya* Turcz., Bull. are the major cultivated species (Coursey, 1967). The genus *Dioscorea* is subdivided into sections. Enantiophyllum contains most of the economically important yam species (*D. rotundata*, *D. alata*, *D. cayenensis*, *D. esculenta* (Lour.) Burkill and *D. polystachya* Turcz.). The vines of yams belonging to this section twine to the right, i.e. in a clockwise direction when viewed from the ground upwards. Species in sections Lasiophyton (*D. dumetorum* (Kunth) Pax and *D. hispida* Dennst.), Opsophyton (*D. bulbifera* L.), Combilium (*D. esculenta*) and Macrogynodium (*D. trifida* L.) twine to the left (Dumont *et al*., 2006). Wilkin *et al*., (2005) constructed phylogenetic analysis of the genus *Dioscorea* based on sequence data from the plastid genes *rbcL* and *matK* where *Dioscorea* sect. *Enantiophyllum* matches the previous classifications of the right-twining and opposite leaves. New species of *Dioscorea* have been discovered such as new species and subspecies of *D. sambiranensis* Wilkin species complex from Madagascar (Wilkin *et al*., 2009a), new endangered species *D. strydomiana* Wilkin from South Africa (Wilkin *et al*., 2010) and *D. kituiensis* Wilkin & Muasya from Kenya (Wilkin *et al*., 2009b).

According to Miege and Demissew (1997), there are eleven species of yams in Ethiopia namely, *D. quartiniana* A. Rich, *D. cochleari-apiculata* De Wild, *D. dumetorum* (Kunth)

Pax, *D. gillettii* Milne-Redh., *D. bulbifera, D. schimperiana* Hochst. ex Kunth, *D. alata*, *D. cayenensis* complex, *D. praehensilis* Benth, *D. abyssinica* Hochst. ex Kunth and *D. sagittifolia* Poir. *D. bulbifera*, *D. alata* and *D. cayenensis* complex are the species used in this study. *D. bulbifera* (aerial yam) is characterized by the production of large numbers of aerial tubers on the axil of a leaf. The stem is cylindrical and twines to the left (Miege and Demissew, 1997). It is the only edible yam that is native to both Asia and Africa. Wild forms of it can be found on both continents (Onueme, 1978). Wild *D. bulbifera* is also found in Ethiopia (Miege and Demissew, 1997). *D. alata* (water yam) is characterised by its angled stem with four or more rows of wings. The stem twines to the right. The leaves are opposite in arrangement (Miege and Demissew, 1997). The stem is usually green, but in some cultivars the wings may be purple or reddish in colour owing to anthocyannin pigments. Many cultivars have varying degrees of purple colouration in the leaves. The extent and distribution of anthocyanin pigmentation on the stem and leaves can be used to distinguish cultivars. *D. alata* is the most widely distributed yam, grown in nearly all parts of the tropics (Onueme, 1978).

Taxonomically, it was not possible to separate *D. cayenensis* and *D. rotundata* from Ethiopia as there are many intermediates between the two species and thus are treated as a species complex under the name *D. cayenensis* (Miege and Demissew, 1997). Ethiopian *D. cayenensis, D. rotundata, D. praehensilis*, *D. abyssinica* and *D. sagittifolia* have been provisionally placed in the *D. cayenensis* complex during the study of Hildebrand (2003). Dumont *et al*., (2006) suggest that *D. rotundata, D. praehensilis* and *D. abyssinica* should be regarded as a single species. All the cultivated forms of the *D. cayenensis* complex are results of the domestication of the wild species *D. praehensilis*, *D. abyssinica, D. burkilliana* and *D.*

satittifolia and wild species such as *D. burkilliana* and *D. mangenotiana* are potential progenitors of the ennobled and pre-ennobled forms of *D. cayenensis* complex (Dumont *et al*., 2006; Mignouna *et al*., 2008). Domestication (ennoblement) in Benin includes taking wild yams into an intense form of cultivation (Dumont *et al*., 2006) while Ethiopian domestication or adoptive transplantation, a special term coined by Hildebrand, (2003) involves moving yams from lowland wooded savanna to highland settings and into home-gardens for easy access rather than for large scale cultivation (Hildebrand, 2009). This could explain the difficulty in clearly differentiating the *D. cayenensis* species complex from South and South Western Ethiopia into distinct species as they are single taxonomic entity as in the case of the wild-managed populations of *D. cayenensis* species complex (Abebe, 2008). This presents an opportunity to study and conserve yams from home-gardens with wild genetic background. In a study involving yams from Southern Ethiopia, except *D. bulbifera*, the remaining landraces were unidentified species or group of species (Tamiru, 2006) that are distinct from the *Dioscorea* species widely cultivated in West Africa (Tamiru *et al*., 2007).

The genus *Dioscorea* includes different ploidy levels even within the same species, and the basic chromomsome number of the genus has been reported as x = 9, 10 and 20 (Gamiette *et al*., 1999; Dansi *et al*., 2000; Dansi *et al*., 2001; Egesi *et al*., 2002; Obidiegwu *et al*., 2009c; Obidiegwu *et al*., 2010).

1.2 MOLECULAR STUDIES AND BREEDING

Despite their economic and socio cultural importance, less is known about the genomics of yams compared to global crops such as rice and maize (Mignouna *et al*., 2008). However, various genetic markers have been used to study genetic variation of cultivated yams, mostly

D. cayenensis- D. rotundata and their wild relatives. Some AFLP and SSR studies failed to clearly differentiate the *D. cayenensis* complex into different species (Tostain *et al.* 2006), including the studies on the Ethiopian yams (Tamiru *et al*., 2007; Abebe, 2008). In a study involving *D. cayenensis- D. rotundata* accessions from Benin, Congo, Côte d' Ivoire, Equatorial Guinea, Gabon, Ghana, Nigeria, Sierra Leone and Togo, Obidiegwu *et al*., (2009b) found highly heterozygous SSR data and concluded that the *D. cayenensis- D. rotundata* are distinct species with intermediate hybrid forms. In another study, RAPD and microsatellite-primed PCR (MP-PCR) clearly separated *D. rotundata* and *D. cayenensis* accessions from Nigeria, Togo, Jamaica, Ivory Coast and Ghana (Ramser *et al*., 1997). The analysis of *D. cayenensis/D. rotundata* complex and 20 cultivars of *D. alata* from Benin using RAPD showed highly significant variation among species, among groups within species, and among cultivars within groups (Zannou *et al*., 2009). RAPD markers are amplified using primers of arbitrary primer sequence which are universal for different species. Polymorphisms are detected frequently but are dominant markers and thus less informative than co-dominant markers to detect heterrozygosity. Microsatellites, ubiquitous simple sequence repeats are codominant and show higher levels of polymorphism (Reiter, 2001).

Recently, new chloroplast DNA primer sets have been used to study the genetic diversity of wild yams and domesticated guinea yams from Benin and revealed that *D. rotundata* contains all the same chlorotypes as wild relatives *D. praehensilis* and *D. abyssinica*, whereas the wild relatives contain additional chlorotypes (Scarcelli *et al*., 2011a). A study of farmers' varieties of yams from Gando ethnic groups of northern Benin revealed 33 different genotypes using 15 microsatellite markers (Scarcelli *et al*., 2011b). *D. cayenensis* species complex into

distinct species of *D. cayenensis* and *D. rotundata* as they are a wild-domesticated continuum with hybrid intermediates (Obidiegwu *et al*., 2009b). Molecular markers failed to differentiate *D. cayenensis* species complex from Ethiopia into distinct taxonomic entities (Tamiru, 2006; Abebe, 2008).

Studies have linked morphological characters with molecular markers. *D. rotundata* cultivars collected from Ghana, Benin, Cameroon, Nigeria, Cote d'Ivoire, Togo, Guinea and Burkina-Faso were characterised morphologically according to the IPGRI/IITA (1997) descriptors for yam and classified into cultivar groups/morphotypes (Dansi *et al*., 1999). AFLP, RAPD and SSR were used to investigate these cultivars. The way AFLP, RAPD, SSR and morphology detected variability varied widely (Mignouna *et al*., 2003). It is speculated that epigenetics might explain the incongruence of morphological and genetic markers, although not tested experimentally (Dumont *et al*., 2006; Mignouna *et al*., 2008). In addition to chloroplast *rbcL* and *matK*, Wilkin *et al*. (2005) observed macro-morphological characters based on underground part, flower, fruit and stem twining direction but the characters were too few to be included in the phylogenetic tree generated from DNA data.

The complete chloroplast genome of *Dioscorea* has been sequenced and is very similar in size, gene content, and gene order to the ancestral angiosperm genome represented by *Amborella*, *Nuphar* and *Nymphaea* except *Dioscorea* has lost one protein-coding gene, rps16 and has a small expansion of the inverted repeat (IR) that has duplicated the trnH-GUG region (Hansen *et al*., 2007). Based on chloroplast genome sequence alignment, genus concept in Dioscoreaceae was found to be different from that of Poaceae and Arecaceae. Levels of divergence between two distant species of *Dioscorea* was in the range of the inter-generic differentiation in Poaceae and Arecaceae, while the genus *Dioscorea* and *Trichopus*

from Dioscoreaceae are so divergent that they are not even alignable for some intergenic spacers (Scarcelli *et al*., 2011a).

Progress has been made in developing molecular markers and mapping the genome. RFLP, AFLP and microsatellite markers have been used so far. Mapping has been done for many important traits like disease resistance (Mignouna *et al*., 2008). A saturated genetic map for anthracnose resistance was developed for *D. alata* (Petro *et al*., 2011). Expressed sequence tags have also been generated (Satya *et al*., 2011). Direct gene transfer into protoplasts has been achieved. *Agrobacterium tumefaciens*-mediated transformation of *D. zingiberensis,* a medicinal crop, has been achieved (Zhu *et al*., 2009).

1.3 PRODUCTION

Yams are grown either for their starchy tubers or medicinal properties, are important crops in the tropics and sub-tropics (Mignouna *et al*., 2008). According to FAO (2009), the highest yam production is in Nigeria followed by Cote d'Ivoire, Ghana, Benin, Togo and Papa New Guinea. West African yam production includes areas in latitudes 4^0N and 10^0N and longitudes 5^0W and 10^0E (Norman, 1995).

Dioscorea cayenensis complex are tropical plants. Their growth is severely restricted at temperatures below 20^0C and the ideal temperature for growth is 25-30^0C. Yams grow best when supplied with ample moisture throughout their growing season. They do best in areas where the rainy season is relatively long. Their tubers are tolerant of dry conditions and they can survive a few months of dry season and sprout before the rains come. Moisture stress after the seedling stage is also well tolerated. Even though the yams survive drought, they do not yield well without adequate moisture. Yams require soils of high fertility, rich in organic

matter, for high yield. Cultivated yams are propagated vegetatively from whole tubers (seed yams), large pieces of tubers (setts) or from minisetts. They can also be propagated from true-seeds though this practice is largely limited to breeding programmes (Onwueme, 1978; Lebot, 2009).

Ethiopia is regarded as an isolated centre of yam production in Africa. Yams are grown with cereals and other root and tuber crops like *Ensete ventricosum* and *Colocasia esculenta* in Southern, South-Western and Western parts of Ethiopia (Westphal, 1975). Farmers in Central and West Africa (Mignouna & Dansi 2003) and South Western Ethiopia (Hildebrand 2003, personal observation) domesticate wild yams. Domestication brings genetic variation to the farmer's field from the wild yams (Dumont *et al*., 2006).

Single and double harvested varieties exist in Ethiopia. For single harvested landraces that normally produce single tubers per plant, the proximal end of each tuber is retained for propagation while the remaining part is consumed. Double harvested landraces produce multiple tubers following first harvest and are ideal planting materials. In southern Ethiopia, single and double harvested varieties are used (Hildebrand, 2003a; Yashu, 2008).

In Wolayita and Gamo-Gofa zones, Southern Ethiopia, the yam is cultivated on an annual cycle of planting in the field starting in October. Planting is delayed till November or December in areas where the dry season is long because soil moisture content is considered important for timing of field planting. It is also easier to loosen the soil that is essential for yam cultivation when soil moisture is high. The first double harvest is in May or June. Farmers use certain signals to harvest the first double harvest, like the senescence of

inflorescences, digging and checking of tubers, soil cracking and wilting of vine tips. Tubers are detached from the corm and the rest is covered with soil for re-growth. Single harvested varieties are harvested at leaf senescence (Tamiru, 2006).

1.4 SITE OF PLANT COLLECTION AND THE PEOPLE

Ethiopia is a country in East Africa with diverse cultures and peoples (Figure 2).

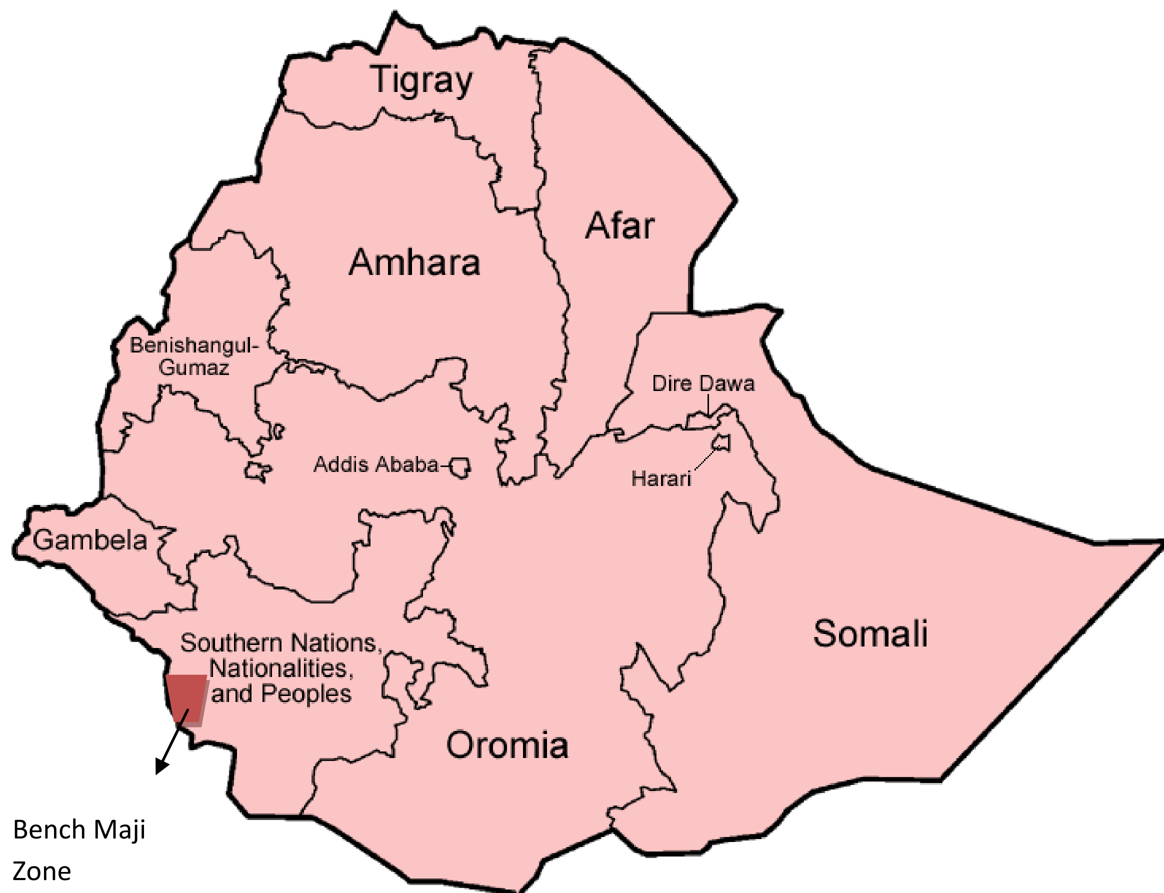

Figure 2 Map of Ethiopia with administrative regions. The study areas, Bench and Sheko district are found in the region called *Southern Nations, Nationalities and Peoples* (SNNP), 560 km South West of the capital Addis Ababa.

The Bench and Sheko people are found in Bench-Maji Zone, which is found in the biodiversity rich south-western part of Ethiopia: Southern Nations, Nationalities and Peoples

Regional State (SNNPR). The people in this area consist of diverse ethnic groups who have their own unique dietary system prepared from various plants (Hildebrand, 2003; Giday, 2007). The majority of the Bench people live in Bench District. The Sheko people inhabit semi-highland and lowland areas of Sheko District.

Bench-Maji Zone is known for its relatively better natural vegetation cover and diverse traditional cultures and languages. Currently, the Zone is divided into nine administrative districts, namely Bench, Shey-Bench, Sheko, Meinit-Goldya, Meinit-Shasha, Gura-Ferda, Maji, Surma and Bero districts. Mizan Teferi (7° 04’ N, 35° 30’ E) is an administrative town for the Zone (Giday, 2007).

According to Demissew and Friis, 2009, the study area falls into the vegetation type moist evergreen montane rainforest and subtypes afromontane rainforest and transitional rainforest. The Afromontane rain forest occurs in the highland areas of south-western Ethiopia at altitudes between 1500 and 2600 m where annual rainfall is between 700 and 1500 mm. The transitional rain forest occurs at altitudes between 500 and 1500 m in areas where annual rainfall is close to 2000 mm. Transitional rain forest is similar to the Afromontane rain forest in its physiognomy and composition with addition of some species from lowland forest (Demissew and Friis, 2009).

1.5 RATIONALE

The reconnaissance survey was done during March, 2010 in Bench and Sheko districts, SW Ethiopia. During the survey, farmers in South-Western Ethiopia informed me that they prefer yams to taro (*Colocasia esculenta*), another root crop cultivated in the area, for consumption but cannot obtain high yields from yam cultivation. This survey revealed that the farmers identify and grow different local varieties of *Dioscorea* species, consume some from the wild

and that domestication of wild yams is still going on in the area. The existence of different local varieties and domestication of wild yams have been reported from Sheko (Hildebrand, 2003a). Different local varieties of yams have also been reported from Bench (Yashu, 2008). However, according to the farmers during survey interview, many varieties have been lost over time due to various constraints of production like disease, reduced rainfall and shortage of stalking due to deforestation. Some young farmers, especially in Bench district are abandoning the tradition of growing yams and replacing them with cash crops; some varieties are only found among the elderly and called *varieties of the elderly* (personal communication; Figure 3). This motivated me to develop a proposal to focus my initial proposal of wild and semi wild edible plants to the cultivated-wild continuum of the yam crop in the area. Another reason is because they are important crops in many parts of the world whose genetics is not very well studied like major crops such as wheat, rice and maize. This presents a gap to apply the genomic techniques developed for other monocot crops to yams and investigate the genome of this monocot, which is a phylogenetically different lineage in relation to grasses, to look at the genome variation within monocots (APG III, 2009; Mignouna *et al*., 2009b).

Figure 3. Different varieties of yam collection by a farmer in a traditional storage. Photo by Kidist Kibret.

To develop new genotypes with ecological adaptation and resistance to pests and diseases, plant breeders will need access to a wide range of diversity. Therefore, a better knowledge of the existing traditional varieties held by farmers is necessary. This is also necessary to conserve the various varieties before they are completely lost and replaced by other crops. Yams are important crops in the context of climate change because the tubers accumulate starch and nutrients during the rainy season and can stay alive for a long time during drought, and then re-grow when they get moisture (Onwueme, 1978; Lebot, 2009). For this reason, morphological, molecular and cytogenetic characterization is needed. *Dioscorea* species

from Ethiopia have been studied morphologically and genetically using AFLP (Tamiru *et al*., 2007; Abebe, 2008) and microsatellite markers (Abebe, 2008). Large scale ploidy screening and DNA content estimations have not been undertaken for *Dioscorea* sp. from Ethiopia and more work is needed to decipher the relationship within the *D. cayenensis* complex and their relation to yams from other parts of the world. Tissue culture techniques have also not been established for Ethiopian yams. This study aims at filling these gaps by analyzing farmers landraces collected from south western Ethiopia.

2. OBJECTIVES

The general objective is to fill gaps in Ethiopian cultivated *Dioscorea* species (*D. alata*, *D. bulbifera* and *D. cayenensis* complex) studies by DNA content estimation, deciphering the relationship within the *D. cayenensis* complex and their relation to yams from West Africa using molecular tools and also establishing tissue culture techniques.

Specific objectives are:

- To estimate nuclear DNA content of *D. alata*, *D. bulbifera* and *D. cayenensis* complex landraces using flow cytometry
- To establish tissue culture techniques for *D. alata*, *D. bulbifera* and *D. cayenensis* complex
- To decipher the relationship within the *D. cayenensis* complex and their relation to other Ethiopian and West African yams using chloroplast SNP markers and including *D. alata* and *D. bulbifera* as outgroups

3. **PLANT MATERIALS USED AND SITE OF COLLECTION**

One of the descriptors recommended to be collected by IPGRI/IITA (1997) is *Passport data* which includes scientific name, local name and place of collection.

Table 1. Code of individual plants and villages of collection. *Kebele is an administrative region below district. #Tree canopy in forest interfered with signal for GPS

Bench District			
Sample codes	**Village**	**Coordinate**	**Altitude (m.a.s.l)**
Be 1-9	Ziamika *Kebele	N6^{0}56.5'; E35^{0}31.6’	1290
Be 10-13	Fanika *Kebele, Jarika area	N06^{0}57’; E35^{0}31’	1338
Be 13-30	Fanika *Kebele around the Dam	N06^{0}57’; E35^{0}30’	1361
Be 31-41	Mashinbaye *Kebele	N06^{0}58’; E35^{0}32.6’	1343
Be 42	Fanika forest	#	#
Sheko District			
Sample codes	**Kebele/goth**	**Coordinate**	**Altitude (m.a.s.l)**
Sh1-12; Sh15-24	Worgu *Kebele, Sheko area	N07^{0}01’; E35^{0}28’	1532
Sh13, 14	Gaizika Forest	N07^{0}02.2’; E35^{0}33’	1515
Sh15-68	Gaizika *Kebele	N07^{0}02’; E35^{0}33’	1570
Sh69-81	Gaizika *Kebele	N07^{0}02.061’; E35^{0}32.83’	1549

The cultivated yam species included in the study are *D. alata*, *D. bulbifera* and a group of yams provisionally placed under *D. cayenensis* complex.

The yam germplasm for this study was provided by the Bench and Sheko ethnic groups, South Western Ethiopia. Over 120 individual tubers were collected in total (Table 2).

Wild inedible *D. bulbifera* (Sh24) and *D. cayenensis* (Sh 13, 14, Be 42) complex were also collected from forest. The *D. cayenensis* complex was planted at Dilla University horticultural site, Southern Ethiopia.

Table 2. List of individual plants included in different studies. Be-from Bench district; Sh-from Sheko district

Morphological characterization	
Species	Individuals
D. cayenensis complex	Be2, Be3, Be4, Be5, Be7, Be34, Be14, Be25, Be30, Be38, Be29, Be40, Sh6, Sh11, Sh15, Sh17, Sh18,Sh19,Sh52, Sh37, Sh38, Sh40, Sh48, Sh49, Sh54, Sh55, Sh61, Sh74, Sh86, Sh54
D. alata	Be35, Be41, Sh1, Sh3, Sh7, Sh19, Sh22, Sh30, Sh43, Sh51, Sh56, Sh60, Sh67, alata 8, alata 10, alata 12, alata 14
D. bulbifera	Sh25c, Sh24e, Sh75d, Sh75a, Sh82d, Sh80d, Bulb, Sh10, Sh80d8, Sh75d8, Be36, Sh76, Be31, Be19, Sh5, Sh25d8
Tissue culture	
Species	Individuals
D. cayenensis complex	Be4, Be14, Sh13, Sh15, Sh52
D. alata	Be 41, alata12, Sh1, Sh3, Sh7
D. bulbifera	Be19, Sh25a, Sh56, Sh57, Sh80
Flow cytometry	
Species	Individuals
D. cayenensis complex	Be3, Be4, Be5, Be14, Be25, Be29, Be34, Be38, Be40, Sh11, Sh18, Sh37, Sh38, Sh48, Sh52, Sh53, Sh54, Sh55, Sh61, Sh86
D. alata	Be12, Be35, Sh7, Sh22, Sh30, Sh43, Sh46, Sh51, Sh56, Sh60, Sh67, Sh91
D. bulbifera	Be37b, Sh24c, Sh24e, Sh75a, , Sh75d, Sh80, Sh82d
Molecular	
Species	Individuals
D. cayenensis complex	Be5, Be29, Be38, Sh11, Sh18, Be40, Sh48, Sh54
D. alata	Be35, Sh19, Sh30, Sh43, Sh60
D. bulbifera	Sh25c, Sh24e, Sh75d, Sh75a, Sh82d, Sh80, bulb

All the collected tubers were not included in the study. Some of them were taken to be grown in glasshouses at the University of Nottingham while the rest were planted at Dilla University

horticultural site, Southern Ethiopia. In addition, not all of the plants grown at University of Nottingham were included in the study. Thus the number codes of the individual plants studied are not continuous (Table 2). All individuals included for molecular study were analyzed for DNA content and their morphology recorded. For each experiment, representatives of species, collection districts and farmers varieties have been included.

4. METHODS

4.1 FIELD BASED STUDY

The sites for study were chosen during the reconnaissance survey in Bench and Sheko districts, SW Ethiopia.

Collection was undertaken to include different varieties recognized by farmers from each species of yam. Methods for the collection of farmers' landraces of these crops were adopted from Christinck *et al*. (2000). Initial information was obtained from local development agents (DAs) because they were actively working with local farmers. Interviews were conducted directly with farmers about farmers who still grow and use traditional landraces. Families having the most representative material and being experts in knowledge were identified by information obtained from interviewing farmers and DAs. These farmers were informed about the purpose of the study and were visited during harvesting time to collect the samples. From each identified yam variety by the farmers, the required tubers for planting were collected. The different farmers' varieties were collected with the hypothesis that they reflect genetic diversity.

Collection was done during a series of field trips between 28 October, 2010 and 22 March, 2011 (Figure 4).

Figure 4. ***D. bulbifera*** **as snack during field work. Photo by Zelalem Ukryans (guide and translator).**

4.2 FLOW CYTOMETRY

4.2.1 Preparation of stock solutions and buffers

Otto I buffer contained 0.1 M citric acid monohydrate and 0.5 % (v/v) Tween 20. Otto II buffer is 0.4M $Na_2HPO_4 .12H_2O$. Propidium Iodide and RNAase stock solutions were prepared as 1 mg/ml and stored in aliquots (Dolezel *et al.* 2007b). Fluorescent beads were used as 1 drop in 1ml water per test.

4.2.2 Setting up the template

First, the flow cytometry software was adjusted so that it detects PI signals. The measurement was changed from logarithmic scale to linear scale as recommended for DNA content measurement. To check instrument linearity, fluorescent beads were used. To check the

procedure, pea nuclei stained with PI were run. Both tests gave acceptable CVs of less than 3%. Nuclei of the standard tomato stained with PI were used to adjust the gain of the instrument so that the histogram of tomato would be the centre of the x-axis. Keeping this gain constant, the histogram at this point was recognized as tomato nuclei and sample histograms during analysis appeared either before or after the tomato histogram depending on the content of DNA.

4.2.3 Standardization

In the case of DNA content estimation, the relative fluorescence is converted into absolute DNA content by including an internal standard whose DNA content is known. An ideal DNA reference standard should have DNA content close to the target species. The ratio between the 2C-value of an analyzed plant and the internal standard should be small to minimize the potential linearity of flow cytometer measurements. However, at least 12% difference in 2C-values of the internal standard and sample should be present to exclude bias due to very close or overlapping peaks (Dolezel *et al*., 2007b). *Lycopersicum esculentum*, 2c=1.96 picogram (Dolezel *et al*., 2007b) has been used as a standard for DNA content estimation of various *Dioscorea* species because it yields peaks close enough but not overlapping to peaks of *Dioscorea* species (Obidiegwu *et al*., 2009a).

Flow cytometry has become a popular method for estimation of absolute DNA content or C-value (Dolezel *et al.,* 2007a). However, there are precautions to be taken to avoid artifactual data. In the 1990's, artifactual intraspecific DNA content variation was reported (Price and Johnston 1996; Price *et al*., 1998), which was later proven to be due to polyphenols interfering with DNA staining (Greilhuber, 1998; Noirot *et al*., 2000; Price *et al*., 2000). Later, the inclusion of β-mercaptoethanol and internal standardization was recommended

(Suda, 2004). Since yams contain phenols (Obidiegwu *et al*., 2009a), β-mercaptoethanol was included in this study. Moreover, nuclei of the sample and the standard were isolated simultaneously (internal standardization) during this study so that the staining of sample and standard DNA by PI is affected equally by any other secondary metabolites released from the leaves. Thus, the errors during calculation of C value based on the standard DNA content are minimized. The other method used is to minimize errors with replication of measurements on different days. This is to take into account the instrument variation on different days, if it exists.

Coefficient of variation (CV) is an attribute of a histogram peak describing its width (expressed as a percentage). It is defined as the standard deviation divided by the mean of a series of fluorescence values, and allows comparing the quality of peaks located on different fluorescence channels. Low CV of about 3 % indicates reliable results. CVs should not exceed 5% (Suda, 2004). Coefficients of variation were ≤ 3% in most analyses and never more than the recommended limit of 5%.

4.2.4 Measurement of DNA content

A simplified two step procedure using Otto I and Otto II buffers as described in Dolezel *et al.* (2007b) was used for the isolation of nuclei from yams. Otto I and Otto II buffers have been used in many studies of yam genome size and ploidy (Dansi *et al*., 2000; Dansi *et al*., 2001; Egesi *et al*., 2002; Obidiegwu *et al*., 2009a; Obidiegwu *et al*., 2009c; Obidiegwu *et al*., 2010). Staining was done using Propidium iodide (PI).

Propidium iodide (PI) and RNase, both 50 µg/ml were added to the Otto II buffer; ß-mercaptoethanol (2 µl/ml) was added to avoid polyphenolics oxidation (Dolezel *et al.* 2007b). Young, healthy and intact but not premature leaves were selected. Approximately 1

mg of leaves of both sample and standard were co-chopped with a new razor blade in a petri dish containing 0.5 ml of ice-cold Otto I buffer. The homogenate was filtered through 20µm pore size nylon mesh filters. The filtered solution was incubated at room temperature for 5 minutes. 1 ml of Otto II buffer supplemented with fluorochrome PI, RNAse and ß-mercaptoethanol was added and incubated in the dark for 5 minutes. This is critical because when incubated for more than 5 minutes after addition of the Otto II buffer, nuclei are not stable (Dolezel *et al.* 2007b). Then, the solution was passed and analysed by a Becton Dickinson LSR II flow cytometer. 5000 particles were analyzed. The relative fluorescence emitted from each nucleus, which is proportional to the DNA content, was measured and analyzed using a Becton Dickinson LSR II flow cytometer.

4.2.5 Data analysis

Data was directly transferred and stored from the flow cytometer on a computer and G1 peak mean of the sample and the standard were obtained using the FACSDiva Version 6.1.3 software. Sample 2C DNA content was calculated as follows:

Sample 2C DNA content = [(sample G1 peak mean)/ (*Lycopersicum esculentum* G1 peak mean)] x *Lycopersicum esculentum* 2C DNA content (pg DNA).

Microsoft excel spreadsheets were used to calculate standard deviation, error and make graphs from nuclear DNA content values. Inter and intraspecific DNA content variation was tested using non-hierarchial cluster analysis and linear mixed models (REML) on the GenStat statistical software (v14).

4.3 CHLOROPLAST SINGLE NUCLEOTIDE POLYMORPHISM

4.3.1 Genomic DNA extraction and quantification

The Sigma Plant Miniprep Kit was used to isolate genomic DNA according to the manufacturers' instruction. Elution used 50 µl of water warmed to 65 ^{0}C. Quantification of genomic DNA was with a Nanodrop spectrophotometer and agarose gel electrophoresis (Figure 5).

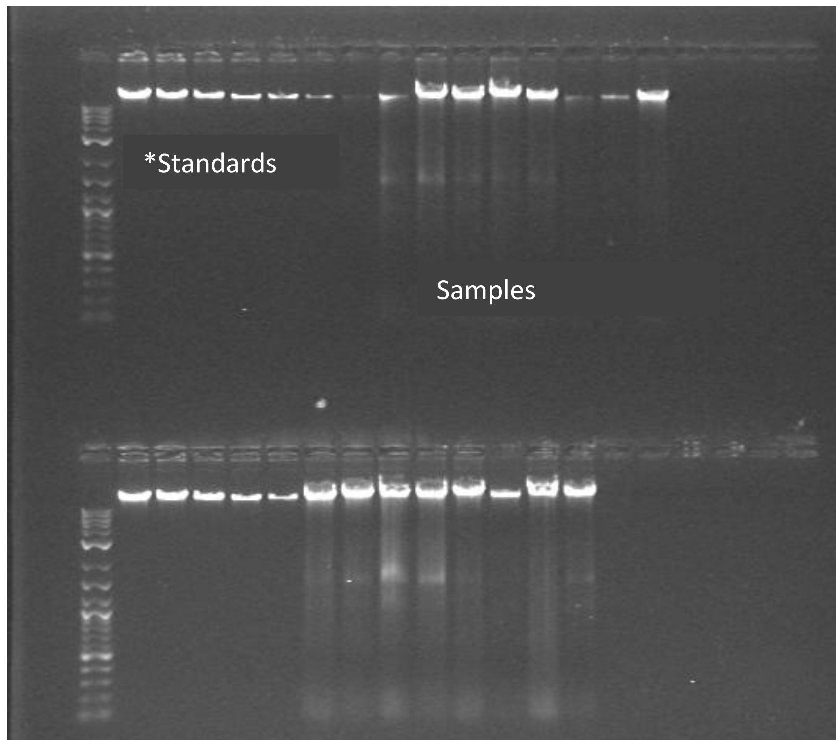

Figure 5. **DNA quantification after extraction with Sigma Plant Miniprep Kit. *Concentration of DNA standards is in the following order: 500, 250, 125, 100, 50 and 25 ng/µl.**

4.3.2 Polymerase chain reaction and sequencing

For PCR reactions, genomic DNA was diluted to 10 ng/µl.

Table 3 Primers used to amplify and sequence different regions of chloroplast (Scarcelli *et al*., 2011a). IGS: inter-genic spacer; IR: inverted repeat; SSC: small single copy region

Name	Location	Type	Forward sequence	Reverse sequence
*rrn*4,5-*trn*N	IR	IGS + Gene	GYCAAGTGGAAGTGCAGTGA	GGTAGAGCGGTYGGCTGTTA
*ycf*1-*rrn*5 Dio	SSC	IGS + Gene	AAAATAGCTCGACGCCAGAA	GGATAATAGATTACCTAGTAGAAAAG
*ndh*H Exon	SSC	Exon	GGACGAATTTTCCATCTCC	CATCAATGCAYGGTGTTCT
ycf-seq-rev	SSC	Sequencing primer	None	GTTCCGTTCCTTCTATTGCATT

Primers for amplification were adopted from Scarcelli *et al*., (2011a) that were used for study of population genetic structure of *D. abyssinica*, *D. praehensilis* and *D. rotundata* (Table 3). Reactions were 20 µl with the amplification profile shown in Table 4.

Table 4 Amplification profile of reactions using the different primers

Name	Initial denaturation	35 cycles of denaturation	Optimized annealing	Elongation	Final elongation
*rrn*4,5-*trn*N	94 ^{0}C, 3 min	94 ^{0}C, 30 sec	62 ^{0}C, 30 sec	72 ^{0}C, 1 min	72 ^{0}C, 10 min
*ycf*1-*rrn*5 Dio	94 ^{0}C, 3 min	94 ^{0}C, 30 sec	59 ^{0}C, 30 sec	72 ^{0}C, 1 min	72 ^{0}C, 10 min
*ndh*H Exon	94 ^{0}C, 3 min	94 ^{0}C, 30 sec	59 ^{0}C, 30 sec	72 ^{0}C, 1 min	72 ^{0}C, 10 min

Amplification (Figure 6) was using the PCR machine Veriti® Thermal Cycler, (Applied Biosystems®).

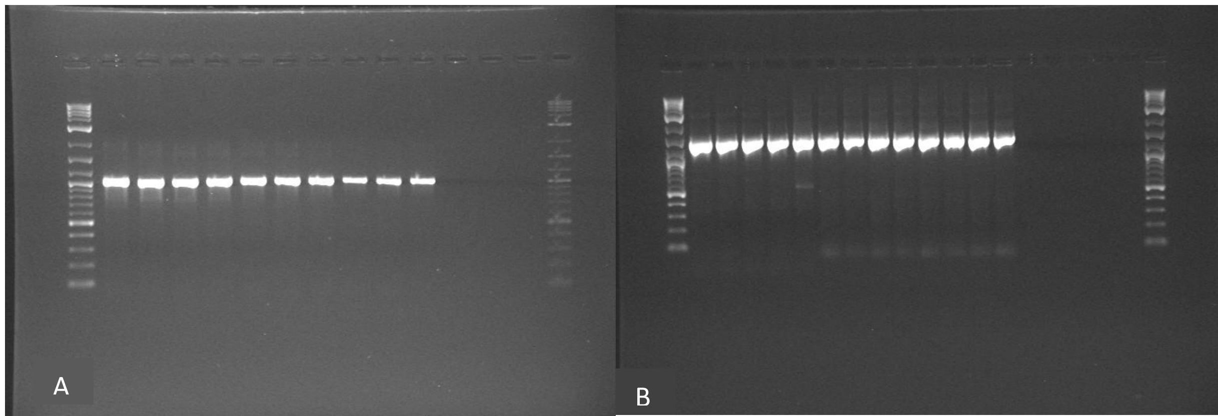

Figure 6 PCR products of A. *ndh*H Exon ~1.0 kb B. rrn4,5-trnN, ~1.5 kb and *ycf*1-*rrn*5 Dio ~1.5 kb

PCR products and primers were sent to *Source BioScience LifeSciences* for sequencing after being diluted according to service provider's specifications. Product of the *ycf*1-*rrn*5 Dio product was sequenced using a primer that gives clear sequence in the region where a SNP differentiating within *D. abyssinica* and *D. praehensilis* Benin yams is located so that it becomes easier to compare *D. abyssinica* and *D. praehensilis* from Benin to yams from Ethiopia.

4.3.3 Analysis of sequences

Sequences were aligned using the CLC genomics workbench (v 4.8). Additional sequences of *D. abyssinica* and *D. praehensilis*, which also include all chlorotypes of *D. rotundata* (Scarcelli *et al.*, 2011a) were downloaded from National Centre for Biotechnology Information (NCBI) through CLC genomics workbench (v 4.8) for comparison with Ethiopian yams.

The quality of sequences was assessed by visually inspecting the chromatogram. Products which showed unacceptable quality were sent for sequencing again. Since the chloroplast genome does not normally recombine, each unique combination of alleles across the chloroplast SNP is scored as a different haplotype. Scarcelli *et al*. (2011a) defined a chlorotype as a combination of SNP located on the chloroplast or a haplotype based on chloroplast SNP. Chlorotypes for Ethiopian yams were scored accordingly. However, the chlorotypes from this study were not fully comparable with Scarcelli *et al*., (2011a) because all of the primers used by their study were not included here.

Distance based, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was constructed using CLC genomics workbench (v4.8) with 1000x bootstrap.

4.4 DESCRIPTORS AND CHARACTERIZATION

IPGRI encourages the collection of data for descriptors on *Passport*, *Management*, *Environment and Site*, *Characterization* (IPGRI/IITA. 1997). This descriptor list is intended to be comprehensive for the descriptors that it contains. This approach assists with the standardization of descriptor definitions. IPGRI does not, however, assume that each curator will characterize accessions of their collection utilizing all descriptors given. Descriptors should be used when they are useful to the curator for management and maintenance of the collection and/or to the users of the plant genetic resources (IPGRI/IITA. 1997).

Passport has been given in section 3.

Management, *Environment and Site*: The underground tubers of *D. alata* and *D. cayenensis* and aerial tubers of *D. bulbifera* were grown in two types of glasshouses at University of Nottingham. Temperature controlled glasshouse and temperature and light duration

controlled glasshouse (Future Crop Glasshouse). The temperature was maintained at 25-30^{0}C for all glasshouses. Day-light length was 16 hour per day for the plants grown in Future Crop Glasshouse. Few *D. alata* and some *D. bulbifera* plants were grown in a 12 hour day-length glasshouse but *D. cayenensis* were not included due to lack of space. Planting was done in pots, except for FutureCrop where planting was directly into soil pits. The soil for pots was 50% compost and 50% sand. Watering was done every day for potted plants and trickle irrigation used for the Future Crop Glasshouse. Potted plants were supplied nutrient solution after a few months of growth. Plants in all glasshouses were sprayed with pesticides and fungicides as required.

Characterization descriptors: These enable an easy and quick discrimination between phenotypes. They are generally highly heritable, can be easily seen by the eye and are equally expressed in all environments. In addition, these may include a limited number of additional traits thought desirable by a consensus of users of the crop (IPGRI/IITA, 1997). Morphological characters of the plants grown in glasshouses at University of Nottingham were recorded according to basic list of descriptors for edible *Dioscorea* species described in IPGRI/IITA (1997), with some additions from the complete list of descriptors. For the most important edible yams, minimum highly discriminating descriptors for characterization are given in a basic list of descriptors for edible *Dioscorea* species described in IPGRI/IITA (1997) to assist curators to detect duplicates in large collections. However the completion of other additional descriptors that are relevant is recommended (IPGRI/IITA. 1997).

For *D. cayenensis-rotundata,* the following descriptors were added to fit the Ethiopian samples in the *D. cayenensis* species complex. Descriptor number 7.1.9 (presence/absence of spines) was added because some Ethiopian samples have. Descriptor number 7.2.9 (position of leaves) was added because Ethiopian samples are alternate at base/opposite above, which

is also mentioned in Miege and Demissew (1997). Since differences were observed, 7.1.18 (mature stem colour) was included. Some descriptors do not differentiate between Ethiopian samples, but were still included. For instance, descriptor number 7.2.12 (mature leaf type simple/compound) is not relevant to Ethiopian samples, and the *D. cayenensis* complex from Ethiopia were reported to have simple leaves (Meige and Demisew, 1997). The descriptor 7.1.40 (colour of spot at spine base) was excluded because no spot was observed at base of spine. None of the *D. alata* samples have spines so descriptors 7.1.34 and 7.1.35 are not applicable. No additional descriptors were needed for *D. alata* and *D. bulbifera*.

Data for flowers, fruits and seeds were not recorded because *D. alata* and *D. bulbifera* never flowered and flower buds of the *D. cayenensis* complex died before opening. Data for underground tubers were not recorded because they are not yet harvested. Descriptors that need measurements were not included due to time constraint.

4.5 TISSUE CULTURE

Three types of media were used: Murashige and Skoog medium (MS), MS with 10 g/L charcoal and Woody Plant medium (WP) with 10 g/L charcoal. Charcoal was added to avoid the negative effect of phenols on plant growth.

Nodal and meristem explants were collected in plastic bag from glasshouse. The explants were sterilized in 10% bleach for about 20 to 30 minutes. The bleach was discarded and the explant rinsed three times with sterile water. The explants were cut into about 1-2 cm length with one or two nodes and inserted into solidified media. These were stored in growth chamber at 25^0C.

5. RESULTS

5.1 FIELD BASED STUDIES

This section is the result of field based observations and discussions with farmers. One of the constraints of yam production in south western Ethiopia is stalks for support of stems. When a tuber is planted, a short cane is put as support for the small emerging vines. When it starts to grow, a longer support is needed. Some varieties of *D. cayenensis* complex and *D. bulbifera* with excessive vine growth are planted under trees. In some areas in south western Ethiopia, aerial tubers of *D. bulbifera* are deliberately scattered in the forest and harvested for consumption when they grow.

My study areas involved remote villages up to 5 hours walk from the main road in Bench and Sheko districts, South Western Ethiopia. In my study areas, wild plants have been transformed to semi-wild plants, and they grow among coffee and corn, while completely wild plants also grow in patches of forests nearby. Thus the folk classifications categorized as wild are in the forest containing wild coffee, and are used as a snack when people go to the forest to harvest coffee beans or honey. Tubers with acceptable quality are taken to be planted in the home-garden. What farmers describe as domestic are the tubers planted in home-garden (personal communication with farmers).

In the villages along the Sheko-Tepi road, farmers told me the following story about domestication: '*When people go to the forest to manage coffee plants or harvest honey, and*

they find a wild yam growing very well, they plant it in home-garden. Some time ago, part of the forest was cleared for planting maize, then the wild yams regrew in the corn field amongst the corn. When people go to cultivate maize and find a good yam variety, they plant it in home-garden'.

A farmer from Bench district told me the following story during my field work: '*My father told me that a long time ago; our forefathers went to the forest to look for liana to build huts. Then they saw the stem of wild yam and cut it for building the house. They were hungry and noticed the tuber. They roasted and ate it. That is how yam domestication began. People started consuming wild yams while working in the forest and brought tubers home for planting.*' and took me to a nearby forest where we found remains of a wild yam tuber which had been consumed by people working in the forest (Figure 7). This has been planted at the yam conservation site in Southern Ethiopia.

Figure 7. Digging wild yam tuber by farmers in Bench district. Photo by Kidist Kibret

Farmers in Sheko mention one of the reasons to domesticate yams is to introduce variation into the domestic yams. Young farmers have reduced the rate of domestication because they think there is enough variation among the yams in the home-gardens. Yam production is more important in Sheko district than in Bench district; many young Sheko farmers plant the crop and having diverse yam varieties is considered a privilege.

5.2 FLOW CYTOMETRY

Histograms were obtained from flow cytometric FACSDiva Version 6.1.3 software (Figure 8). Coefficients of variation were $\leq$ 4% in most analyses and never more than the recommended limit of 5% (Suda, 2004). Nuclei isolated from plant tissues and stained usually emit two distinct peaks of fluorescence, one corresponding to G_0 + G_1 phase cells (with 2C DNA content) and the other G_2 + M phase cells (with a 4C DNA content). The latter produces twice the fluorescence, as they contain twice the DNA content. Nuclei from some tissues display only one peak, e.g. non-dividing nuclei subject to a G_1 developmental cell cycle block form a single peak at the 2C DNA value (Suda, 2004). In total, 42 individuals were measured for DNA content. Of these, 13 were *D. alata*, 21 were *D. cayenensis* complex and 7 were *D. bulbifera*. In total, 67 analyses were done, including the replications. Mean and standard deviations were computed using Microsoft excel (Table 5, Figure 9).

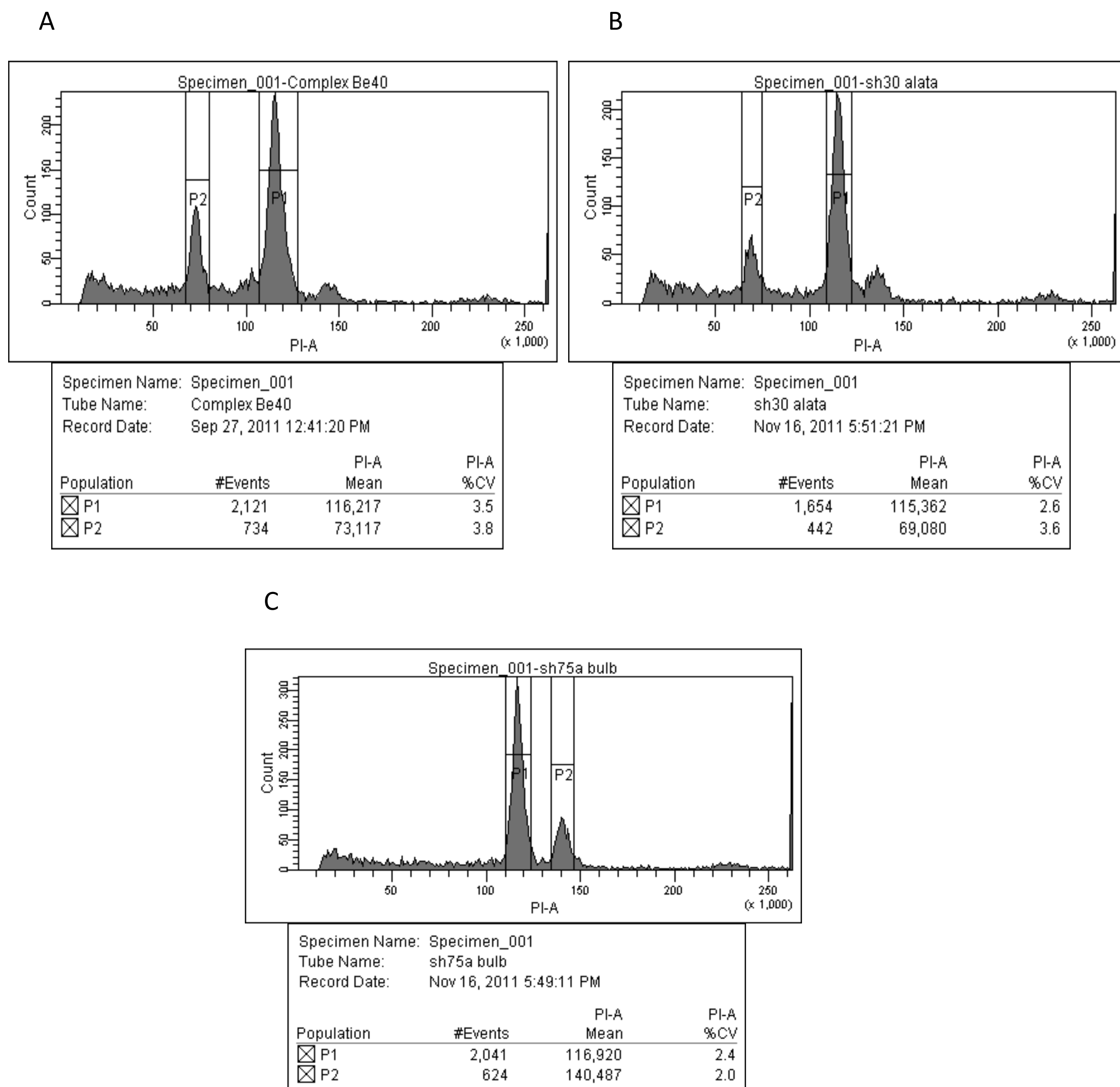

Figure 8. Typical histogram output of FACSDiva Version 6.1.3 software from flow cytometric analysis of different species of yams. P1 is tomato G1 nuclei peak whose position was pre-set during setting up the template and kept constant for all analyses. P2 is the test sample G1 nuclei: 2C DNA content of A. *D. cayenensis* complex (1.275 ± 0.02, 4x) and B. *D. alata* (1.153 ± 0.02, 4x) are lower than tomato (2C= 1.96 pg) and C. *D. bulbifera* (2.374 ± 0.02, 6x) is greater than tomato.

Table 5. DNA content of individual plants. *Mean nuclear DNA content and standard deviations are given only for individual plants measured with replication.

Code	Species	2C value (pg)	Standard deviation within individual
Be12	*D. alata*	1.12	0
Sh46	*D. alata*	1.125	0
Sh22	*D. alata*	1.13	0
Sh 43	*D. alata*	1.14	0
Sh91	*D. alata*	1.14	0
Sh 67	*D. alata*	1.15	0
Sh7	*D. alata*	1.16	0
Sh56	*D. alata* with 2 replications	1.160*	0.057
Sh51	*D. alata*	1.166	0
Sh30	*D. alata*	1.168	0
Be 35	*D. alata* with 2 replications	1.170*	0
Sh43	*D. alata* with 2 replications	1.176*	0.023
Sh60	*D. alata* with 2 replications	1.180*	0.028
Be40	*D. cayenensis*	1.233	0
Be29	*D. cayenensis*	1.247	0
Be38	*D. cayenensis*	1.248	0
Sh 54	*D. cayenensis* with 4 replications	1.249*	0.012
Be 5	*D. cayenensis* with 2 replications	1.254*	0.028
Sh 18	*D. cayenensis* with 2 replications	1.258*	0.003
Be 14	*D. cayenensis* with 4 replications	1.265*	0.01
Sh54	*D. cayenensis* with 3 replications	1.266*	0.031
Sh61	*D. cayenensis*	1.27	0
Sh48	*D. cayenensis* with 2 replications	1.248*	0.002
Sh52	*D. cayenensis*	1.28	0
Sh53	*D. cayenensis*	1.286	0
Sh11	*D. cayenensis* with 3 replications	1.288*	0.014
Be34	*D. cayenensis*	1.288	0
Be3	*D. cayenensis* with 3 replications	1.290*	0
Be4	*D. cayenensis* with 4 replications	1.287*	0.015
Sh37	*D. cayenensis*	1.29	0
Sh38	*D. cayenensis*	1.3	0
Sh55	*D. cayenensis*	1.3	0
Sh86	*D. cayenensis*	1.3	0
Be25	*D. cayenensis* with 3 replications	1.301*	0.022
Sh24e	*D. bulbifera*	2.334	0
Sh75a	*D. bulbifera*	2.35	0
Sh82d	*D. bulbifera*	2.378	0
Sh24c	*D. bulbifera* with 3 replications	2.379*	0.016
Be37b	*D. bulbifera*	2.38	0
Sh80	*D. bulbifera*	2.4	0
Sh75d	*D. bulbifera*	2.4	0

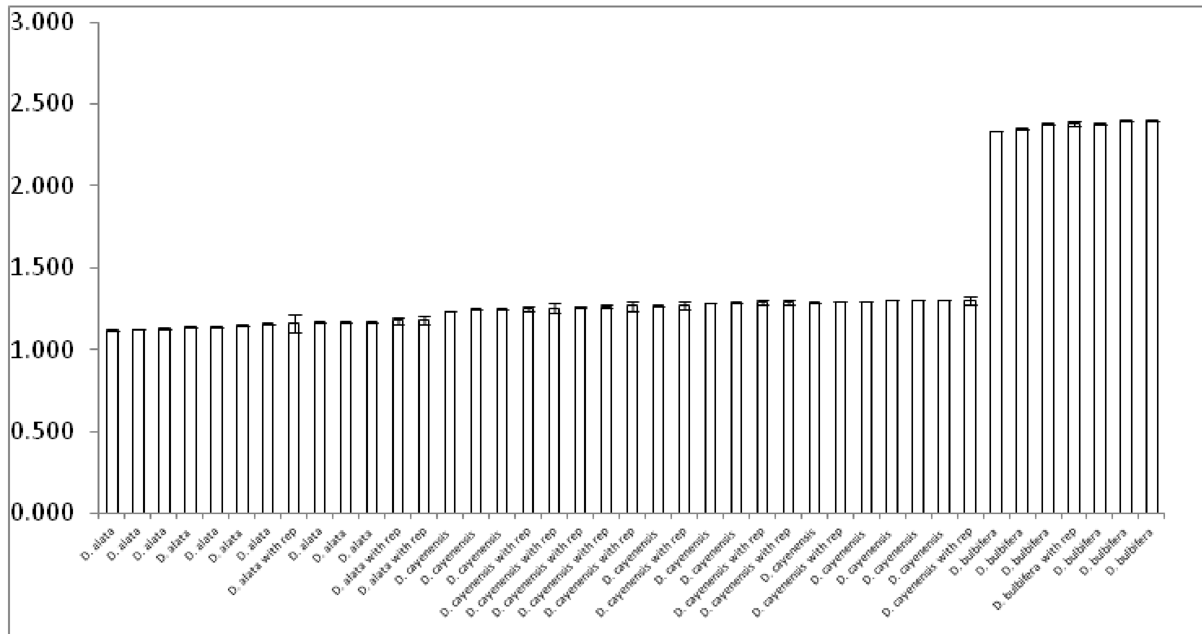

Figure 9. 2C DNA content ± SD of individual plants from table 5. Mean nuclear DNA content is given for individual plants measured with replication (with rep).

The raw data of all the 67 analyses were grouped using non-hierarchial clustering in GenStat with the assumption that the data contains three groups. This gave output by grouping the data according to species. Non-hierarchial clustering GenStat output gave three class means correlating to each species with similar values to the overall mean for each species from excel data (Table 6).

Table 6. Overall mean 2C DNA content and class mean values of each species.

Species	Overall mean 2C DNA content of each species (computed from table 6 values)	Class mean values from non hierarchical clustering, GenStat output
D. alata	1.153 ± 0.02	1.156
D. cayenensis complex	1.275 ± 0.02	1.276
D. bulbifera	2.374 ± 0.02	2.378

Non-hierarchial cluster analysis forms a grouping of a set of units into a predetermined number of groups, using an iterative algorithm that optimizes a chosen criterion, starting from an initial classification, in this case three groups. However, the optimal grouping was 7 suggesting that intraspecific DNA content detection was not reliable and could have been masked by technical error.

Intraspecific DNA content variation was further tested using linear mixed models (REML). The Response Variable was DNA content, Fixed Model was species and Random Model was individual plants. The random model is used to describe those factors for which the values present in an experiment can be considered drawn from some large homogenous population. Linear mixed models correctly computed estimates and standard errors even when observations cluster under higher entities. Since the residual variance estimate (0.000425) was greater than estimated variance components (0.0001251), this shows that technical error variance as slightly higher than intraspecific difference, meaning that intraspecific variation could not be inferred from the current data.

C-values calculated from overall mean 2C DNA content and converted to mega base pair (Mbp) are given in Table 7.

Table 7. C-value calculated from overall mean 2C DNA content. 1pg = 980 Mbp (Bennett and Leitch 2010).

Species	C-value (pg)	C-value (Mbp)
D. alata	0.576	564
D. cayenensis complex	0.637	624
D. bulbifera	1.187	1163

5.3 ANALYSIS OF SEQUENCES

Sequences aligned are shown in Figures 10-12. In total, 5 individuals of *D. alata* and *D. bulbifera*, 6 individuals of *D. cayenensis* complex from Ethiopia, sequences of two *D. praehensilis* and two *D. abyssinica* downloaded from NCBI were included in the analysis.

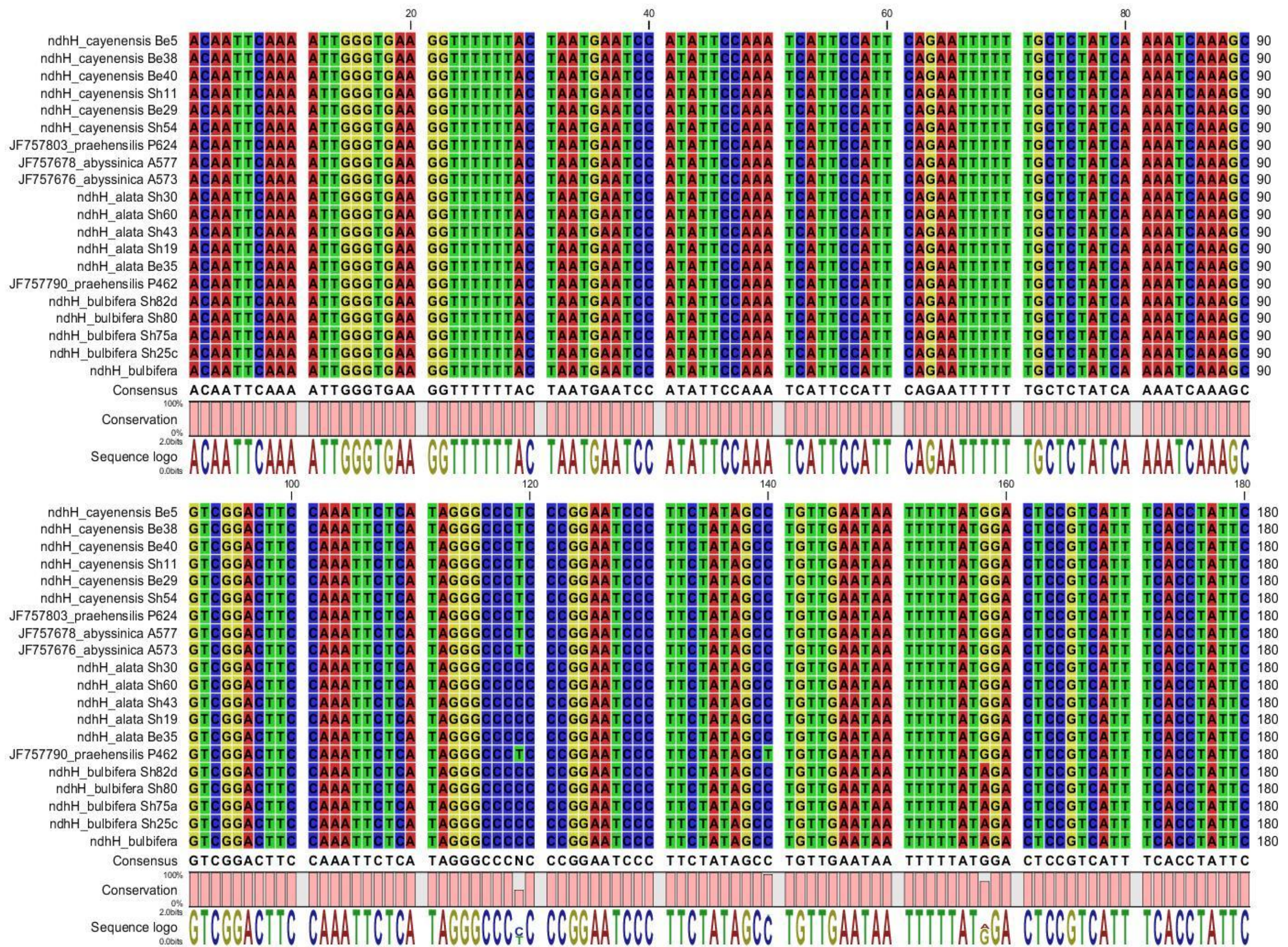

Figure 10. Alignment of sequences from *ndhH-Exon* primer. Labels contain part of primer name or NCBI accession number followed by species name and individual plant code.

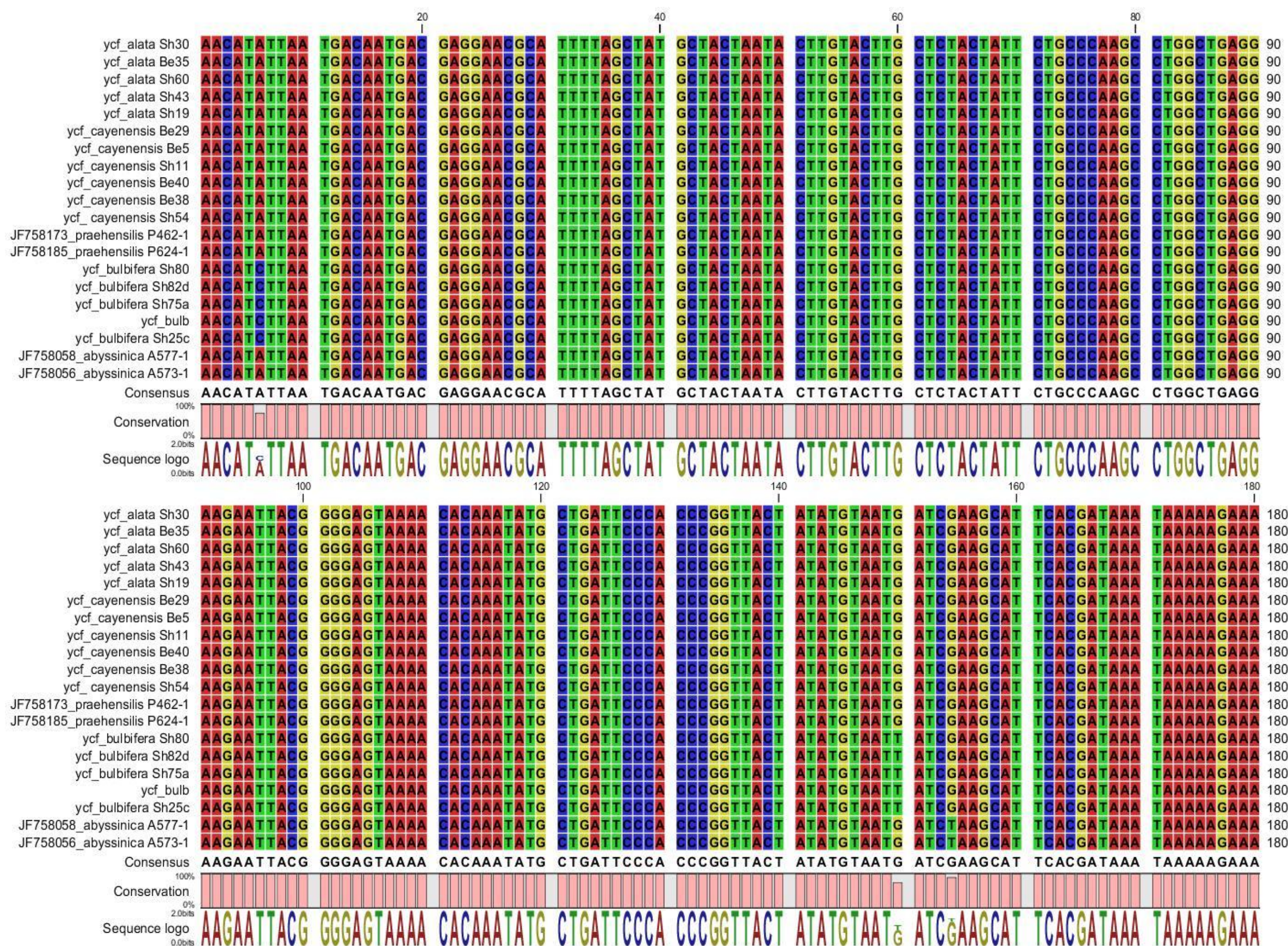

Figure 11. Alignment of sequences from *ycf1-rrn5* primer. Labels contain part of primer name or NCBI accession number followed by species name and individual plant code.

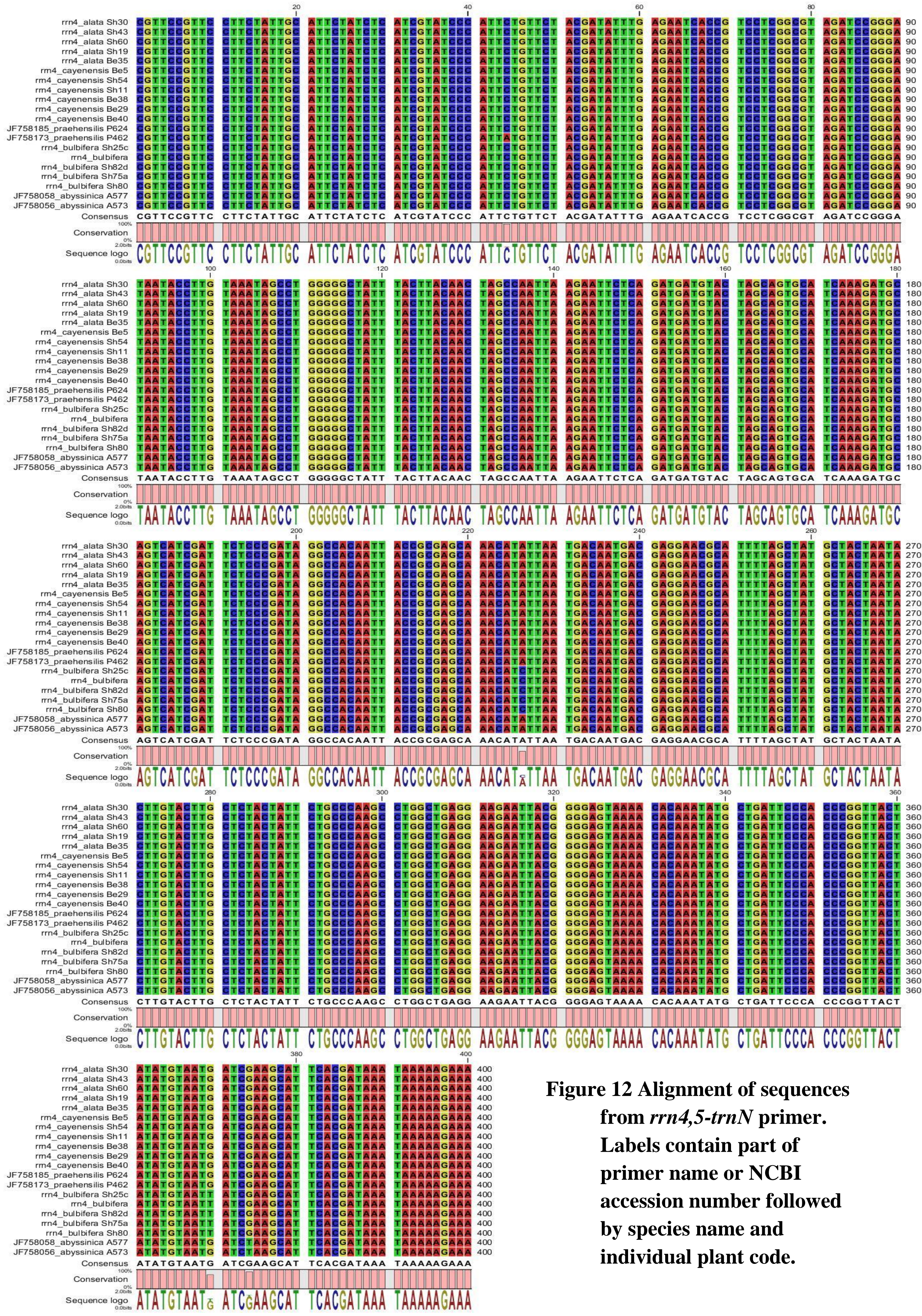

Figure 12 Alignment of sequences from *rrn4,5-trnN* primer. Labels contain part of primer name or NCBI accession number followed by species name and individual plant code.

Table 8. Alleles

ndhH		ycf		rrn4	
1	TCG	1	AGTGG	1	CAGG
2	CCG	2	CGTTG	2	CCTG
3	TTG	3	AGTGT	3	AAGG
4	CCA			4	CAGT

Table 9 Chlorotypes

			Primers			
Species	Individual	ycf	ndhH	rrn4	Chlorotype	Source of sequence and locality of plants
cayenensis	Be29	1	1	1	**1**	This study, SW Ethiopia
cayenensis	Be38	1	1	1	**1**	This study, SW Ethiopia
cayenensis	Be5	1	1	1	**1**	This study, SW Ethiopia
cayenensis	Sh11	1	1	1	**1**	This study, SW Ethiopia
cayenensis	Be40	1	1	1	**1**	This study, SW Ethiopia
cayenensis	Sh54	1	1	1	**1**	This study, SW Ethiopia
alata	Sh30	1	2	1	**2**	This study, SW Ethiopia
alata	Sh60	1	2	1	**2**	This study, SW Ethiopia
alata	Sh43	1	2	1	**2**	This study, SW Ethiopia
alata	Sh19	1	2	1	**2**	This study, SW Ethiopia
alata	Be35	1	2	1	**2**	This study, SW Ethiopia
bulbifera	Sh80	2	4	2	**3**	This study, SW Ethiopia
bulbifera	Sh82d	2	4	2	**3**	This study, SW Ethiopia
bulbifera	bulb	2	4	2	**3**	This study, SW Ethiopia
bulbifera	Sh25C	2	4	2	**3**	This study, SW Ethiopia
bulbifera	Sh75a	2	4	2	**3**	This study, SW Ethiopia
praehensilis	P624	1	1	1	**1**	Scarcelli *et al* (2011a), Benin, West Africa
praehensilis	P462	1	3	3	**4**	Scarcelli *et al* (2011a), Benin, West Africa
abyssinica	A577	3	1	4	**5**	Scarcelli *et al* (2011a), Benin, West Africa
abyssinica	A573	3	1	4	**5**	Scarcelli *et al* (2011a), Benin, West Africa

Genotyping was done based on single nucleotide polymorphism from the alignment of sequences (Table 9).

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used to construct phenetic tree from combined alignment of sequences from the three primers, *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN. D. bulbifera* split was supported by high bootstrap value of 99.3% from Enantiophyllum subgenus (Figure 13).

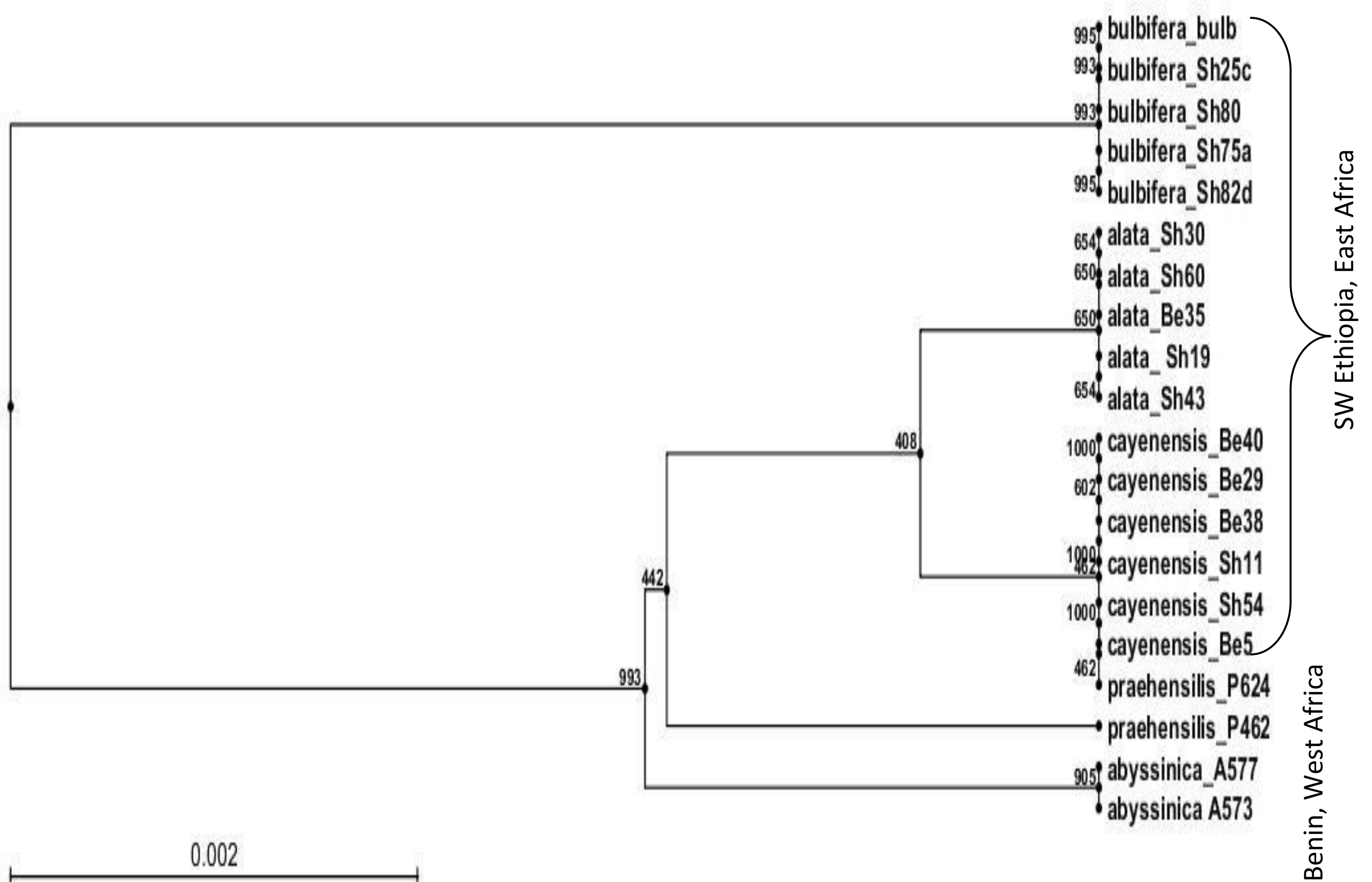

Figure 13. UPGMA tree constructed from combined analysis of aligned sequences from the three primers, *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN.*

5.4 MORPHOLOGICAL CHARACTERIZATION

5.4.1 *D. cayenensis* complex

Of the descriptors included in the study, mature leaf types are all simple, spine shapes are all straight and no samples have coalescent spines. All samples have spines on the stem only, except Sh52, which has a few spines on the petioles in addition to the ones on the stem (Figure 14). The other differentiating descriptors were young and mature stem colour, young and mature leaf colour, mature stem waxiness and presence/absence of spines (Table 10).

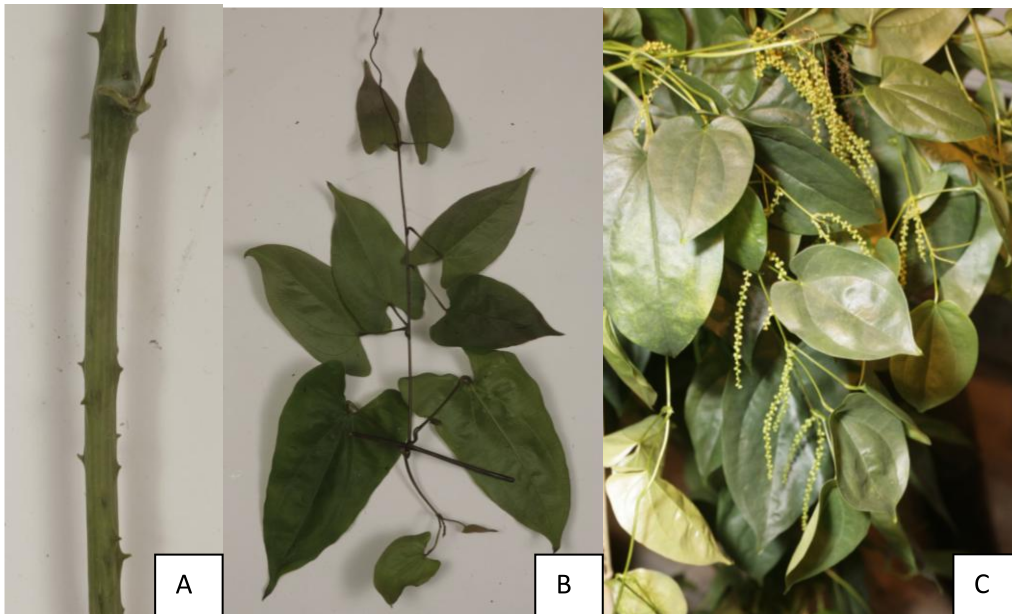

Figure 14. Morphologies used as descriptors for *D. cayenensis* complex. A. Be4 spine, B. Sh11 brown stem, C. Sh48 green stem and flower buds

Table 10. Characterization of *D. cayenensis* complex. Description of descriptor numbers: 7.1.4 Young stem colour; 7.1.18 Mature stem colour; 7.1.24 Mature stem waxiness; 7.1.9 Presence/absence of spines; 7.1.36 Mature stem spine position; 7.1.37 Mature stem spine shape; 7.1.39 Mature stem coalescent spines; 7.2.12 Mature leaf type; 7.2.15 Mature leaf colour; 0 absent, 1 present; NA not applicable

Code	7.1.4	7.1.18	7.1.24	7.1.9	7.1.36	7.1.37	7.1.39	7.2.12	7.2.15
Be14	Dark brown	Green	1	1	Stem	Straight	0	Simple	Green
Sh52	Green	Brownish green	1	1	Stem & petiole	Straight	0	Simple	Dark green
Sh37	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Be4	Green	Green	1	1	Stem	Straight	0	Simple	Green
Sh61	Dark brown	Dark brown	0	1	Stem	Straight	0	Simple	Green
Be34	Dark brown	Dark brown	0	1	Stem	Straight	0	Simple	Green
Be3	Dark brown	Dark brown	0	1	Stem	Straight	0	Simple	Green
Be25	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Sh15	Brownish green	Green	0	0	NA	NA	NA	Simple	Green
Sh86	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Be2	Dark brown	Dark brown	0	1	Stem	Straight	0	Simple	Green
Sh37	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Sh74	Dark brown	Dark brown	0	1	Stem	Straight	0	Simple	Green
Sh38	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Sh40	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Sh6	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Be7	Dark brown	Purple	0	0	NA	NA	NA	Simple	Green
Sh19	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Sh49	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Be30	Green	Green	0	1	Stem	Straight	0	Simple	Green
Sh17	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Sh55	Dark brown	Green	0	1	Stem	Straight	0	Simple	Green
Sh48	Green	Brownish green	1	0	NA	NA	NA	Simple	Green
Be29	Green	Brownish green	0	0	NA	NA	NA	Simple	Green
Sh11	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Be38	Dark brown	Dark brown	0	0	NA	NA	NA	Simple	Green
Be40	Green	Brownish green	0	1	Stem	Straight	0	Simple	Green
Sh18	Green	Brownish green	0	0	NA	NA	NA	Simple	Green
Be5	Green	Brownish green	1	0	NA	NA	NA	Simple	Green
Sh54	Green	Green	0	0	NA	NA	NA	Simple	Green

5.4.2 *D. alata*

When descriptors involving measurement are excluded, the ones differentiating between the *D. alata* mostly involve colour differences of different parts. Spines and aerial tubers are absent in all the samples (Table 11, Figure 15).

Table 11. Characterization of *D. alata.* Description of descriptor numbers: 7.2.3 Young leaf colour; 7.2.4 Young leaf margin colour; 7.2.56 Young leaf vein colour 7.2.6 Young leaf petiole colour; 7.2.7 Young leaf petiole wing colour; 7.2.15 Mature leaf-colour; 7.2.16 Mature leaf-vein colour; 7.2.18 Mature leaf margin; 7.2.37 Mature leaf- petiole colour; 7.2.38 Mature leaf- petiole wing colour; GP-Green with purple edges; PG-all green with purple at both ends

Code	7.2.3	7.2.4	7.2.5	7.2.6	7.2.7	7.2.15	7.2.16	7.2.18	7.2.37	7.2.38
Sh43	purple	purple	purple	purplish green	purple	purplish green	purple	purple	green	purple
Sh19	purple	purple	Green	PG	purple	Green	green	green	PG	purple
Sh3	green	green	Green	green	green	Green	green	green	green	GP
Sh1	purple	purple	purple	purplish green	purple	Green	green	green	Purplish green	GP
Sh67	green	green	Green	green	green	Green	green	green	green	GP
Sh56	green	green	Green	green	green	Green	green	green	green	GP
Sh7	purple	purple	purple	green	purple	Green	green	green	green	GP
Sh51	purple	purple	purple	purplish green	purple	Green	green	green	green	GP
Sh22	green	green	Green	green	green	Green	green	green	green	GP
Be41	green	green	Green	green	green	Green	green	green	green	GP
a8	green	green	Green	green	green	Green	green	green	green	GP
a10	green	green	Green	green	green	Green	green	green	green	GP
a12	green	green	Green	green	purple	Green	green	green	green	GP
a14	green	green	Green	green	purple	Green	green	green	green	GP
Be35	green	green	Green	green	green	Green	green	green	green	GP
Sh30	green	green	Green	green	green	Green	green	green	green	GP
Sh60	green	green	Green	green	green	Green	green	green	green	GP

Figure 15. Morphologies used as descriptors for *D. alata.* A. Sh19 young shoot, purple leaf, purple spotted shoot, B. Sh19 mature shoot, green leaf C. Sh43 young shoot, D. Sh43 mature shoot, E. Sh60 young and mature shoot with green stem and wing with purple tip

5.4.3 *D. bulbifera*

Among the descriptors used, aerial tuber colour was differentiating within this species (Table 12). Vigour could only be assessed in a uniform environment so it was not possible to compare between different glasshouses. The colour of the young and mature stem, leaf, stem and petiole were green for every individual. The aerial tubers have different shapes but all these are irregular (Figure 16). Although not mentioned in the basic descriptor of IPGRI/IITA

(1997) and not quantitatively measured, farmers' varieties classified them as ones having small and big tubers. Moreover, these size differences were observed when grown in the same glasshouse.

Table 12. Characterization of *D. bulbifera.*

Code	Aerial tuber flesh colour (7.5.8)
Sh25c	Yellowish
Sh75d	White with purple
Sh75a	White with purple
Sh82d	Yellowish
Sh80d	Yellowish
Bulb	Yellowish
Sh10	White with purple
Sh80d8	White with purple
Sh75d8	White with purple
Be36	White with purple
Sh76	Purple with white
Be31	Yellowish
Be19	White with purple
Sh5	White with purple
Sh25d8	Yellowish

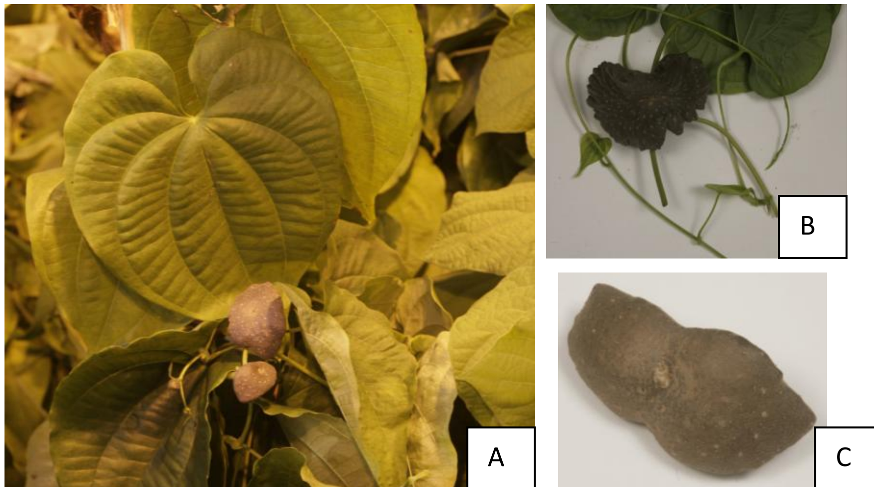

Figure 16. Different shapes of *D. bulbifera* aerial tubers which are all irregular. A. Sh24c, wild B. Sh82, variety with big tuber. C. typical shape of most edible varieties

5.5 TISSUE CULTURE

The explants with continued growth and which form complete root and shoots were followed up for 5 months as shown in Table 13. All three species were able to root, shoot and produced aerial tubers without the use of growth regulators (Table 14). The highest proportion of plants from all three species grew in woody plant medium with charcoal (Table 14)

Table 13. Growth of different yam species on different growth media. MS-Murashige and Skoog medium without charcoal; WPC-woody plant medium with charcoal; MSC-Murashige and Skoog medium with charcoal

Species	Code of individual	Medium	Growth period
D. alata	alata12	MS	root and shoot in 1 month
	Sh3	MS or WPC	shoot and rooted in 1 month
	Sh7	WPC	shoot and rooted in 1 month
	Be 41	MSC	shoot and rooted in 1 month
	Sh1	MSC	shoot and rooted in 1 month
D. bulbifera	Sh80	MSC	aerial bulb in 10-12 days; Root, shoot in 2 months
	Sh25a	MS	aerial bulb in 13 days; Root, shoot in 1 month
	Be19	MS	only aerial bulb and shoot, no root
	Sh56	WPC	aerial bulb in 13 days; Root, shoot in 1 month
	Sh57	MS	aerial bulb13 days; shoot and rooted in 4 month
D. cayenensis complex	Be4	MSC	shoot and rooted in 1 month
	Be4	MS	None
	Sh15	MS & WPC	shoot and rooted in 1 month
	Be14	MS	shoot and rooted in 4 month
	Be14	MSC	shoot and rooted in 1 month
	Sh52	MS	shoot and rooted in 2 months
	Sh13	MS	shoot and rooted in 4 month

Table 14. Proportion of plants grown in different media.

Species	Media	Percentage
D. alata	MS	60
	WPC	69.2
	MSC	37.5
D. bulbifera	MS	33.3
	WPC	60
	MSC	25
D. cayenensis complex	MS	30.8
	WPC	50
	MSC	44.5

6. DISCUSSION

6.1 FIELD BASED STUDIES

It has been previously reported that Sheko farmers from South Western Ethiopia take wild yams and grow them in home-gardens and the folk classifications involve location as wild and domestic rather than specific morphological characters (Hildebrand, 2003a, b; 2009). The study of Hildebrand (2003a) included a completely remote village near a natural forest (personal communication with farmers who assisted Hildebrand). My study areas involved remote villages up to 5 hours walk from the main road in Bench and Sheko districts, South Western Ethiopia. In my study areas, wild plants have been transformed to semi-wild plants, and they grow among coffee and corn, while completely wild plants also grow in patches of forests nearby.

Farmers in Sheko mention one of the reasons to domesticate yams is to introduce variation into the domestic yams. Interestingly, the similar aim of domestication to increase variety of yams in cultivation is practised among farmers in Benin (Dumont *et al.*, 2006). Yam production is more important in Sheko district than in Bench district; many young Sheko farmers plant the crop and having diverse yam varieties is considered a privilege (personal observation). This could explain the highest genetic diversity found in Sheko population by microsatellite study of Abebe, 2008. Cultural reasons for domestication give an insight into the importance of yam cultivation. A story about domestication from Guraferda, SW Ethiopia mentions a child eating yams from the forest and growing faster than the other children (Hildebrand, 2003a), indicating the nutritional value of yams.

6.2 FLOW CYTOMETRY

The DNA content of Ethiopian yams has not been investigated before. The current study estimates of that the 2C DNA content is 1.153 ± 0.02 for *D. alata,* which is close to the report of 2C=1.15 ± 0.008 by Obidiegwu *et al.,* (2009). The estimate from this current study of 2C =1.275 ± 0.02 for *D. cayenensis* complex is comparable to the DNA content 2C=1.25 that was reported for the tetraploid *D. abyssinica, D. praehensilis* and *D. cayenensis-rotundata* complex in the angiosperm C-value database (Hamon *et al*., 1992; Bennett and Leitch 2010) rather than 2C=1.53 ± 0.11 reported for *D. rotundata* (Obidiegwu *et al.* 2009). The estimate of 2C=2.374 ± 0.02 for *D. bulbifera* from this study is close to *D. bulbifera* reported to be 2C=2.4 ± 0.022 (Obidiegwu *et al.,* 2009). Differences in inter-laboratory DNA amount estimates are not significantly different when Propidium Iodide (PI), a DNA intercalating dye, and laser equipped flow cytometer are used according to Dolezel *et al*.(1998;, Hamon *et al*. (1992); Obidiegwu *et al*. (2009))

Flow cytometry inferred ploidy by comparison of current estimates to published literature suggests *D. cayenensis* complex to be tetraploid (Obidiegwu *et al.* 2009; Bennett and Leitch 2010), *D. alata* to be a tetraploid (Obidiegwu *et al* 2009) and *D. bulbifera* to be a hexaploid (Obidiegwu *et al* 2009). However, chromosome counting of representative samples is needed to validate the ploidy levels inferred from the flow cytometric analysis of this study.

The *D. cayenensis* complex is a provisional name for the set of sub-Saharan yam species whose taxonomic relations are currently being examined: *D. cayenensis, D. rotundata, D. abyssinica, D. prehaensilis* and *D. sagittifolia* (Wilkin, 2001; Hildebrand, 2003). Cultivated Ethiopian yams belonging to this group are not clearly differentiated into species but suggested to be single taxonomic entity of wild-managed populations of the *D. cayenensis*

species complex (Abebe, 2008) with domestication still going on in the area (Hildebrand, 2003). The estimate from this study, 2C =1.275 ± 0.02 for the of wild-managed population *D. cayenensis* complex is closer to previous reports of 2C=1.25 for *D. abyssinica, D. praehensilis* and *D. cayenensis-rotunda*, rather than 2C=1.53 ± 0.11 reported for *D. rotundata* cultivated in Nigeria. Further confirmation using chromosome counts and segregation of marker loci are needed to confirm the ploidy levels.

The 2C-values of *Dioscorea* species in the angiosperm database range from 0.95 to 13.50 pg (Bennett and Leitch, 2010). C-values of *D. alata* and the *D. cayenensis* complex are very small and that of *D. bulbifera* is small compared across all land plants (Leitch *et al*., 2005). The 2C DNA content estimation of *D. alata*, *D. bulbifera*, *D. dumetorum*, *D. esculenta* and *D. rotundata* ranged from 0.702 ± 0.004 pg for G_1 nuclei of diploid *D. dumetorum* to 2.573 ± 0.020 pg for G_1 nuclei of octoploid *D. cayenensis* (Obidiegwu *et al*., 2009a). The highest 2C value from the genus reported is that of *D. elephantipes* (2C= 13.50) (Table 15) although this order is poorly represented in terms of C-value data (Leitch *et al*., 2010)

Table 15. 2C-values of *Dioscorea* species reported. *Recorded in the angiosperm C-value database (Bennett and Leitch, 2010). 1 pg ≈ 980 Mbp (Bennett and Leitch, 2010).

Species	Ploidy level	2C (pg)	1 C (Mbp)	Original Reference
D. togoensis	4	0.95*	465	Hamon *et al*., 1992
D. alata	4	1.15*	562	Arumuganathan and Earle, 1991
D. abyssinica	4	1.25*	611	Hamon *et al*., 1992
D. mangenotiana	4	1.25*	611	Hamon *et al*., 1992
D. praehensilis	4	1.25*	611	Hamon *et al*., 1992
D. cayenensis-rotundata	4	1.25*	611	Hamon *et al*., 1992
D. sylvatica	Not mentioned	1.70*	831	Bharathan *et al*., 1994
D. cayenensis-rotundata	6	1.75*	856	Hamon *et al*., 1992
D. alata	8	2.12*	1037	Dansi *et al*., 2005
D. cayenensis-rotundata	8	2.60*	1271	Hamon *et al*., 1992
D. villosa	Not mentioned	4.80*	2347	Bharathan *et al*., 1994
D. elephantipes	Not mentioned	13.50*	6602	Zonneveld *et al*., 2005
D. dumetorum	2	0.70 ± 0.004	343	Obidiegwu *et al*. (2009)
D. dumetorum	3	1.06	519	Obidiegwu *et al*. (2009)
D. alata	4	1.15 ± 0.008	563	Obidiegwu *et al*. (2009)
D. alata	8	1.96	960	Obidiegwu *et al*. (2009)
D. rotundata	4	1.426 ± 0.008	698	Obidiegwu *et al*. (2009)
D. cayenensis	6	1.53 ± 0.110	749	Obidiegwu *et al*. (2009)
D. cayenensis	8	2.57 ± 0.020	1259	Obidiegwu *et al*. (2009)
D. esculenta	4	2.10 ± 0.230	1029	Obidiegwu *et al*. (2009)
D. bulbifera	4	2.40 ± 0.022	1176	Obidiegwu *et al*. (2009)

Many studies involving chromosome counts and flow cytometric ploidy analysis of various *Dioscorea* species estimate ploidy levels based on a basic chromosome number of 10 or 9, with different ploidy levels observed even within the same species (Gamiette *et al*., 1999; Dansi *et al*., 2000; Dansi *et al*., 2001; Egesi *et al*., 2002; Obidiegwu *et al*., 2009c; Obidiegwu *et al*., 2010). For instance, the majority of cultivated *D. cayenensis* complex were found to be

tetraploid, some hexaploid, a few octoploid as well as a mixploid individual (Obidiegwu *et al*., 2009c). Tetraploids, hexaploid and octoploids were found during flow cytometric analysis of ploidy levels among *D. alata* (Obidiegwu *et al*., 2010). In another study, ploidy level analysis of *D. alata* showed that the majority of plants were hexaploid with a smaller percentage of tetraploids (Egesi *et al*., 2002). Extra chromosomes have been also been reported in studies involving chromosome counts (Gamiette *et al*., 1999; Dansi *et al*., 2005).

In segregating populations of *D. rotundata*, the AFLP markers segregated in the same patterns as diploids ($2n=2x=40$) (Mignouna *et al*., 2002). Segregation analysis using isozyme and microsatellite markers led to the conclusion that *D. rotundata* is a diploid species (Scarcelli *et al*., 2005). Microsatellite segregation analysis provided the genetic evidence to establish the diploidy of *D. alata* plants with $2n = 2x = 40$ chromosomes and to support the hypothesis that plants with $2n = 40$, 60 and 80 chromosomes are diploids, triploids and tetraploids, respectively, suggesting that the basic chromosome number of *D. alata* is 20 (Arnau *et al*., 2009).

The small DNA content possessed by many of the *Dioscorea* species and recent indications of higher basic chromosome numbers and reduced ploidy levels makes it ideal for sequencing the genome and it could become a model organism to represent a different lineage to grasses within monocots. Even if polypoidy exists, the recent advent of techniques to sequence polyploid genomes (Bancroft *et al*., 2010) could make genome sequencing feasible in the future.

All families in Dioscoreales have small to very small chromosomes and high levels of polyploidy with up to 2*n* = 140 having been reported in Dioscoreaceae but the order Dioscoreales is poorly represented by DNA content data (Leitch *et al*., 2010). Given the high levels of polyploidy, it was assumed by Leitch *et al*. (2010) that the upper limit of C value for the order is unlikely to be much larger than that of *D. elephantipes*. Whether *Dioscorea* species are recent or ancient polyploids has not been studied yet. The fact that DNA content within the genus does not increase in direct proportion to ploidy level and detection of tetrasomic behaviour suggests that they could well be ancient polyploids that have undergone diploidization. Polyploidy-induced differential elimination of genome specific sequences can facilitate homologous chromosome pairing (Shaked *et al*., 2001; Liu and Wendel, 2002). Logically, polyploids should have higher C- values than diploids, with the C- values of polyploids increasing in direct proportion to ploidal level. This expectation holds true in synthetic polyploids and newly formed polyploids (Soltis *et al*, 2003). However, Leitch and Bennet (2004) found that the mean 1C DNA amounts tended to decrease with increasing ploidal level. This shows potential DNA loss following polyploid formation. It could be explained at least partly by homoeologous recombination after intraspecific hybridization leading to DNA loss (Leitch and Bennett, 2004).

6.3 ANALYSIS OF SEQUENCES

Amplification of *D. alata* using primers *ndhH-Exon, ycf1-rrn5 and rrn4,5-trnN* and the use of these primers for genetic diversity studies of *D. alata* and *D. bulbifera* has been carried out in the current study for the first time. This study demonstrated that SNPs revealed by sequencing with *ndhH-Exon, ycf1-rrn5 and rrn4,5-trnN* can successfully be used to study not only the diversity of the crop-wild relatives' complex of *Dioscorea* but also *D. alata* and *D. bulbifera*. Scarcelli *et al*., (2011a) tested amplification of *ccsA-Exon, ccsA-ndhD, psbD-Exon,*

ndhH-Exon, ycf1-rrn5 and rrn4,5-trnN on *D. abyssinica*, *D. praehensilis*, *D. rotundata*, *D. dumetorum*, *D. bulbifera* and used them to study population genetic structure of closely related *D. abyssinica*, *D. praehensilis*, *D. rotundata*. Here we present not only successful amplification with primers *ndhH-Exon, ycf1-rrn5 and rrn4,5-trnN* on *D. alata* but also the use of the primers to identify SNPs from it. This study also demonstrated that the primers to identify SNPs from and *D. bulbifera*.

In addition to sequences generated in his study, *Dioscorea* species sequences from *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN* available on NCBI were included in the alignment. Sequences of *D. abyssinica* and *D. praehensilis* from *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN* (Scarcelli *et al*., 2011a) were aligned to sequences generated from this study.

The two SNPs (***G→T*** and ***C→A***) revealed by *rrn4,5-trnN* and ***C→T*** revealed by *ndhH-Exon* (Scarcelli *et al*., 2011a) were also found in this study. ***C→A***, *rrn4,5-trnN* differentiated one P462, *D. praehensilis* (***A***) from the rest of sequences aligned. In the case of ***G→T***, *rrn4,5-trnN,* ***G*** is unique to *D. abyssinica* (Scarcelli *et al*., 2011a), while the rest are ***T***. Additional SNPs were revealed by this study. ***G→T***, *rrn4,5-trnN* with ***T*** unique to *D. bulbifera*; ***C→A***, *rrn4,5-trnN*, ***C*** unique to *D. bulbifera*. In case of ***C→T*** revealed by *ndhH-Exon* (Scarcelli *et al*., 2011a) ***T*** was unique to P462, *D. praehensilis* while the rest of sequences in the current study had ***C***. Additional SNPs revealed by the current study were ***C→T***, *ndhH-Exon* with ***C*** unique to *D. alata* and *D. bulbifera*, ***T*** unique to *D. cayenensis* complex from Ethiopia and *D. praehensilis* and *D. abyssinica* from Benin. In case of the change ***A→G***, *ndhH-Exon* ***A*** is unique to *D. bulbifera* while the rest of the samples had ***G***. SNPs already reported by Scarcelli *et al*. (2011a) and new in this study are shown in Table 16

Table 16. SNPs found in this study. *Also found in Scarcelli *et al*. (2011a)

Primers	Change, Position (n^{th} nucleotide on alignment)			
ndhH-Exon	***C→T***, 119	****C→T***, 140	***A→G***, 158	
rrn4,5-trnN	****C→A,*** 44	***C→A,*** 226	***G→T,*** 370	****G→T,*** 374
ycf1-rrn5	***C→A***, 6	***G→T***, 150	***G→T***, 154	

Scarcelli *et al*., (2011a) defined a chlorotype as a combination of SNP located on the chloroplast or a haplotype based on chloroplast SNP. Using regions of chloroplast genome amplified by primers *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN* in this study, five chlorotypes were found from SNPs including the SNPs from Scarcelli *et al*. (2011a). However, the chlorotypes from this study were not fully comparable with Scarcelli *et al*., (2011a) because all of the primers used by their study were not included here. Based on five primers, namely, *ccsA-Exon, ccsA-ndhD, psbD-Exon, ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN*, Scarcelli *et al*., (2011a) found five chlorotypes within *D. rotundata*, *D. abyssinica* and *D. praehensilis*. Out of these five primers three of them, namely *ndhH-Exon, ycf1-rrn5* and *rrn4,5-trnN* were used in this study and chlorotypes defined in this study were based on the three primer products. Among samples included in this study, all *D. cayenensis* complex individuals contained chlorotype 1, all *D. alata* contained chlorotype 2 and all *D. bulbifera* contained chlorotype 3.

One individual of West African *D. praehensilis* contained the chlorotype 1 in this study, the same as Ethiopian *D. cayenensis* complex. Both West African *D. abyssinica* contain chlorotype 5 in this study. Among chlorotypes defined by Scarcelli *et al*., (2011a), chlorotype 1 was found in some individuals from each species of *D. rotundata*, *D. abyssinica* and *D. praehensilis*. Some individuals of *D. abyssinica* had chlorotype 2 and some chlorotype 3. Chlorotypes 4 and 5 were specific to *D. praehensilis* (Scarcelli *et al*., 2011a). The occurrence

of an individual from *D. praehensilis* with the same chlorotype as samples of the*D. cayenensis* complex in this study, the occurrence of *D. praehensilis* and *D. abyssinica* with the same chlorotypes as *D. rotundata* in Scarcelli *et al*., (2011a) has also been reported earlier. Using chloroplast DNA simple sequence repeats (cpSSR) it was found that *D. rotundata* and *D. cayenensis* had the same chlorotypes as *D. praehensilis* and some morphotypes of *D. abyssinica* (Chair *et al*., 2005). In another study, RFLP analysis of chloroplast DNA revealed that two cultivated species (*D. rotundata* and *D. cayenensis*) display the same chloroplast genome type, type A, as the three wild species *D. praehensilis*, *D. liebrechtsiana* and *D. abyssinica* (Terauchi, *et al*., 1992).

Using UPGMA analysis of aligned sequences, *D. bulbifera* was separated from the rest of the species included in this study and was supported by high bootstrap values of 99.3 %. This is congruent with the phylogenetic study of the genus *Dioscorea* using *matK* and *rbcL* genes and morphological characters which placed *D. bulbifera* and *D. alata* in different clades. *D. bulbifera* fell into compound leaved clade while *D. alata* fell into the Enantiophyllum subclade (Wilkin *et al*., 2005). However, the *D. cayenensis–D. rotundata* complex, *D. praehensilis* and *D. abyssinica* were not included in the phylogenetic analysis of Wilkin *et al*. (2005). In this study, *D. alata* and *D. cayenensis* from Ethiopia were closely related but supported with only a 40.8% bootstrap value. Taxonomically, *D. alata, D. cayenensis-D. rotundata* complex, *D. abyssinica* and *D. praehensilis* are placed in the same subgenus Enantiophyllum while *D. bulbifera* is placed in Opsophyton (Onueme, 1978). The *D. cayenensis* complex and *D. alata* share were domesticated from wild yams belonging to the same subgenus Enantiophyllum, the *D. cayenensis* complex was domesticated in Africa and *D. alata* in Asia (Dumont *et al*., 2006). The *D. cayenensis* complex taxa, *D. abyssinica* and *D. praehensilis* did not form a discrete cluster from *D. alata* so this needs further

investigation by sequencing the west African yams rather than aligning the sequences from NCBI.

6.4 MORPHOLOGICAL CHARACTERIZATION

Morphological characters of the plants grown in glasshouses at University of Nottingham were recorded according to the basic list of descriptors for edible *Dioscorea* species described in IPGRI/IITA (1997), with some modifications where needed. However, not all characters were recorded such as underground tubers and flowers. The variation in vegetative parts is similar to criteria used to differentiate different varieties by farmers (Hildebrand, 2003a; Tamiru, 2006; Yashu, 2008; personal communication with farmers).

Previously, morphology of different varieties of yams from Ethiopia were studied from the farmer's point of view. A study conducted in Gedeo, Sidama, Wolayta and Gamo-Gofa Zones in Southern Ethiopia by Tamiru (2006) revealed farmers in the area recognized 37 yam landraces on their farm. However, only 5 of these varieties could be correlated with a well defined species, i.e. *D. bulbifera*. The remaining landraces form a yet unidentified species or group of species. Hildebrand (2003a) estimated there are at least 23 folk taxa of yams classified by the Sheko and Dizi people in SW Ethiopia. Yashu (2008) recorded 30 local varieties classified by the Bench people. Yashu (2008) recorded 30 local varieties classified by the Bench people but no attempt was made to correlate these to scientific taxonomy during his study.

Sheko and Bench farmers have morphological criteria for differentiating different yam groups and subgroups, many of which are similar to scientific classification in Meige and Demisew (1997). Relationships between scientific and folk taxonomy of *Dioscorea* species classified by the Sheko and Dizi people in SW Ethiopia have been found by Hildebrand

(2003a). Many of the characters that farmers use to differentiate different varieties are similar to the yam descriptors developed by IPGRI/IITA (1997), and these characters have been reported to show clear genetic inheritance (IPGRI/IITA 1997). According to Yashu, (2008), Yasind/Karkabat/Shah boy is the name given to the wild yam with Ongubai characterized by its angled stem and Oake known by its aerial tuber, based on folk taxonomy. When compared to descriptions of species from the flora of Ethiopia, the description Yasind/Karkabat/Shah boy matches the wild *D. cayenensis* complex, Oake *D. bulbifera* and Ongubai, *D. alata* (Meige and Demisew, 1997; Table 16; Figure 13).

Table 16. Correlation of folk taxonomy from Bench district with scientific name of yams

Species (Meige and Demisew, 1997)	**Folk taxonomy major group described by Yashu, (2008)**	**Folk taxonomy subgroups described by Yashu, (2008)**
D. cayenensis complex	Boy	Banda Boy, Tsid Boy, Don boy, Tsenah
D. cayenensis complex	Chebsha	
D. cayenensis complex	Tolubay	
D. cayenensis complex	Yasind /Karkabat	
D. bulbifera (Cultivated)	Oake	Don (Neep) Oake and Tiab Oake
D. bulbifera (Wild)	Oake	Balakay Oake
D. alata	Ongubay	Ongubay dal, Ongubay zenkuru

The farmers in Southern and South Western Ethiopia classify yam varieties according to the absence or presence of spines, leaf size, colour and shape, maturity time, vigour, canopy size, presence or absence of toxicity, number of harvests, tolerance to drought, pests and diseases, tuber taste and colour. Bench farmers identify the major group *Oake* by its aerial tubers and *Ongubay* by its angular stem, which are similar morphological criteria to the scientific classification. *Ongubay* is also characterized as an early maturing yam. For the *Oake*

subgroups, *Tiab Oake* is the edible variety which produces large aerial tubers and *Balakay Oake* is the wild yam with toxic non-edible tubers. Members of the major group '*Boy*' are the *D. cayenensis* complex. According to the farmers, the *Boy* sub-group is differentiated by the morphology, colour of the tuber and some culinary properties and taste. Farmers referred to wild yams as *Yasind/Karkabat* during the field work for this study. They also characterize yams as flowering and those that never flower. For example, *Ongubai* is classified as a yam that never flowers and the wild *Yasind/Karkabat* as one that flowers and gives seeds (Personal communication; Hildebrand 2003a; Tamiru, 2006; Yashu 2008).

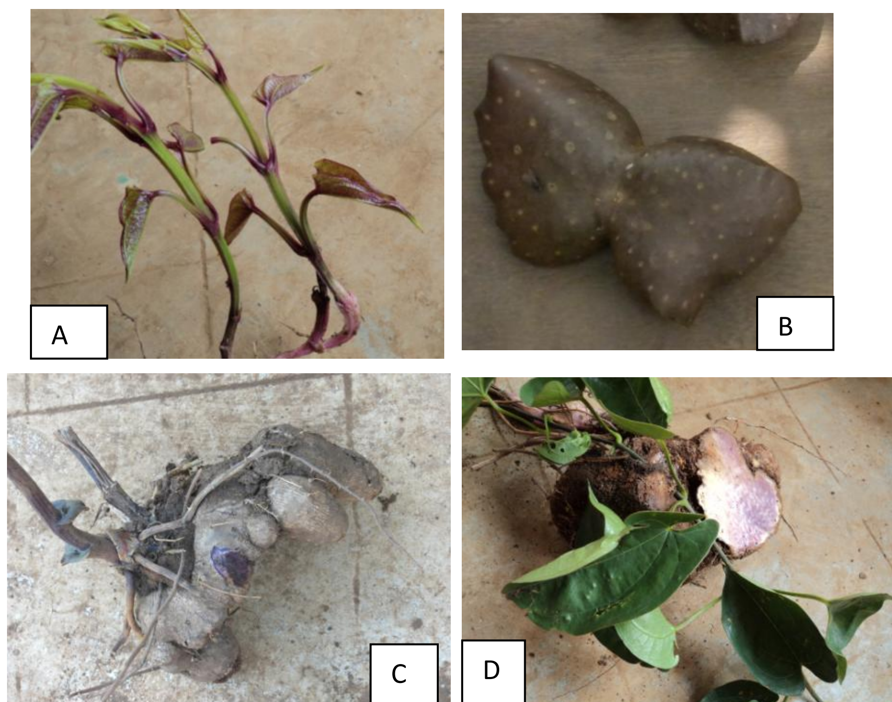

Figure 17. Pictures of yams taken during field trips after identification of local names by farmers. A. a variety of *D. alata* with angled stem and red pigment (local name *Ongubay zenkuru*, sheko). B. Aerial bulb of edible *D. bulbifera* (local name *Ama*, Sheko; Oak, Bench) C. *D. cayenensis* complex with purple stem and purple tuber (local name, *tsidboy/tsana*). D. *D. cayenensis* complex with white and purple variegated tuber, green leaf and green stem, local name Surukachi (Sheko). Photo by Kidist Kibret.

The completion of all relevant descriptors is recommended. All descriptors of IPGRI/IITA (1997) need to be investigated in the future to either be included or modified according to the Ethiopian samples. Additional characters from other morphological observations would also be useful. In addition to the IPGRI/IITA descriptors (1997), other references need to be used in order to decide the characters to be investigated and to gather sufficient morphological data to be correlated to molecular studies. Characters affected by environment should also be investigated. This would be important for future variety development and breeding of the crop.

6.5 TISSUE CULTURE

In this study, it was found that in addition to MS medium, woody plant medium with charcoal can also be used to grow yams in culture. All three species showed better and faster growth in woody plant medium with charcoal when compared to MS medium. Charcoal was added to avoid the negative effect of phenols on plant growth as yams are known to contain phenols. Previously, MS medium, Murashige and Skoog (1962) has been recommended for the tissue culture of yams (Mignouna *et al*., 2009a). MS medium with different combinations of growth hormones has been used successfully for *D. alata* (Adeniyi *et al*., 2008; Royero *et al*., 2010) and *D. rotundata* (Adeniyi *et al*., 2008). For *D. bulbifera*, activated charcoal has been used with MS medium (Narula *et al*., 2007).

In previous studies (Adeniyi *et al*., 2008; Royero *et al*., 2010), *Dioscorea* species were induced to grow roots and aerial tubers using growth regulators. Here, they are reported to grow roots and aerial bulbs without growth regulators, with the highest proportion being in woody plant medium with charcoal. The growth of *D. bulbifera* aerial tubers in culture has commercial importance to produce diosgenin, which can be made into various steroids

(Narula *et al*., 2007). Microtubers produced with tissue culture techniques have the potential to be used for propagation and conservation of yams (Balogun, 2009).

6.6 MULTIPLE APPROACHES TO STUDY GENETIC DIVERSITY OF CULTIVATED YAMS FROM SW ETHIOPIA

The major cultivated species of yams in SW Ethiopia are *D. alata*, *D. bulbifera* and the *D. cayenensis* complex. The genetic diversity of these species, especially the *D. cayenensis* complex needs to be studied for improvement as well as conservation. There is taxonomic confusion regarding *D. rotundata* and *D. cayenensis* which are domesticated from wild yams native to Africa. In Nigeria, the two are clearly differentiated as *D. rotundata* being the white yam and *D. cayenensis* the yellow yam by farmers (Dumont *et al*., 2006). Taxonomically, it was not possible to separate the *D. cayenensis* and *D. rotundata* from Ethiopia as there are many intermediates between the two species and thus are treated as a species complex under the name of the *D. cayenensis* complex (Miege and Demissew, 1997), which were found to consist of wild and managed populations of a single taxonomic entity using AFLP and microsatellite markers (Abebe, 2008) and found to be genetically distinct from West African yams (Tamiru *et al*., 2007).

Chloroplast sequence, DNA content and morphological observations were used to assess the diversity of cultivated and managed yams from SW Ethiopia. DNA amount is an important biodiversity character in monocots (Bennett and Leitch 2000), although data is scarce for *Dioscorea* species (Bennett and Leitch 2010). The DNA content of *D. alata* and *D. bulbifera* from SW Ethiopia is comparable to those from West Africa. This supports the theory that *D. alata* was introduced into Ethiopia, and *D. bulbifera* is taxonomically similar to those from the rest of Africa. DNA content of the *D. cayenensis* complex is closer to the wild *D.*

praehensilis and *D. abyssinica* yams whose DNA content has been measured under the name of the *D. cayenensis–D. rotundata* complex, the name given to yams not clearly differentiated as either *D. cayenensis* or *D. rotundata* but definitely not *D. alata* or *D. bulbifera* (Hildebrand, 2003a; Dumont *et al*., 2006). This supports the Ethiopian *D. cayenensis* complex to be single taxonomic entity of wild and managed yams.

One individual of *D. praehensilis* from West Africa had the same chlorotype as the Ethiopian *D. cayenensis* complex. The other West African *D. praehensilis* and both *D. abyssinica* accessions have different chlorotypes. Given the problem of clearly differentiating between the morphology of *D. abyssinica, D. praehensilis,* the *D. cayenensis/rotundata* complex, the *D. praehensilis* with same chlorotype as the Ethiopian *D. cayenensis* complex needs further investigation whether these two individuals are actually from the same taxonomic entity. Farmers in West and Central Africa classify wild yams as 'the ones that can be domesticated' and 'the ones that cannot be domesticated'. Domestication involves taking wild yams with well developed vegetative organs and small and bitter tubers from shaded forest and planting them in fertile soil with organic matter and no shade. The farmers report the domesticated yams will have shorter vegetative parts and large tubers with improved taste after a few years of domestication (Dumont *et al*., 2006). Farmers in SW Ethiopia also have criteria for selecting 'the ones that can be domesticated' and further screen them by growing them in the homegarden for a few years. The ones that 'look like domestic yams' through time are retained and 'the ones that continue to look like wild yam' are discarded (personal communication with farmers). Chair *et al*., (2005) reported that the yams under domestication, the *D. praehensilis* and the *D. rotundata-cayenensis* complex and some morphotypes of *D. abyssinica* had the same chlorotypes. Whether these are the same

taxonomic entity with different gene expression in different environments or heritable genetic or epigenetic variation as a result of change in environment needs to be investigated.

As Scarcelli *et al*., (2011a) did not include *D. alata* and *D. bulbifera* in the analysis, comparison between Ethiopian and West African *D. alata* and *D. bulbifera* was not possible. Additional SNPs to the report of (Scarcelli *et al*., 2011a) were revealed through analysis of yams from Ethiopia. To further investigate the diversity revealed by chloroplast sequence diversity, more regions of the chloroplast genome should be included in future studies.

Morphometric observations suggest that the Ethiopian *D. cayenensis* complex cannot be defined by the IPGRI/IITA (1997) basic list of descriptors for *cayenensis/rotundata*. For instance, presence/absence of spines on stem had to be added before 'shape of spine' because some of the Ethiopian samples have spines and some do not The descriptor 'opposite/alternate' is not applicable because some of the Ethiopian samples have both opposite and alternate leaves on the same plant. Further investigation of all characters is needed, including flowers if possible.

7. CONCLUSION AND RECOMMENDATION

DNA amount and chlorotypes show that Ethiopian *D. cayenensis* complex are same taxonomic entity as certain morphotypes of *D. praehensilis* and *D. abyssinica*. Farmers in West, Central and East Africa domesticate wild yams 'that can be domesticated' by changing the environment from forest to fertile land in homegardens and fields. These wild yams are transformed within 2-3 years from small, bitter tuber to big and tasty tuber. This change involves phenomena other than genetic variation because many studies have shown certain morphotypes of *D. praehensilis* and *D. abyssinica* have same chlorotypes as *D. cayenensis/rotundata* complex suggesting that they are same taxonomic entity. On the other hand, some morphotypes of *D. praehensilis* and *D. abyssinica* have different chlorotypes than *D. cayenensis/rotundata* complex. Whether these are the 'ones that cannot be domesticated' and are different taxonomic entity needs further investigation.

The following recommendations are made for future work with yam chloroplast marker and cytogenetics:

- Additional primers are needed to relate between *D. abyssinica, D. praehensilis,* and *D. cayenensis/rotundata* complex
- Extra Ethiopian taxa need to be included to relate between Ethiopian and other African *D. cayenensis/rotundata* complex
- More sequences of *Dioscorea* spp. from other countries need to be included to compare with Ethiopian

- This study involved Sheko population from south western Ethiopia. Other populations from Ethiopia need to be included
- Relation of morphology, genetic diversity and the cause of change in morphology during domestication need further investigation
- Chromosome count of a representative individual is needed to relate DNA amount with chromosome number.

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