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Genetic Diversity Assessment of Yams (*Dioscorea* spp.) from Ethiopia Using Inter Simple Sequence Repeat (ISSR) Markers

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This is to certify that the thesis prepared by Kedra Mohammed, entitled: Genetic Diversity Assessment of Yams (*Dioscorea* spp.) collected from Ethiopia Using Inter Simple Sequence Repeat (ISSR) Markers and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology complies with the regulations of the University and meets the standard with respect to originality and quality.

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ABSTRACT

Genetic Diversity Assessment of Yams (*Dioscorea* spp.) from Ethiopia Using Inter Simple Sequence Repeat (ISSR) Markers

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The genetic diversity and relationships of 70 accessions of yam belonging to *Dioscorea cayenensis/Dioscorea rotundata* complex (55), *Dioscorea bulbifera* (13) and *Dioscorea alata* (2 as a reference) were assessed using six ISSR primers. DNA was extracted from a bulk of two plants per accession using a modified CTAB method. Six ISSR primers amplified 77 fragments with 75 (97.40%) polymorphism at genus level. The genetic diversity, estimated by Gene diversity and Shannon's index were 0.36 and 0.53, respectively, revealing a high level of genetic variation at genus level. At species level 75 bands were amplified for *D. cayenensis/D. rotundata* complex, out of which 71 were polymorphic accounting for 92.2% polymorphism. Gene diversity and Shannon's index for *D. cayenensis/D. rotundata* complex were 0.33 and 0.49, respectively. In case of *D. bulbifera* a total of 64 bands were scored, out of which 55 were found to be polymorphic which resulted in 71.4% polymorphism. Gene diversity and Shannon's index for this species were 0.24 and 0.47, respectively. Genetic diversity analysis of *D. cayenensis/D. rotundata* complex accessions showed that Gedeo were the most diverse among populations and South among groups. Analysis of molecular variance (AMOVA) indicated the presence of higher proportion of variation within species (63.9%) than among species (36.1%) which could be due to presence of several shared bands. AMOVA for *D. cayenensis/D. rotundata* complex also showed higher within population variation (53.6) than among populations (46.4). This could be due to gene flow through seed materials exchange among local farmers. Cluster analysis showed grouping of most of the accessions according to their species. In addition, cluster analysis for relationship between *D. cayenensis/D. rotundata* complex accessions showed grouping of some of the accessions according to their population but it failed to produce clear species boundary between *D. cayenensis/D. rotundata* complex. The results suggested that there exists a high level of genetic diversity in yams of Ethiopia to be exploited for future improvement (breeding) of the crop.

Keywords/phrases: *D. bulbifera*, *D. cayenensis/D. rotundata* complex, Genetic diversity, Yam

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TABLE OF CONTENTS **Page**

LIST OF FIGURES viii

LIST OF TABLES ix

LIST OF ABBREVIATIONS x

1. INTRODUCTION 1

2. LITERATURE REVIEW 5

2.1. Description of yams 5

2.2. Origin and distribution 5

2.3. Classification of yams 8

2.3.1. Taxonomic classification 8

2.3.2. Morphological character based classification 11

2.4. Cultivation system 12

2.5. Nutritional importance 13

2.5.1. Antinutrient composition 14

2.6. Importance of genetic diversity for sustainable use of plant genetic resource 14

2.6.1.Genetic markers for diversity analysis 15

2.6.2. Morphological markers 15

2.6.3. Biochemical Marker 16

2.6.3.1. Allozymes (Isozymes) 16

2.6.4. Molecular markers for diversity analysis 18

2.6.4.1. Non PCR-based techniques 20

2.6.4.1.1. Restriction Fragment Length Polymorphism (RFLP) 20
2.6.4.2. PCR based markers 21
2.6.4.2.1. Random Amplified Polymorphic DNA (RAPD) 21
2.6.4.2.2. Amplified Fragment Length Polymorphism (AFLP) 22
2.6.4.2.3. Simple sequence repeat (SSR) 23
2.6.4.2.4. Inter Simple Sequence Repeat (ISSR) 25
3. OBJECTIVES OF THE STUDY 27
3.1. General objective 27
3.2. Specific objectives 27
4. MATERIALS AND METHODS 28
4.1. Plant material 28
4.2. DNA Extraction 29
4.3. Test gel and electrophoresis 31
4.4. DNA concentration and quality checking 31
4.5. Primer screening and PCR optimization 31
4.6. PCR amplification and agarose gel electrophoresis 34
4.7. Data scoring and analysis 34
5. RESULTS 37
5.1. Banding patterns of ISSR primers 37
5.2. Species specific ISSR bands in *D. cayenensis/D. rotundata complex* and *D. bulbifera* 38
5.3. Genetic diversity within and among species 39

5.4. Analysis of Molecular Variance (AMOVA) 45

5.5. Cluster analysis 48

5.6. Principal Coordinate (PCO) analysis 57

6. DISCUSSION 62

6.1. Application of ISSR marker in *Dioscorea* species genetic diversity assessment .. 62

6.2. Partitioning of genetic diversity in yams (*Dioscorea* spp.) 65

6.3. Genetic relationships within and among species 66

6.4. Relationship between *D. cayenensis/D. rotundata* complex accessions 68

7. CONCLUSIONS 70

8. RECOMMENDATIONS 72

9. REFERENCES 73

10. APPENDICES 91

LIST OF FIGURES **Page**

Figure 1. ISSR fingerprint generated for some *Dioscorea* spp. accessions using ISSR-Primer 848. 37

Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient among 55 *D. cayenensis/D. rotundata* complex, 13 *D. bulbifera* and 2 *D. alata* accessions as a reference. 52

Figure 3. Neighbor-joining dendrogram for 55 *Dioscorea cayenensis/D. rotundata* complex, 13 *D. bulbifera* and 2 *D.alata* accessions using six ISSR-primers.. 53

Figure 4. UPGMA dendrogram based on Jaccard's similarity coefficient among 55 *D. cayenensis/D. rotundata* complex accessions.. 54

Figure 5. Neighbor-joining dendrogram constructed based on Jaccard's similarity coefficient for 55 accessions of *D. cayenensis/D. rotundata* complex.. 55

Figure 6. UPGMA based dendrogram for eight populations of *Dioscorea* spp. (seven *D. cayenensis/D. rotundata* complex; one *D. bulbifera*)..... 56

Figure 7. Two-dimensional plot obtained from principal coordinate analysis of 70 (55 *D. cayenensis/D. rotundata* complex; 13 *D. bulbifera*; 2 D. *alata*) accessions using Jaccard's coefficient of similarity.. 58

Figure 8. Three-dimensional plot obtained from principal coordinate analysis of 70 (55 *D. cayenensis/D. rotundata* complex; 13 *D. bulbifera*; 2 *D. alata*) accessions using Jaccard's coefficient of similarity.. 59

Figure 9. Two-dimensional plot obtained from principal coordinate analysis of 55 *D. cayenensis/D. rotundata* complex accessions using Jaccard's coefficient of similarity.. 60

Figure 10. Three-dimensional plot obtained from principal coordinate analysis of 55 *D. cayenensis/D. rotundata* complex accessions using Jaccard's coefficient of similarity.. 61

LIST OF TABLES **Page**

Table 1. The main section under the genus *Dioscorea* and corresponding cultivated species including their common names, origin and ploidy level.. 9

Table 2. Number of accessions of *Dioscorea* spp. collected from each region. 29

Table 3. List of primers, primer sequence, annealing temperature, amplification pattern and repeat motives used for optimization and screening. 33

Table 4. List of ISSR primers and their banding pattern in *D. cayenensis/D. rotundata* complex *and D. bulbifera* 39

Table 5. Level of genetic diversity revealed by six ISSR primers in *D. cayenensis/D. rotundata* complex, *D. bulbifera* and the combination of the two species 41

Table 6. Genetic diversity at the genus level 43

Table 7. Genetic diversity within and among populations and groups of Guinea yam (*D. cayenensis/D. rotundata* complex) 44

Table 8. Analysis of molecular variance (AMOVA) at the genus level. 46

Table 9. AMOVA for Guinea yam (*D. cayenensis/D. rotundata* complex) with and without grouping. 47

Table 10. Pair wise Jaccard's similarity coefficient based comparisons among eight populations of *Dioscorea spp.* collected from Ethiopia..... 51

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
CTAB	Cetyl trimethyl ammonium bromide
EIB	Ethiopian Institute of Biodiversity
GBS	Genotyping by Sequencing
H	Nei's Gene Diversity
I	Shannon Information Index
ISSR	Inter Simple Sequence Repeat
NJ	Neighbor Joining
NPL	Number of Polymorphic Loci
PAGE	Polyacrylamide Gel Electrophoresis
PCO	Principal Coordinate
PGR	Plant Genetic Resource
PPL	Percent of Polymorphic Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SNNPRS	Southern Nation Nationalities People Regional State

SSR	Simple Sequence Repeat
TBE	Tris Borate EDTA
TE	Tris EDTA
UPGMA	Unweighted pair group method with arithmetic mean

1. INTRODUCTION

Tuber crops are those with the edible carbohydrate rich storage organs that develop wholly or partly from underground stems (Okigbo, 1989). Thus, the storage organ of tuber crops is of stem origin. Many of the developing world's poorest producers and most food insecure households are highly dependent on roots and tubers contributing to their livelihoods as source of food, nutrition and cash income **(**Alexandratos, 1995). They are the second most important food crops after cereals (Asha and Nair, 2002). These crops also play important roles to the diet and food security of a large section of the Ethiopian Population (EIB, 2012).

Yams are one of the tuber crops which belongs to the genus *Dioscorea* in the family *Dioscoreaceae*. The Dioscoreales are considered to be one of the earliest angiosperms that have originated in Southeast Asia, but followed a divergent evolution in three continents separated by the formation of the Atlantic Ocean and desiccation of the Middle East (Hahn, 1995). This resulted in the occurrence of the major food species in three isolated centers: Africa, Southeast Asia and tropical America (Alexander and Coursey, 1969).

The genus *Dioscorea* comprises over 600 species (Sesay *et al*., 2013) out of which only 10 are cultivated. Their chromosome number ranges from 2n = 20 to 2n = 140 (Coursey 1967; Asiedu 1997). These are: *Dioscorea alata* L., *Dioscorea esculenta* Lour, *Dioscorea batatas* Decne or *D. opposite* Thumb. originating from Asia, *Dioscorea bulbifera* L., *D. cayenensis/D. rotundata* complex, *Dioscorea dumetorum* Kunth. originating from Africa,

Dioscorea trifida L. originating from America, *Dioscorea nummularia* Lam. and *Dioscorea pentaphylla* L. originating from both Asia and Oceania (Girma *et al*., 2012). Others are grown for medicinal purposes (Wu *et al*., 2005; Lebot, 2009).

Yams (*Dioscorea* L. spp.) are the fourth ranked and most important tuber crops in economic terms next to potatoes (*Solanum tuberosum* L.), cassava (*Manihot esculenta* Crantz), and sweet potatoes (*Ipomoea batatas* (L.) Poir.) (Mignouna *et al*., 2005). Yams serve as the staple carbohydrate source for millions of people (Adejumo *et al.*, 2013). They are cultivated in tropical countries, especially in West Africa, which produces over 95 % of the output (FAO, 2010).

Yams (*Dioscorea* spp.) are one of the most important native tuber crops of Ethiopia (EIB, 2009) and ranks 10th in the world in terms of their production (FAOSTAT, 2013). They are widely distributed and are amongst the main root and tuber crops grown by subsistence farmers in Southern, Southwestern and Western parts of the country (Muluneh *et al*., 2008). About 23 indigenous yam types belonging to at least four *Dioscorea* species were reported in Sheko, Southwest Ethiopia (Hildebrand *et al*., 2002). The cultivated species of yam, *D. bulbifera* (aerial yam), *D. abyssinica* and *D. schimperiana* are native to Ethiopia (Westphal, 1975). Yams in Ethiopia are hardly known by the scientific community and the country is rather considered as an isolated center of yam cultivation (Norman *et al*., 1995).

DNA fingerprinting has become an important tool for cultivar identification in plant breeding and for germplasm management (Zhou *et al.*, 2008). A DNA molecular marker in essence detects nucleotides sequence variation at a particular location in the genome. The

genetic variation/diversity must be found between the parents of the chosen cross for the marker to be informative among their offspring and to allow its pattern of inheritance to be analyzed. DNA markers can generate fingerprints, which are distinctive patterns of DNA fragments resolved by agarose gel electrophoresis and detected by staining or labeling.

The advent of the PCR was a breakthrough for molecular marker techniques and made possible many fingerprinting methods. Among all markers, RAPD, ISSR, AFLP and most recently genotyping by sequencing (GBS) markers are the most widely applied probably because they do not require the knowledge of genome sequences and the protocols used are relatively simple, rapid, cost effective and highly reproducible (Srivastava *et al.*, 2004; Vijayan *et al.*, 2005; Elshire *et al*., 2011).

Different types of DNA molecular assays have been applied in yam, including Isozyme (Dansi *et al.*, 2000a), RAPD (Asemota *et al.*, 1995; Dansi *et al.*, 2000b; Mignouna *et al.*, 2005), RFLP (Terauchi *et al*., 1992), SSR (Mignouna *et al.*, 2003), AFLP (Mignouna *et al*., 2003, and Malapa *et al.*, 2005), 18S rRNA gene sequencing (Liu *et al.*, 2001) and ISSR (Zhou Y. *et al.*, 2008; Nascimento *et al*., 2013). Until recently, only few studies: AFLP (Muluneh Tamiru, 2007; Wendawek Abebe *et al*., 2012) and SSR (Atnafua, 2014) have been conducted on yam germplasms of Ethiopia using molecular markers. Few other studies were also conducted based on morphological and agronomic traits (Muluneh Tamiru *et al*., 2008; Tewodros Mulualem, 2013).

Previous researchers on yams of Ethiopia suggested the need for further studies with inclusion of areas which were not included in their studies for better understanding of the

existing diversity. For instance, yams accessions from Ilu Ababora were not included in any of previous studies of Muluneh Tamiru *et al*. (2008); Wendawek Abebe *et al*. (2012); Tewodros Mulualem (2013) and Atnafua (2014). It is also much better to analyze the existing diversity using additional markers which provide us with further information. Besides, there is no previous report on ISSR marker for analysis of genetic diversity of yams (*Dioscorea species*) from Ethiopia. Hence, the present study aimed at assessing the level and pattern of inter and intra species genetic diversity and relationships among yams (*Dioscorea* spp.) accessions of major growing areas of Ethiopia using inter simple sequence repeat (ISSR) markers. This will be of great importance to supplement actions to be taken toward improvement and conservation of the crop.

2. LITERATURE REVIEW

2.1. Description of Yam (*Dioscorea* species)

Yam plants are herbal or woody, climbing with tuberous, starch rich storage organ. They are perennials with strongly marked annual cycle of growth. Typical *Dioscorea* species produce annual stems which climb by twining through the undergrowth and trees. The leaves are borne on long petioles usually simple, and cordate or accuminate. The flowers are small and borne in long racemes, with male and female flower always being separate and usually borne on separate plants (Coursey, 1983; Miege and Sebsebe Demissew, 1997). The areal storage organ of *Dioscoreaceae* is bulbil. The organ of dormancy and economically most important part of the plant is the storage tuber in the soil. The yam roots grow more or less horizontally within the soil and lie close to the soil surface (Onwueme and Charles, 1994).

2.2. Origin and distribution

The name “yam” is probably derived from the tribal African word “niam”, or “enyame”, meaning “to sample” or “taste” and it is also known as igname (French) or Ñamé (Spanish) (Alexander and Coursey, 1969). Yam belongs to the genus *Dioscorea* in the family *Dioscoreaceae*. The family is believed to be among the earliest angiosperms and probably originated in Southeast Asia (Coursey, 1967; Wilkin, 1998). Their ploidy level varies from 2n=20 to 2n=140 with basic chromosome number of either 9 or 10 (Table 1) (Coursey 1967; Asiedu 1997).

The various *Dioscorea* species apparently followed a divergent evolutionary course in three continents separated by the formation of the Atlantic Ocean and desiccation of the Middle East (Hahn, 1995). Accordingly, the major food species originated in three isolated centers: Africa, Southeast Asia and South America (Alexander and Coursey, 1969). These centers are also considered as areas for independent yam domestication, and represent considerable diversity (Asiedu *et al*., 1997). The genus had spread worldwide by the end of the Cretaceous period (approximately 75 million years ago) (Alexander and Coursey, 1969) and with migrating people from Asia to the south pacific as early as 3500 BC (Coursey, 1975). Nigeria is the main cultivation centre in the savannah region of West Africa, where more than 90 percent of the crop is grown.

White yam (*Discorea rotundata*) is believed to be indigenous to the area stretching from Côte d'Ivoire to Cameroon and is generally considered to be the best edible yam in that region. The yellow yam (*Dioscorea cayenensis*) is also indigenous in West Africa, whereas the water yam (*Dioscorea alata*) has originated in South-East Asia (Coursey 1967; Asiedu 1997).

Among the tropical tuber crops, cultivated guinea yam (*Dioscorea cayenensis/Dioscorea rotundata* complex) is one of the most important, especially in the so-called 'yam belt' of West Africa where they are indigenous (Dansi *et al.*, 2000a; Mignouna *et al*., 2003). They are most commonly cultivated in the world and represent 95% of worldwide cultivation (Mignouna *et al.*, 2003). Guinea yams (*D. cayenensis/D. rotundata* species complex) constitute the predominant starchy staple in sub-Saharan Africa (Fu *et al*., 2011; Demuyakor *et al*., 2013).

D. abyssinica and *D. preahensilis* are indicated by Miege and Sebsebe Demissew (1997) as parents (possible ancestors) for several cultivars of *D. cayenensis*/*D. rotundata* complex. *D. preahensilis* is widely distributed throughout the Western, Central and Eastern parts of Africa including Ethiopia (Dumont *et al*., 2006). *D. abyssinica* is widely distributed in Southern, Western and Northern parts of Ethiopia (Miege and Sebsebe Demissew, 1997) and reported to have originated in Ethiopia (Franklin and Lucien, 1978).

D. bulbifera is the only species believed to be native to both Asia and Africa (Wilkin, 2001). It is sole species of the genus that is distributed in wild and cultivated state in both Asia and Africa (Teranchi *et al*., 1991). This species is characterized by the formation of many bulbils at the bases of its petioles. Unlike other *Dioscorea* species, the organ of *D. bulbifera* which is used as food in most cases is the bulbil (Teranchi *et al*., 1991). The native range of *D. bulbifera* in Africa includes: the east tropical Africa countries of Tanzania and Uganda; the Southern African countries of Zambia, Zimbabwe, Malawi, Mozambique and Namibia; Cameroon in West-central tropical Africa; and, the West tropical Africa countries of Benin, Burkina Faso, Ivory Coast, Ghana, Guinea, Liberia, Nigeria, Senegal and Sierra Leone (Coursey, 1967; Wilkin, 2001).

D. bulbifera is reported by Westphal (1975) as one of the *Dioscorea* species that are native to Ethiopia. It is distributed across diverse agro-geographical areas in South and South-western parts of Ethiopia (Tewodros Mulualem, 2013). Hildebrand *et al.* (2002) also reported about 23 other indigenous yam types belonging to at least four *Dioscorea* species in Sheko, Southwest Ethiopia. Yams (*Dioscorea* spp.) are therefore, one of the most important native tuber crops of the country (EIB, 2009). It is a traditional crop that has

long been cultivated in Wolayita and Gamo-Gofa zones as co-staple with enset (*Ensete ventricosum* (Welw.) Cheesman), cereals, and other root and tuber crops in South and Southwestern parts of Ethiopia (Westphal, 1975). As the crop is adapted to dry season planting (mainly at the onset of the dry season in October) early harvests in May fill a seasonal gap in food supply when cereals are not ready for harvest. Yam is preferred to the other root and tuber crops because it is an important cash crop, generating additional income for farm households (Muluneh *et al*., 2008).

2.3. Classification of yams (*Dioscorea* species)

2.3.1. Taxonomic classification

Yam is a monocotyledonous angiosperm, which belongs to the order *Liliflorae*, family *Dioscoreaceae,* and genus *Dioscorea.* It is among the most primitive angiosperms and contains over 600 species, of which only 10 are cultivated (Sesay *et al.,* 2013). The genus is subdivided into sections (Table 1), under which the various species are classified. The section Enantiophyllum is the largest in terms of number of species, and includes the most important species of *D. alata*, *D. rotundata* and *D. cayenensis*. Other members of this section are *D. opposita*, *D. japonica* and *D. transversa* (Asiedu *et al*., 1997). Vines that twine to the right, i.e. in clockwise direction when viewed from the ground upwards, characterize members of section Enantiophyllum. On the other hand, vines twining to the left distinguish species in sections Lasiophyton (*D. dumetorum* and *D. hispida*), Opsophyton (*D. bulbifera*), Combilium (*D. esculenta*) and Macrogynodium (*D. trifida*) (Onwueme and Charles, 1994)

Table 1. The main sections under the genus *Dioscorea* and corresponding cultivated species including their common names, origin and ploidy level.

Section	**Twining direction**	**Species**	**Common name**	**Origin**	**Ploidy level**
Enantiophyllum	To the right	*D. alata* L.	Water yam: Greater yam: Winged yam	SE Asia	2n= 20, 30, 40, 50, 60, 70, 80
		D. rotundata Poir.	White guinea yam: White yam	W Africa	2n=40, 80
		D. cayenensis Lam.	Yellow Guinea yam: Yellow yam	W Africa	2n=36, 54, 60, 63, 66, 80, 120, 140
		D. opposita Thumb.	Cinnamon yam	China	2n=40
		D. japonica Thumb.	Chinese yam	Japan	2n=40
		D. transversa R. Br.		SE Asia	-

Lasiophyton	To the left	*D. dumetorum* (kunth.) Pax	Bitter yam: Trifoliate yam: Cluster yam	Africa	2n= 36, 40, 45, 54
Opsophyton		*D. hispida* Dennst.	Asiatic bitter yam	SE Asia India	2n= 40, 60.
		D. bulbifera L.	Aerial yam: potato yam	Africa Tropical Asia	2n= 30, 40, 50,60. 70, 80, 100
Combilium		*D. esculenta* (Lour.) Burkill	Lesser yam: Asiatic yam	Indochina Oceanea	2n=30, 40, 60, 90, 100
Macrogynodium		*D. trifida* L. f.	Cush-cush yam	Tropical America	2n=54, 72, 81

Source: Coursey, 1967; Asiedu, 1997

2.3.2. Morphological character based classification

On the basis of morphological characters, the genus *Dioscorea* were first divided in to six different sections (Alexander and Coursey, 1969) (Table 1). However, the morphological characters do not allow the distinction between species such as *D. cayenensis* and *D. rotundata*, and also between cultivars. *D. rotundata* may in fact be a subspecies of *D. cayenensis* or alternatively may have originated from *D. praehensilis* (Coursey, 1967).

Different views exist as to the taxonomy of Guinea yams (*D. cayenensis/D. rotundata* complex). Both forms were either considered to represent one single and highly evolved species (Martin and Rhodes, 1978), or two clearly separable species, which differ mainly from the colour of the tuber flesh and the vegetative cycle length (Onyelagha and Lowe, 1985), or two well-separated clusters with some intermediate forms (Hamon and Toure, 1990). On the other hand, Miege and Sebsebe Demissew (1997) reported that the criteria used to differentiate the two species *D. cayenensis* and *D. rotundata* are not satisfactory as the original diagnosis are not complete enough to define them precisely. Moreover, many forms are intermediate between the two species and thus impossible to delimit the two species satisfactorily. The main character used in the past, the flesh colour of the tuber, i.e, as white-fleshed (*D. rotundata*) or Yellow-fleshed (*D. cayenensis*) did not work. The two taxa are thus better treated as *D. cayenensis/D. rotundata* complex (Miege and Sebsebe Demissew, 1997).

Based on the presence and absence of spines on stems & roots, number of male and female inflorescence, stem length, twining direction (Left/Right) and flesh color, Atnafua (2014) grouped Ethiopian yams into six species namely *D. alata*, *D.bulbifera*, *D. abyssinica*, *D.*

praehensilis, D. rotundata and *D. cayenensis*. However, in study conducted by Wendawek Abebe *et al*. (2012), *D. rotundata* and *D. cayenensis* are kept together as species complex (*D. cayenensis/D. rotundata* complex).

2.4. Cultivation system

Yams, like most African crops, are generally intercropped in the traditional farming system. They are normally grown in high rainfall areas with distinct wet and dry seasons; they grow best on loose, fertile, well drained soils. Production is labour intensive because of the need for soil mounding before planting, for staking, weeding and above all for harvesting. A major constraint on yam cultivation is the high cost and sometimes the unavailability of planting materials. The cost of planting materials can account for up to 60 percent of production inputs. High perishability and losses during storage (up to 50 percent) are further constraints. Yam production systems have largely evolved within a subsistence economy and are not suited to large-scale mechanized farming. Yam production in Africa is concentrated in areas within 15 degrees of the equator (CGIAR 1996).

In Ethiopia, Yams are grown in the altitude range of 1140 to 2200 m.a.s.l. and in a wide range of soils mainly in clay, clay loam, sandy and sandy loam types. Yam is planted in October (in most parts of Southern Ethiopia), November & December (in Southwestern and Western part of the country) at the onset of the dry season making use of soil moisture reserves from the preceding rains (Hildebrand, 2003).

2.5. Nutritional importance

Yam is of higher nutritional value than some other root and tuber crops such as cassava. Its protein content is about 3 to 5% and higher as compared to 1 to 2% in cassava (Charles *et al*., 2005). Its tubers could be a source of energy, primarily, as their dry material predominantly consists of carbohydrates. Its tubers contain vitamin C, musin (glycoprotein), minerals (K, P, Ca, Mg, Fe, Cu, Co), phytosterols and steroidal saponins (furostanol and spirostanol glycosides), etc. Its tubers are processed into various types of food, including pounded yam, boiled yam, roasted or grilled yam, fried yam slices, yam balls, mashed yams, yam chips, and yam flakes. Fresh yam tubers are also peeled, chipped, dried, and milled into flour that is used to prepare dough called amala or telibowo (in West Africa) (Mahalakshmi *et al.*, 2007). In Ethiopia it is mostly consumed boiled which is mashed and mixed with butter and fermented milk. Roasting of tubers is also practiced on a rare phenomenon. Furthermore, the crude protein, crude fat, crude fiber and ash contents of yams are in the range of 6.7% – 7.9%, 1.0% – 1.2%, 1.2% – 1.8%, and 2.8% – 3.8%, respectively (Zhou *et al.*, 2008).

In Ethiopia, early maturing cultivars of yam are harvested starting from May (the main food deficient period), which enable yams to fill the gap in food shortage and hence play a vital role in food security (Muluneh Tamiru, 2006). It is an ideal candidate for food and nutritional security in countries like Ethiopia.

2.5.1. Antinutrient composition

Most yams have excellent eating quality, although parts of it can be poisonous if ingested uncooked (Dansi *et al.*, 1999). Some species of yams, particularly wild forms, are toxic and/or unpalatable, taste bitter and cause vomiting and diarrhoea when large amount are ingested without proper processing or eaten raw (Webster *et al*., 1984). There are many non-nutrient substances present in roots and tubers. Some of these substances are potentially toxic or may adversely affect the bioavailability of nutrients (Eka, 1985). Many wild yam species also contain toxic and/or other bioactive chemicals and some of these are cultivated for pharmaceutical products (Coursey, 1967).

2.6. Importance of genetic diversity for sustainable use of plant genetic resource

Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants (Agarwal *et al.*, 2008). Understanding the molecular basis of the essential biological phenomena in plants is crucial for the effective conservation, management, and efficient utilization of plant genetic resources (PGR). In particular, an adequate knowledge of existing genetic diversity, where in plant population it is found and how to best utilize it, is of fundamental interest for basic and applied sciences. It is important for rational breeding program to improve crop productivity.

Genetic diversity refers to the extent of genetic variability exists within and among populations adapted in various geographic regions/areas. Determining genetic diversity can be based on morphological, biochemical and molecular types of information.

2.6.1. Genetic markers for diversity analysis

The Genetic markers used in genetics and plant breeding can be classified into two categories: classical markers and DNA markers (Xu, 2010). Classical markers include morphological markers, cytological markers and biochemical markers. DNA markers have developed into many systems based on different polymorphism detecting techniques or methods including southern blotting – nuclear acid hybridization, PCR – polymerase chain reaction, and DNA sequencing (Collard *et al*., 2005).

2.6.2. Morphological marker

Morphological markers represent genetic polymorphisms which are easily identified by naked eye and manipulated. Therefore, they are usually used in construction of linkage maps by classical two- and/or three point tests. Some of these markers are linked with other agronomic traits and thus can be used as indirect selection criteria in conventional breeding (Liu, 1991). However, morphological markers available are limited, and many of these markers are not associated with important economic traits (e.g. yield and quality) (Jiang, 2013).

Morphological traits (phenotypic) have direct correspondence with genes controlling the trait (such as shape, colour, size or height). They can be used as reliable indicators for specific genes. On the other hand, several limitations were examined with morphological markers during genetic studies where visible markers often possessed deleterious effects on the studied organisms and that they were also rare (Paterson, 1996). Morphological

characters that have been traditionally used for plant identification may lead to unreliable determinations due to environmental influences (Solar *et al.*, 1991).

Morphological characterization does not require expensive technology but large tracts of land are often required for these experiments, making it possibly more expensive than molecular assessment. These traits are often susceptible to phenotypic plasticity; conversely, this allows assessment of diversity in the presence of environmental variations (Mondini *et al.*, 2009).

2.6.3. Biochemical markers

2.6.3.1. Allozymes (isozymes)

Biochemical markers, especially the electrophoretic profile of isozymes and proteins, have been widely used in identification of crop varieties (Dadlani, 2007). Isozymes are alternative forms or structural variants of an enzyme that have different molecular weights and electrophoretic mobility but have the same catalytic activity or function. Isozymes reflect the products of different alleles rather than different genes because the difference in electrophoretic mobility is caused by point mutation as a result of amino acid substitution (non synonymous mutation) (Xu, 2010).

When a mutation happen in protein coding DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because changes in electric charge and conformation can affect the migration rate of proteins in an electric field and results in allelic variation. The allelic variation can be detected by gel electrophoresis and

subsequent enzyme specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (e.g. nitro-blue tetrazolium) (Kumar *et al.,* 2009).

Isozymes are generally made up of subunits, and the various subunits give rise to enzymes with the same catalytic activity. Major functions of isozymes are controlling metabolic activities of the organism. Different genes code for isozymes, while each gene may have different alleles at the same locus, coding for slightly modified proteins as a subclass of the isozyme called allozymes (Abdullah, 2001).

In 1968, the term allozyme was coined by Prakash (Buth, 1984). Allozymes display co-dominant expression, so that plants which are heterozygous at an isozyme locus may be distinguished from either homozygote. When a plant is heterozygous at a locus for a monomeric enzyme, two allozyme bands will be produced, whereas three bands will be produced if the enzyme is dimeric, because the allelic product at dimeric loci can associate at random producing both homodimer and a heterodimer (Moore and Durham, 1996). Both forms of data are important in molecular systematics, and both involve proteins that can be separated on the basis of net charge and size (Murphy *et al*., 1990).

Isozymes are becoming biochemical markers of choice for initiating or advancing genetic studies of plants in early 1960s. Since isozymes are direct gene products, the banding patterns obtained, so called zymograms, can be effectively correlated to the genetic make-up of the particular sample. These zymograms are analogous to “fingerprints”. Electrophoretic isozyme characters have proved to be highly useful for solving various problems of plant taxonomy, genetic diversity and phylogenetic systematics at intrageneric

and specific levels (Karaca, 2013). Isozymes have been proven to be reliable genetic markers in breeding and genetic studies of plant species (Heinz, 1987), due to their consistency in their expression, irrespective of environmental factors. Isozymes markers have been successfully used in several crop improvement programmes (Vallejos, 1983; Glaszmann *et al.*, 1989; Baes and Custsem, 1993; Chhabra *et al*., 2001).

However, they have their own drawbacks due to the fact that the number of detectable isozymes are limited and they are typically tissue and developmental stage specific (Park *et al*., 2009), have restricted number of suitable allozyme loci in the genome, the requirement of fresh tissue, and sometimes limited variation (Weising *et al*., 2005).

2.6.4. Molecular markers for diversity analysis

The concept of genetic markers is not a new one; in the nineteenth century, Gregor Mendel employed phenotype-based genetic markers in his experiments. Later, phenotype-based genetic markers for *Drosophila melanogaster* led to the founding of the theory of genetic linkage, a phenomenon that occurs when particular genetic loci or alleles for genes are inherited jointly. The limitations of phenotype-based genetic markers led to the development of DNA-based molecular markers. A molecular marker can be defined as a genomic locus, detected through probe or specific starters (primer) which, in virtue of its presence, distinguishes unequivocally the chromosomal trait which it represents as well as the flanking regions at the 3' and 5' extremity (Barcaccia *et al.*, 2000).

Molecular markers, due to their stability, cost-effectiveness and ease of use provide an immensely popular tool for a variety of applications including genome mapping, gene

tagging, genetic diversity, phylogenetic analysis and forensic investigations (Grover and Sharma, 2014).

No marker is superior to all others for a wide range of applications. The most appropriate genetic marker depends on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and knowhow, time constraints and financial limitations. An ideal molecular marker technique should have the following criteria: (1) be polymorphic and evenly distributed throughout the genome, (2) provide adequate resolution of genetic differences, (3) generate multiple, independent and reliable markers (4) simple, quick and inexpensive, (5) need small amounts of tissue and DNA samples, (6) have linkage to distinct phenotypes, (7) require no prior information about the genome of an organism, (8) Codominant inheritance (determination of homozygous and heterozygous states of diploid organisms), (9) Easy access (availability) and Easy exchange of data between laboratories and (10) Selective neutral behaviour. Unfortunately, no molecular marker technique can fulfill the above criteria of ideal molecular marker. Techniques differ from each other with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost (Agarwal *et al.,* 2008; Kumar *et al.,* 2009; Bagali *et al.,* 2010).

Basic marker techniques can be classified into two categories: (1) non-PCR-based techniques or hybridization based techniques and, (2) PCR-based techniques (Agarwal *et al*., 2008).

2.6.4.1. Non PCR based marker

2.6.4.1.1. RFLP

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of particular Restriction Endonucleases, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another (Kumar *et al*., 2009).

RFLP technique is mainly based on the special class of enzyme i.e. Restriction Endonucleases (Chittaranjan and Albert, 2008; Kumar *et al*., 2009). DNA polymorphism is detected by hybridizing a chemically labelled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profile or electrophoresis resolution in gel matrix.

The RFLP markers are relatively highly polymorphic, codominantly inherited, highly replicable and allow the simultaneous screening of numerous samples. Regardless of its advantages, it is not very widely used because it is time consuming, involves expensive and radioactive/toxic reagents and requires large quantity of high quality genomic DNA. The requirement of prior sequence information for probe generation increases the complexity of the methodology. These limitations led to the conceptualization of a new set of less technically complex methods known as PCR-based techniques (Agrawal *et al.*, 2008 and Mondini *et al.*, 2009).

2.6.4.2. PCR based markers

2.6.4.2.1. RAPD

Random Amplification of Polymorphic DNA (RAPD) is a modification of the PCR in which a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome. The assumption made is that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels (1.5- 2.0%) and visualized by ethidium bromide staining (Kumar and Gurusubramanian, 2011).

The standard RAPD technology (Williams *et al.*, 1990) utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR.

RAPDs are viewed as having several advantages over RFLPs . When the primer of intermediate size (on the order of 10 base pairs) are used, multiple amplified fragments are usually present for each set of primers in each genome. Since it makes use of standard agarose gel and ethidium bromide staining for visualization of amplified fragments, it eliminates the need for radio labeled probes (Lynch and Milligan, 1993). The major advantage of RAPD includes: no prior sequence information required, it is simple, efficient

and inexpensive, vast range of potential primers that can be used (Kumari and Thakur, 2014).

Despite these advantages, RAPD analysis presents some practical problems. The profiling is dependent on the reaction conditions, hence may vary within two different laboratories and as several discrete loci in the genome are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (it is dominant) (Bardakci, 2001).

2.6.4.2.2. AFLP

To overcome the limitation of reproducibility associated with RAPD, AFLP technology (Zabeau and Vos, 1992; Vos *et al.*, 1995) was developed. It combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers and gel analysis of amplified fragments. The primer pairs used for AFLP usually produce 50–100 bands per assay. Number of amplicons per AFLP assay is a function of the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size and complexity. The AFLP technique generates fingerprint of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping. The technique can be used to distinguish closely related individuals at the sub-species level (Althoff *et al.*, 2007).

AFLP is highly reliable and reproducible (Mueller *et al.,* 1996; Lin *et al.,* 1996; Powell *et al.,* 1996b; Jones *et al.,* 1997); it does not require any DNA sequence information from the organism under study; it is information-rich due to its ability to analyze a large number of polymorphic loci simultaneously (effective multiplex ratio) with a single primer combination on a single gel as compared to RAPDs, RFLPs and microsatellites (Powell *et al.,* 1996b; Milbourne *et al.,* 1997; Russell *et al.,* 1997); and co-migrating AFLP amplification products are mostly homologous and locus specific (Vandervoort *et al.,* 1997; Waugh *et al.,* 1997; Qi *et al.,* 1998), with exceptions in polyploidy species.

Furthermore, it requires more number of steps to produce the result, it requires template DNA free of inhibitor compounds that interferes with the restriction enzyme. The technique requires the use of polyacrylamide gel in combination with AgNO staining, radioactivity, or fluorescent methods of detection, which will be more expensive and laborious than agarose gels. It involves additional cost to purchase both restriction and ligation enzymes as well as adapters. Like RAPD, most AFLP loci are dominant, which does not differentiate dominant homozygotes from heterozygotes. This reduces the accuracy of AFLP markers in population genetic analysis, genetic mapping, and marker assisted selection (Semagn *et al*., 2006).

2.6.4.2.3. SSR

Microsatellites or short tandem repeats or simple sequences repeats are monotonous repetitions of very short nucleotide motifs, which occur as interspersed repetitive elements in all eukaryotic genomes (Tautz and Renz, 1984). These consist of sequences of repetitions, comprising basic short motifs generally between 2 and 6 base-pairs long.

Polymorphisms associated with a specific locus are due to the variation in length of the microsatellite, which in turn depends on the number of repetitions of the basic motif. Variations in the number of tandemly repeated units are mainly due to strand slippage during DNA replication, where the repeats allow matching via excision or addition of repeats (Schlotterer and Tautz, 1992).

The development of microsatellite markers involves several distinct steps. These include: Microsatellite library construction, identification of unique microsatellite loci, identifying a suitable area for primer design (conserved region), obtaining a PCR product, evaluation and interpretation of banding patterns, and assessing PCR products for polymorphism (Roder *et al.,* 1998). However, due to advancement of sequencing technology this days, development of SSR markers are less expensive and labor intensive.

The primary advantage of microsatellites as genetic markers is that they are co-dominant markers. Further advantages include, high polymorphism rates, high abundance and a broad distribution throughout the genome (Wright and Bentzen, 1994; Morgante *et al.,* 2002), easy to automate, multiallelic and no radioactive labeling is required (kumar *et al.,* 2009).

However, low frequency of SSRs in plants also hinders the large scale isolation of SSRs (Powell *et al.,* 1996a). Moreover, SSR markers developed for one species generally exhibit less transferability across same or different taxa which necessitate the development of species specific markers (Roa *et al.,* 2000; Kindiger, 2006). Another important problem associated with microsatellites is the occurrence of null alleles. The potential cause is poor

primer annealing caused by nucleotide sequence divergence, inconsistent DNA template quality or low template quantity (Ellegren, 2004) or mutations/Indels in the primer binding sites (Pemberton *et al.,* 1995). This leads to complications in the determination of allelic and genotypic frequencies and an underestimation of heterozygosity (Kumar *et al*., 2009). Moreover, homoplasy is also another problem when applying microsatellites as a reliable tool for phylogenetic analysis because co-migrating alleles considered to be identical in state are not necessarily identical by descent (Estoup *et al*., 2002).

2.6.4.2.4. ISSR

ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeats of different sizes. The microsatellite repeats used as primers for ISSRs can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primers used can be either unanchored (Meyer *et al*., 1993; Gupta *et al*., 1994; Wu *et al.,* 1994) or more usually anchored at 3` or 5` end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al.,* 1994).

ISSRs usually use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher primer specificity. The annealing temperature depends on the GC content of the primer used and ranges from 45 to 65^{o}C. The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis.

ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis which is the advantage of random markers (Joshi *et al*., 2000). The primers are not proprietary and can be synthesized by anyone. The technique is simple, quick, and the use of radioactivity is not essential. ISSR markers usually show high polymorphism (Kojima *et al*., 1998). Moreover, low quantities of template DNA are required (5–50ng per reaction). In contrast to other molecular markers, the target sequences for ISSR primers are abundant throughout the eukaryotic genome and evolve rapidly. Consequently, ISSR markers help in revealing a much higher number of polymorphic fragments than RAPD (Random Amplified Polymorphic DNA) markers. In addition, the ISSR reaction is more specific than RAPD reaction (Williams *et al.*, 1990, Fang and Roose, 1997).

ISSR is mostly dominant marker, though occasionally it exhibits as codominance (Kumar *et al*., 2009). Much larger numbers of fragments per primer and relatively low cost, have been widely used for DNA fingerprinting, population genetics and phylogenetic studies and so on (Zhou *et al.*, 2007). ISSR technique has been used in the detection of genetic variation among Chinese yam (*Dioscorea opposita* Thunb) cultivars (Zhou *et al*., 2008) and Amerindian yam (*Dioscorea trifida* L.) (Nascimento *et al*., 2013).

3. OBJECTIVES OF THE STUDY

3.1. General objective:

The general objective of this study was to assess the level and pattern of inter and intra species genetic diversity and relationships among yams (*Dioscorea* spp.) collected from different parts of Ethiopia using ISSR markers.

3.2. Specific objectives:

The specific objectives of the study were to:

- Analyze genetic diversity and relationship among *D. cayenensis/ D. rotundata* complex and *D. bulbifera* accessions.
- Evaluate the amount of genetic variation and relationship among *D. cayenensis/D. rotundata* complex accessions.
- Reveal the genetic structure of *D. cayenensis/D. rotundata* complex and *D. bulbifera*.
- Reveal the genetic structure of *D. cayenensis/D. rotundata* complex populations specifically.
- Determine level of genetic similarity between all accessions.

4. MATERIALS AND METHODS

4.1. Plant material

The yam plant used in this study were kindly provided by Ethiopian Institute of Biodiversity (EIB), Choche field gene bank, which is located in Oromia Regional State, Jimma Zone, Goma Woreda, at approximately 1600 m.a.s.l., $7^{o}54'$ N and $36^{o}39'$ E. It has a total area of 21 hectares, and was established two decades ago mainly for conserving coffee genetic resources of the country. Moreover, 12 species of horticultural genetic resources including Yam (*Dioscorea* spp.) are being conserved at the field genebank.

A total of 70 accessions (55 *D. cayenensis/D. rotundata* complex; 13 *D. bulbifera*; 2 *D. alata*) belonging to 7 populations (3 from Oromia and 4 from SNNPRS) were used for the study (Appendix 1). Due to their sample size, all the *D. cayenensis/D. rotundata* complex (55) and *D. bulbifera* (13) were used for population genetics analysis and AMOVA while the *D. alata* (2) accessions were included only in tree/dendrogram and Principal Coordinate Analysis (PCO).

Table 2. Number of accessions of *Dioscorea* spp. collected from each region.

No	Populations	GL	Regions	No of accessions
1	Semen Omo	South	SNNPRS	8
2	Gedeo	South	SNNPRS	8
3	Jimma	SW	Oromia	6
4	East wellega	West	Oromia	13
5	Ilu Ababora	SW	Oromia	12
6	Sheka- keffa	SW	SNNPRS	4
7	Hadiya- Kembata	South	SNNPRS	5

SNNPRS = Southern Nation Nationalities People Regional State; Semen Omo = current Wolayta and Gamo Gofa; GL= Geographic Location; SW = SouthWest

4.2. DNA extraction

Genomic DNA was extracted following the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol (Borsch *et al.,* 2003) with slight modification. The CTAB (50mM Cetyl trimethyl ammonium Bromide, 0.4mM polyvinylpyrolidone, 0.1M Tris: PH=8, 20mM EDTA, 2M NaCl, 0.03M beta-Mercapto-ethanol) solution was warmed in water bath at 65°C. Eventually, dried and bulked leaf materials per samples were added to eppendorf caps with two beads per eppendorf and pulverized thoroughly using mixer mill (MM400, Retsch®) for 3 minutes. After pulverization, 700µl warm CTAB solution was added to the eppendorf containing powdered samples, dissolved powder was incubated for 30 minutes at 65°C in water bath and finally centrifuged for 7 minutes at 13000rpm. The supernatant was transferred to a new eppendorf tube and 700µl CTAB solution was added to the tissue

pellet for the second extraction and stirred slightly with a new 100 µl pipette tip, incubated for 30 minutes at 65°C again in the water bath. A total of 600 µl chloroform was added immediately to the cap with supernatant and shaken upside down carefully for approximately 10 minutes and then centrifuged for 7 minutes at 13000 rpm. After centrifugation, the supernatant (only clear liquid) was transferred to a new eppendorf-cap and chloroform extraction step was repeated. Cold isopropanol (4°C), approximately 2/3 of the solution volume was added to the supernatant, shaken carefully and allowed to freeze for more than 2 hours at -20°C before centrifugation for 15 minutes at 13000 rpm and aspirating the liquid using yellow tips without touching the pellet. A total of 200µl of 70 % ethanol was added to the pellet and allowed to centrifuge for 15 minutes at 13000 rpm. Finally, ethanol was aspirated by using yellow tips and the pellet was dried in a laminar hood for 15 minutes and dissolved in 1×TE (1mM Tris HCl, 0.1µM EDTA) and stored at 4°C. Later 0.83M of cooled NH_4Ac-solution was added to the TE suspended DNA pellet and mixed carefully. Eventually, 300µl cold absolute ethanol was added to the solution, mixed carefully and frozen for more than 2 hours at -20°C, centrifuged for 35 minutes at 13000 rpm and fluid was aspirated carefully. Then, 200µl of 70% ethanol was added and the inner cap surface was rinsed by turning the cap and centrifuged for 15 minute at 13000 rpm and the liquid was aspirated and the pellet was dried at room temperature. Then, dried pellet was dissolved in 1×TE (1mM Tris HCl, 0.1µM EDTA) and the last four steps were repeated with 0.33M NaAC solution instead of 0.83M NH_4Ac solution (4°C, half the volume) then, centrifuged 15 minute at 13000 rpm and liquid was aspirated and the pellet was dried in hood. Lastly the dried pellet was dissolved in 1×TE (1mM Tris HCl, 0.1µM EDTA) and stored for further use for PCR.

4.3. Test gel and electrophoresis

For test gel, 1% agarose gel was prepared by 1×TBE buffer (0.09M Tris, 0.002M EDTA and 0.09M boric acid) using microwave oven for about 1minute. Ethidium bromide (2µl) was added to the boiled agarose and mixed well before casting on a gel tray. A total of 2µl genomic DNA with 6×loading dye (0.004M Bromophenol Blue, 4.1M Glycerol and 0.02M Tris HCl) was loaded and subjected to electrophoresis in BIORAD min-sub® (from Biometra® cell GT at 80V standard power pack P25) for 45 minutes and visualized under UV using gel documentation system (Biosens SC750). The gel was photographed using Canon camera attached to gel documentation system (Biosens SC750).

4.4. DNA concentration and quality checking

Although the quality and concentration of DNA can be estimated depending on the gel picture generated, nanodrop was used as a confirmation. The concentration of DNA was within a range of 190-3264ng/µl. The ratio of absorption (260/280) was found to be within a range of 1.8 to 2. Thus, the DNA samples were in good quality for PCR work. They were diluted with ddH_2O at ratio of 1:5 for further use.

4.5. Primer screening and PCR optimization

The ISSR marker assay was conducted at Plant Genetics Research Laboratory of the Department of Microbial Cellular and Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia. A total of 15 ISSR primers were screened for their ability to generate clear, reproducible, polymorphic and high resolution bands, in four (two from *D. cayenensis/D. rotundata* complex; two from *D. bulbifera*) randomly selected accessions.

Out of fifteen candidate ISSR- primers, six best with clear, reproducible and polymorphic bands (ISSR-811, ISSR-818, ISSR-844, ISSR- 848, ISSR-873 and ISSR-880) were selected and used for analysis of genetic diversity of *Dioscorea* spp. (Table 3). Various combinations (at different concentrations) of PCR components were tested to find out optimum concentrations of the PCR reaction components and the one with clear band was used.

Table 3. List of primers, primer motif, annealing temperature, repeat motives and amplification quality used for optimization and screening

ISSR-Primers	Primer motif	T^o (^oC)	Repeat motif	Amplification quality
809	$(AG)_8G$	48	Di-nucleotide	No amplification
810	$(GA)_8T$	45	Di-nucleotide	Not reproducible, Not polymorphic
811	$(GA)_8C$	48	Di-nucleotide	Polymorphic, reproducible
812	$(GA)_8A$	45	Di-nucleotide	Not reproducible, Not polymorphic
815	$(CT)_8G$	48	Di-nucleotide	Not reproducible, Not polymorphic
818	$(CA)_8G$	48	Di-nucleotide	Polymorphic, reproducible
824	$(TC)_8G$	48	Di-nucleotide	Not reproducible, Not polymorphic
844	$(CT)_8RC$	48	Di-nucleotide	Polymorphic, reproducible
848	$(CA)_8NG$	48	Di-nucleotide	Polymorphic, reproducible
866	$(CTC)_6$	55	Tri-nucleotide	Not reproducible, Not polymorphic
873	$(GACA)_4$	45	Tetra-nucleotide	Polymorphic, reproducible
874	$(CCCT)_4$	45	Tetra-nucleotide	Not reproducible, Not polymorphic
876	$(GAYA)_4$	45	Tetra-nucleotide	No amplification
880	$(GGAGA)_3$	45	Penta-nucleotide	Polymorphic, reproducible
881	$(GGGTG)_3$	48	Penta-nucleotide	Not reproducible, Not polymorphic

N = (A, T,G,C); R = (A, G); Y = (C, T)

4.6. PCR amplification and agarose gel electrophoresis

PCR amplification was carried out in a 25 µl reaction mixture containing 16.7µl sterile deionized H_2O, 1mM each dNTPs, 2.5µl 10X reaction buffer S (100mM Tris-HCl, 500mM KCl, 0.1% Tween 20 and 15mM $MgCl_2$), 2mM $MgCl_2$, 8pmol primer, 2unit Taqpolymerase and 50-100ng diluted template DNA. The polymerase chain reactions were conducted in Biometra T3 Thermocycler with amplification program: a preheating and initial denaturation at 94^oC, for 4 minutes, then for 40 x 15 seconds at 94^oC, 1 minute primer annealing at primer annealing temprature (45^oC/48^oC), primer extension at 72^oC for 1:30 minute and final extension at 72^oC for 7 minutes. The PCR products (10µL for each sample) together with 6X loading dye (0.004 M Bromophenol Blue, 4.1 M Glycerol, 0.02M Tris HCl) were loaded on 1.67% agarose gel with 100bp ladder as a size standard (on the first lane of the gel) and subjected to electrophoresis in BIORAD min-sub® (from Biometra® cell GT at 80V standard power pack P25) for about 2 hours and visualized under UV light using gel documentation system (Biosens SC750), stained by immersing in 1µg/ml ethidium bromide solution. The image of both stained and destained was photographed with gel documentation system (Biosens SC750).

4.7. Data scoring and analysis

Each band formed after electrophoresis of ISSR-PCR result (amplified DNA) was considered as independent locus and recorded as discrete characters, presence ‘1’ or absence ‘0’ and ‘?’ for missing or ambiguous data for all accessions.

For analysis, scored data were subjected to different softwares like POPGENE version1.32 (Yeh *et al.*, 1999), which was used to calculate genetic diversity for each species (*D. cayenensis/D. rotundata* complex; *D. bulbifera*) and populations within *D. cayenensis/D. rotundata* complex as number of polymorphic loci, percent polymorphism, Nei's gene diversity and Shannon's information index.

Analysis of molecular variance (AMOVA) was used to calculate variation among and within species as well as among and within populations and groups of *Dioscorea* spp. using Areliquin version 3.01 (Excoffier *et al.*, 2006).

NTSYS-pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.*, 1999) software's were used to calculate Jaccard's similarity coefficient (S) by using the following formula:-

$$S_{ij} = \frac{a}{a+b+c}$$

Where, 'a' is the total number of bands shared between individuals i and j

'b' is the total number of bands present in individuals i but not in individuals j

'c' is the total numbers of bands present in individuals j but not in individuals i

The Unweighted Pair Group Method with Arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the accessions and generate phenogram using NTSYS-pc version 2.02 (Rohlf, 2000). The Neighbor Joining (NJ) method (Saitou *et al.*, 1987; Studier *et al.*, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.*, 1999).

To visualize patterns of variation among individual samples on two dimensional (2D) and three dimensional plot (3D), a principal coordinated (PCO) analysis was performed based on Jaccard's similarity coefficient (Jaccard, 1908). The calculation of Jaccard's similarity coefficient and construction of 2D and 3D plots were done using PAST software version 1.18 (Hammer *et al.*, 2001). The first three axes generated by PAST were used to plot the three dimensional PCO with STATISTICA version 6.0 software (Hammer *et al.*, 2001; Statistica Soft, Inc.2001).

5. RESULTS

5.1. Banding patterns of the ISSR primers

Six ISSR primers namely 811, 818, 844, 848, 873 and 880, were selected based on the presence of well defined, informative and good resolution bands. A total of 77 bands with a size ranging from 200bp to 3000bp and an average of 13 bands per primer were obtained with six ISSR primers on 70 accession of *Dioscorea* spp. (including *D. alata*) The highest number of scorable bands (17) was generated by penta-nucleotide ISSR-primer 880 whereas di-nucleotide ISSR-primers 844 and 811 generated the least number of scorable bands (11). The remaining ISSR-primers 818, 848 and 873 generated 13, 13 and 12 bands, respectively. Out of the six ISSR primers used, gel electrophoresis pattern obtained using primer ISSR-848 is illustrated in Figure 1. For the rest of the primers, gel pictures are shown in appendix 2.

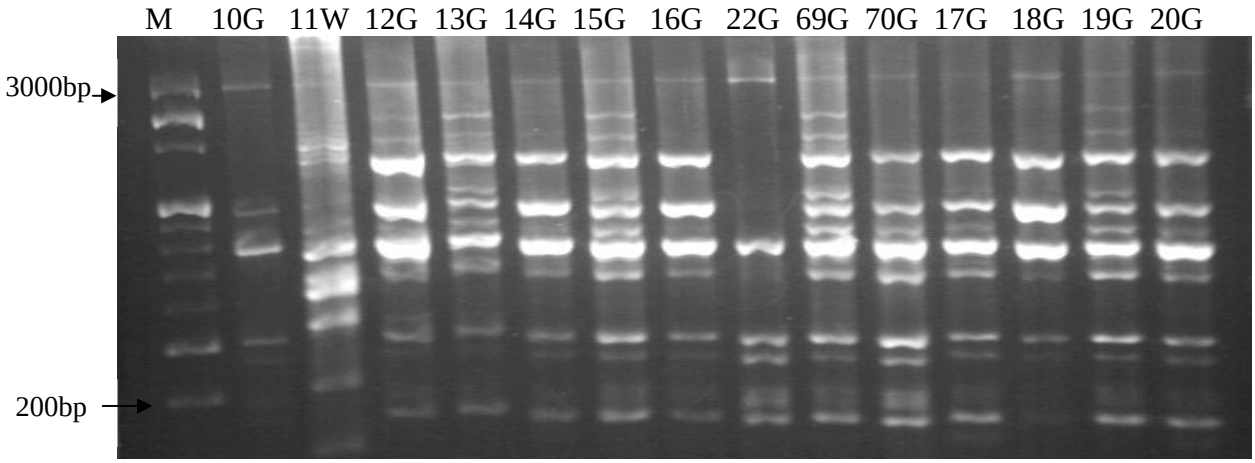

Figure 1. ISSR fingerprint generated for some *Dioscorea spp.* accessions using ISSR-Primer 848. Key: G= Guinea yam (*D. cayenensis/D.rotundata* complex; W= winged yam (*D. alata*)

For *D. cayenensis/D. rotundata* complex, a total of 75 bands were generated with number of bands produced by each primer ranging from 10 to 16, with the average bands per primer being 12 The highest number of bands was again amplified by primer 880 while primer 844 amplified the lowest number of bands (Table 4).

In *D. bulbifera* accessions, a total of 64 bands with 7 to 13 bands for each primer with an average of 11 bands per primer were resulted from the six ISSR primers used. Primer 880 and 818 produced the highest number of bands, while the lowest number of bands was amplified by primer 811 (Table 4).

5.2. Species specific ISSR bands in *D. cayenensis/D. rotundata* complex and *D. bulbifera*

Out of the six ISSR primers used, five ISSR primers showed species specific bands (Table 4). A total of 13 bands specific to *D. cayenensis/D. rotundata* complex were generated. Primer 811 and 880 showed the highest number of specific bands to this species (four bands). Only two bands specific to *D. bulbifera* were amplified by six primers. Two bands specific to *D. bulbifera* were generated by primers 844 and 880. ISSR primer 818 generated no specific bands to both species whereas primers 811, 848 and 873 generated specific bands to only *D. cayenensis/D. rotundata* complex (Table 4).

Table 4. List of ISSR primers used and their banding pattern in *D. cayenensis/D. rotundata* complex and *D. bulbifera.*

primers	Repeat motif	No of scorable bands			No of species specific bands	
		G	A	G and A	G	A
811	$(GA)_8C$	11	7	11	4	0
818	$(CA)_8G$	13	13	13	0	0
844	$(CT)_8RC$	10	8	11	3	1
848	$(CA)_8NG$	13	12	13	1	0
873	$(GACA)_4$	12	11	12	1	0
880	$(GGAGA)_3$	16	13	17	4	1
Total		75	64	77	13	2
Average		**12**	**11**	**13**	**2.16**	**0.33**

*G = Guinea yam (*D. cayenensis/D. rotundata *complex), A = Aerial yam (*D. bulbifera*),*

G and A = Combination of Guinea and Aerial Yam , N = (A, T,G,C); R = (A, G).

5.3. Genetic diversity within and among *Dioscorea* species

All six primers generated a total of 77 scorable bands, out of which 75 were polymorphic with 97.40% polymorphism. Furthermore, four primers (811, 844, 848 and 880) generated 100% polymorphism while, the rest two primers (818 and 873) showed 92.31% and 91.67% polymorphism, respectively. The overall gene diversity (h) for the six primers was 0.36, while Shannon's information index (I) was 0.53 (Table 5). Primer 880 showed the

highest gene diversity (0.39) and Shannon's information index (0.57) while, primer 811 showed the least gene diversity (0.29) and Shannon's information index (0.45) (Table 5).

In *D.cayenensis/D. rotundata* complex, a total of 75 bands were scored, of which 71 were polymorphic, equivalent to 92.21%. Two primers, 811 and 848, showed 100% polymorphism in this species, while primer 818 showed the least polymorphism, 76.92% (Table 5). The overall gene diversity (h) and Shannon's information index (I) for the six ISSR primers were 0.33 and 0.49, respectively (Table 5). From among six primers, primer 880 showed the highest gene diversity (0.37) and Shannon's information index (0.54), whereas primer 811 showed the least gene diversity (0.27), and the least Shannon's information index (0.43) was obtained from primer 811 and 818 (Table 5).

In *D.bulbifera,* a total of 64 bands were amplified on a total of 13 individuals, out of which 55 were polymorphic accounting for 71.43% polymorphism. The highest polymorphism (84.62%) was obtained from primer 848, while the least polymorphism (58.33%) was obtained from primer 873. Over all gene diversity (h) and Shannon's information index (I) for the six ISSR primers were 0.24 and 0.47, respectively, for *D.bulbifera*. The higher values for gene diversity (0.32) and Shannon's information index (0.47) were obtained from primer 848, while the lowest gene diversity (0.18) and Shannon's information index (0.28) were obtained from primer 873 (Table 5).

Table 5. Level of genetic diversity revealed by six ISSR primers in *D. cayenensis/D. rotundata* complex, *D. bulbifera* and the combination of the two species.

	Guinea Yam				Aerial Yam				Guinea Yam and Aerial Yam Combined			
Primer	NPL	PPL	h	I	NPL	PPL	h	I	NPL	PPL	h	I
811	11	100	0.27	0.43	7	63.64	0.19	0.30	11	100	0.29	0.45
818	10	76.92	0.29	0.43	10	76.92	0.27	0.41	12	92.31	0.35	0.51
844	10	90.91	0.35	0.51	7	63.64	0.29	0.41	11	100	0.37	0.55
848	13	100	0.35	0.52	11	84.62	0.32	0.47	13	100	0.38	0.56
873	11	91.67	0.35	0.52	7	58.33	0.18	0.28	11	91.67	0.36	0.53
880	16	94.12	0.37	0.54	13	76.47	0.22	0.35	17	100	0.39	0.57
Over all	71	92.21	0.33	0.49	55	71.43	0.24	0.47	75	97.40	0.36	0.53

NPL = Number of Polymorphic Loci; PPL = Percent of Polymorphic Loci; h = Nei's Gene Diversity; I = Shannon's Information Index

At the genus level, *D. cayenensis/D. rotundata* complex was more polymorphic (71 bands; 92.21 %) than *D. bulbifera* (55 bands; 71.43%). The same holds true for gene diversity and Shannon's information index with *D. cayenensis/D. rotudata* complex showing the highest value of gene diversity (0.33) and Shannon's information index (0.49) than *D. bulbifera* (Table 6).

For *D. cayenensis/D. rotundata* complex, Gedeo population showed the highest polymorphism (38 bands; 49.35%), while population from East Wellega showed the lowest

polymorphism with 20 bands and 25.97% polymorphism. Gene diversity and Shannon information index showed the same pattern with the highest values of 0.21 and 0.30, respectively, for population from Gedeo; while the least gene diversity (0.103) and Shannon's information index (0.15) being for population from East Wellega (Table 7).

For populations within *D cayenensis/D. rotundata* complex grouped in to geographic location such as South, Southwest and West, it was found that the South group showed highest polymorphism (61 bands; 79.22%) followed by Southwest (59 bands; 76.62%) and West (20 bands; 25.97%) (Table 7). Similarly, the highest gene diversity (0.29) and Shannon's information index (0.43) were obtained for South group followed by Southwest (0.28 gene diversity and 0.41 Shannon's information index) and West (0.103 gene diversity and 0.15 Shannon's information index).

Table 6. Genetic diversity at the genus level.

Analysis	n	NPL	PPL	h	I
Genus level					
Guinea Yam	55	71.0	92.21	0.33	0.49
Aerial Yam	13	55.0	71.43	0.24	0.47
Over all	68	75	97.40	0.36	0.53
Average	**34**	**63**	**81.82**	**0.28**	**0.48**

*n = Sample Size; NPL = Number of Polymorphic Loci; PPL = Percent of Polymorphic Loci; h = Nei's Gene Diversity; I = Shannon's Information Index; Guinea Yam (*D. cayenensis/D.rotundata *complex); Aerial Yam (*D. bulbifera*).*

Table 7. Genetic diversity within and among populations and groups of Guinea yam (*D. cayenensis/D. rotundata* complex).

Guinea yam	n	NPL	PPL	H	I
Populations					
Semen Omo	8	33	42.86	0.17	0.25
Gedeo	7	38	49.35	0.21	0.30
Jimma	6	23	29.87	0.108	0.16
East Wellega	13	20	25.97	0.103	0.15
Ilu ababora	4	36	46.75	0.18	0.26
Sheka-Kefa	4	31	40.26	0.17	0.25
Hadiya-Kembata	5	32	41.56	0.17	0.25
Over all	47	70	90.91	0.32	0.48
Average	**6.71**	**30.42**	**39.51**	**0.16**	**0.23**
Groups					
South	20	61	79.22	0.29	0.43
Southwest	14	59	76.62	0.28	0.416
West	13	20	25.97	0.103	0.15
Over all	47	70	90.91	0.32	0.48
Average	**15.67**	**46.66**	**60.60**	**0.224**	**0.332**

n = Sample Size; NPL = Number of Polymorphic Loci; PPL = Percent of Polymorphic Loci; h = Nei's Gene Diversity; I = Shannon's Information Index

5.4. Analysis of molecular variance (AMOVA)

AMOVA was conducted on the ISSR data generated using six ISSR primers; four di-nucleotide, one tetra-nucleotide and one penta-nucleotide for the combination of the two species: *D. cayenensis/D. rotundata* complex and *D. bulbifera* together and for *D. cayenensis/D. rotundata* complex separately with and without grouping. The analysis was carried out by computation of the distance between 'haplotypes' as each individual data pattern considered as one 'haplotype' and computing variance components for each level (i.e. presence 1, absence 0) (Exocoffer *et al*.,1992). AMOVA result obtained from the combination of the two species showed that most of the total genetic variation (63.87%) was found within species than among species (36.12%) (Table 8).

For *D. cayenensis/D. rotundata* complex without grouping, the percentage of genetic variation within population was higher (53.56%) than among population (46.44%) (Table 9). On the other hand, the partitioned genetic diversity using grouped populations showed that 50.873% of the total variation was attributed to within populations while the remaining diversity were found to be distributed among populations within groups (25.25%) and among groups (23.87%).

Table 8. Analysis of molecular variance (AMOVA) at the genus *Dioscorea* level.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-Value*
Guinea Yam and Aerial Yam combined						
Among species	1	98.767	6.400	36.128		P< 0.001
Within species	66	596.896	11.317	63.871		P< 0.001
Total	67	695.663	17.718		0.361	

*d.f = degree of freedom; * significance tests after 1023 permutations; Guinea Yam* = D. cayenensis/D. rotundata *complex; Aerial Yam* = D. bulbifera

Table 9. AMOVA for Guinea yam (*D. cayenensis/D. rotundata* complex) with and without grouping.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-Value*
Guinea yam (*D. cayenensis*/*D. rotundata* complex*)*						
Among populations	6	219.969	1.96	46.444		P< 0.001
Within populations	40	212.121	7.24	53.555		P< 0.001
Total	46	432.090	11.591		0.464	
Guinea yam (*D. cayenensis*/*D. rotundata* complex*)*						
Among groups	2	137.030	2.912	23.872		P< 0.001
Among populations within groups	4	83.939	3.081	25.254		P< 0.001
Within populations	40	212.121	6.208	50.873		P<0.001
Total	46	432.090	11.591		0.491	

*d.f = degree of freedom, * significance tests after 1023 permutations*

5.5. Cluster analysis

Neighbor joining (NJ) and Unweighted Pair-Group Methods Using Arithmetic Averages (UPGMA) clustering methods on the basis of Jaccard's coefficient of similarity were conducted to investigate the relationship among the 70 accessions of the three species (55 *D. cayenensis/D. rotundata* complex, 13 *D. bulbifera* and 2 *D. alata* (as a reference) together and 55 accessions of *D. cayenensis/D. rotundata* complex separately.

Three major clusters were identified from the dendrogram derived from UPGMA analysis of ISSR data for all the 70 accessions (Figure 2). In the first major cluster, only accessions belonging to *D. bulbifera* were grouped together. The two accessions of *D. alata* were clustered in the second major cluster (Major II), while the rest were grouped in to the third major cluster (Major III). Major cluster III showed several hierarchical sub-clusters in which some of the accessions tend to cluster according to their respective populations. Accordingly, the population from East Wellega clearly formed its own separate cluster. Samples from Gedeo also tend to cluster together with some accessions from elsewhere (exact origin unknown) which might be collected from Gedeo or adjacent area. Most of the accessions from Jimma also clustered together while population of Semen Omo tends to intermix with Hadiya-Kembata population but other accessions belonging to this major cluster did not show a clear grouping.

The tree generated from neighbor joining showed three major clusters (I, II and III) and two outliers. Major cluster I contains three accessions from Jimma; Major cluster II contains five accessions from Gedeo, one accession from Jimma and two accessions of unknown collection area; Major cluster III was further divided in to two sub-clusters: sub-

cluster I contains inter mix from Semen Omo and Hadiya-Kembata, sub-cluster II contains several hierarchical clusters in which only accessions from East Wellega clearly formed its own cluster. Unlike in UPGMA dendrogram, accessions belonging to *D. bulbifera* and *D. alata* didn't form their own major cluster but they have formed their respective group under sub-cluster II of major cluster I (Figure 3).

The UPGMA dendrogram constructed to show genetic relationship among 55 accessions of *D. cayenensis/D. rotundata* complex showed four major clusters (Major I, Major II, Major III and Major IV) (Figure 4). The first major cluster consists of two accessions (one from Gedeo and another from unknown collection area). Major cluster II contains two accessions (one from Ilu Ababora and one from Semen Omo). In major cluster III there are seven accessions with two accessions from Ilu Ababora forming their own sub-group. The rest five accessions (two from sheka-Kefa and three from unknown areas) formed the other sub-group. Major cluster IV is divided in to two sub-clusters (sub-cluster I and sub-cluster II) with each cluster divided into several sub-clusters. In sub-cluster I accessions from East Wellega formed their own distinct group. Accessions from Jimma and Gedeo also tended to form their respective group. Accessions from Semen Omo tended to intermix with that of Hadiya-Kembata under sub-cluster II. Although there is slight difference in clustering, almost the same grouping was recovered by the tree generated from neighbor joining analysis (Figure 5) which showed four major clusters (I, II, III and IV) and one accession from Gedeo as an outlier. Cluster I contains eight accessions; five accessions from Gedeo, one accession from Jimma and the other two accessions from unknown collection area; Cluster II contains one accessions from Jimma and another accession from Gedeo; Cluster

III containing three accessions from Jimma; Cluster IV containing two sub-clusters with sub-cluster I containing four accessions; two accessions from Sheka-Kefa and two accessions of Unknown area. Sub-cluster II containing several hierarchical clusters in which accessions from East Wellega clearly formed its own cluster, accessions from Semen Omo tends to intermix with accessions from Hadiya-Kembata, with no other accessions forming clear group based on their geographic origin.

In addition, population based dendrogram (Figure 6) constructed using UPGMA showed Jaccard's similarity coefficient for seven populations of *D. cayenensis/D. rotundata* complex and one population of *D. bulbifera* ranging from 0.301 to 0.746, with the highest similarity value (0.746) being between Semen Omo and Hadiya-Kembata populations of *D. cayenensis/D. rotundata* complex and the least similarity value (0.301) being among Semen Omo population of *D. cayenensis/D. rotundata* complex and Ilu Ababora population of *D. bulbifera* (Table 10).

Table 10. Pair wise Jaccard's similarity coefficient based comparisons among eight populations of *Dioscorea* spp. collected from Ethiopia.

	O- G	Ged-G	Jim-G	Well-G	I. Ab-A	SH-KF G	H-K G	I. Ab-G
O-G	1.000							
Ged-G	0.617	1.000						
Jim-G	0.664	0.740	1.000					
Well-G	0.553	0.605	0.709	1.000				
I. Ab-A	0.301	0.404	0.395	0.366	1.000			
SH-KFG	0.549	0.585	0.635	0.553	0.362	1.000		
H-K G	0.746	0.593	0.647	0.550	0.314	0.592	1.000	
I. Ab-G	0.475	0.532	0.566	0.502	0.310	0.535	0.464	1.000

*O = Semen Omo; Well = East Wellega; I. Ab = Ilu Ababora; SH-KF = Sheka-Keffa; Jim = Jimma; Ged = Gedeo; H-K = Hadiya-Kembata; G after population name = Guinea Yam (*D. cayenensis/D. rotundata *complex; A after population name = Aerial Yam (*D. bulbifera*)*

Key:

- O = Semen Omo
- H-K = Hadiya-Kembata
- G = Gedeo
- SH-KF = Sheka-Kefa
- J = Jimma
- W = East Wellaga
- I = Ilu Ababora
- G after number = Guinea yam (*D. cayenensis/D. rotundata* complex)
- A after number = Aerial yam (*D. bulbifera*)
- W after number = Winged yam (*D. alata*)

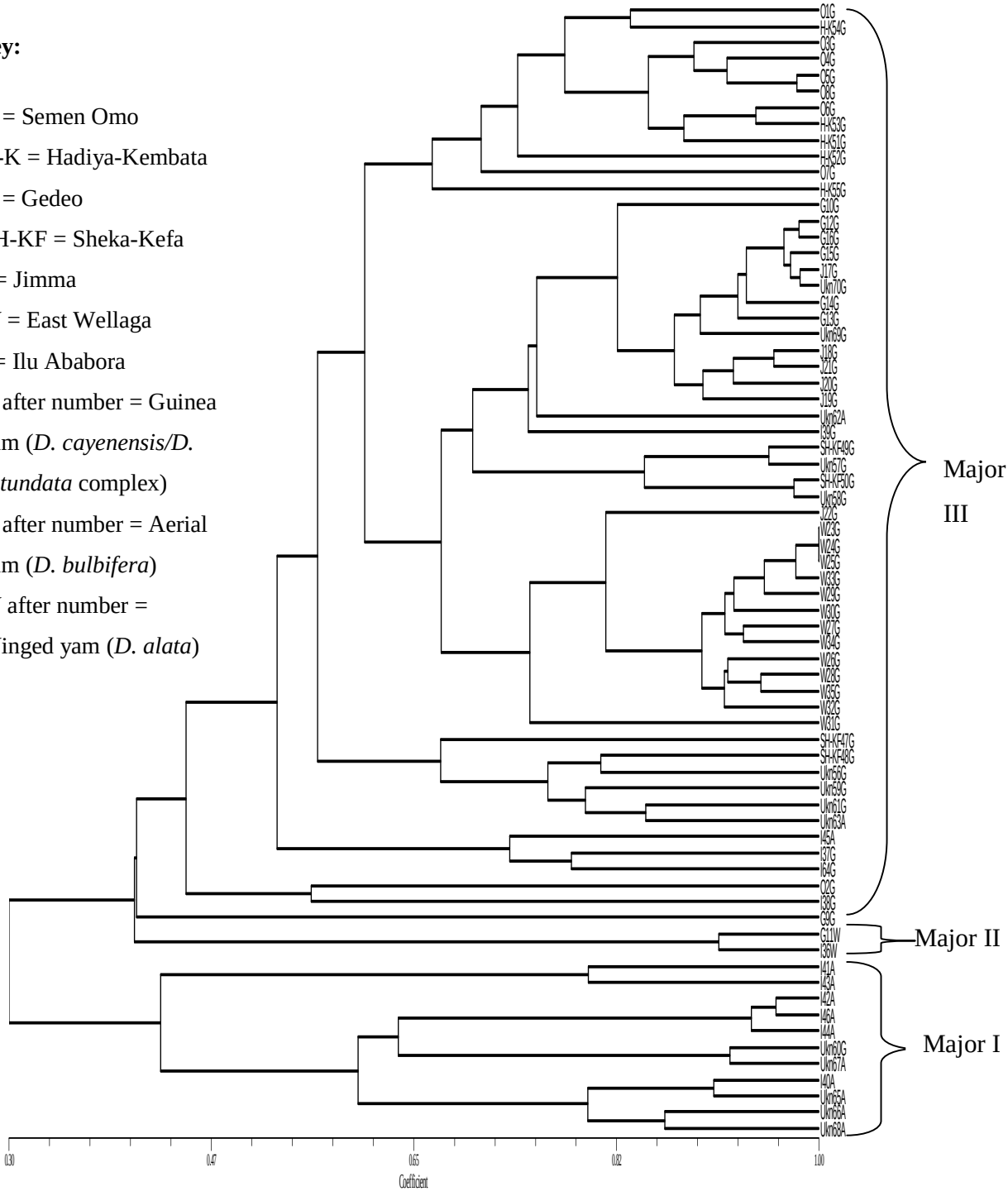

Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient among 55 *D. cayenensis/D. rotundata* complex , 13 *D. bulbifera* and 2 *D. alata* accessions.

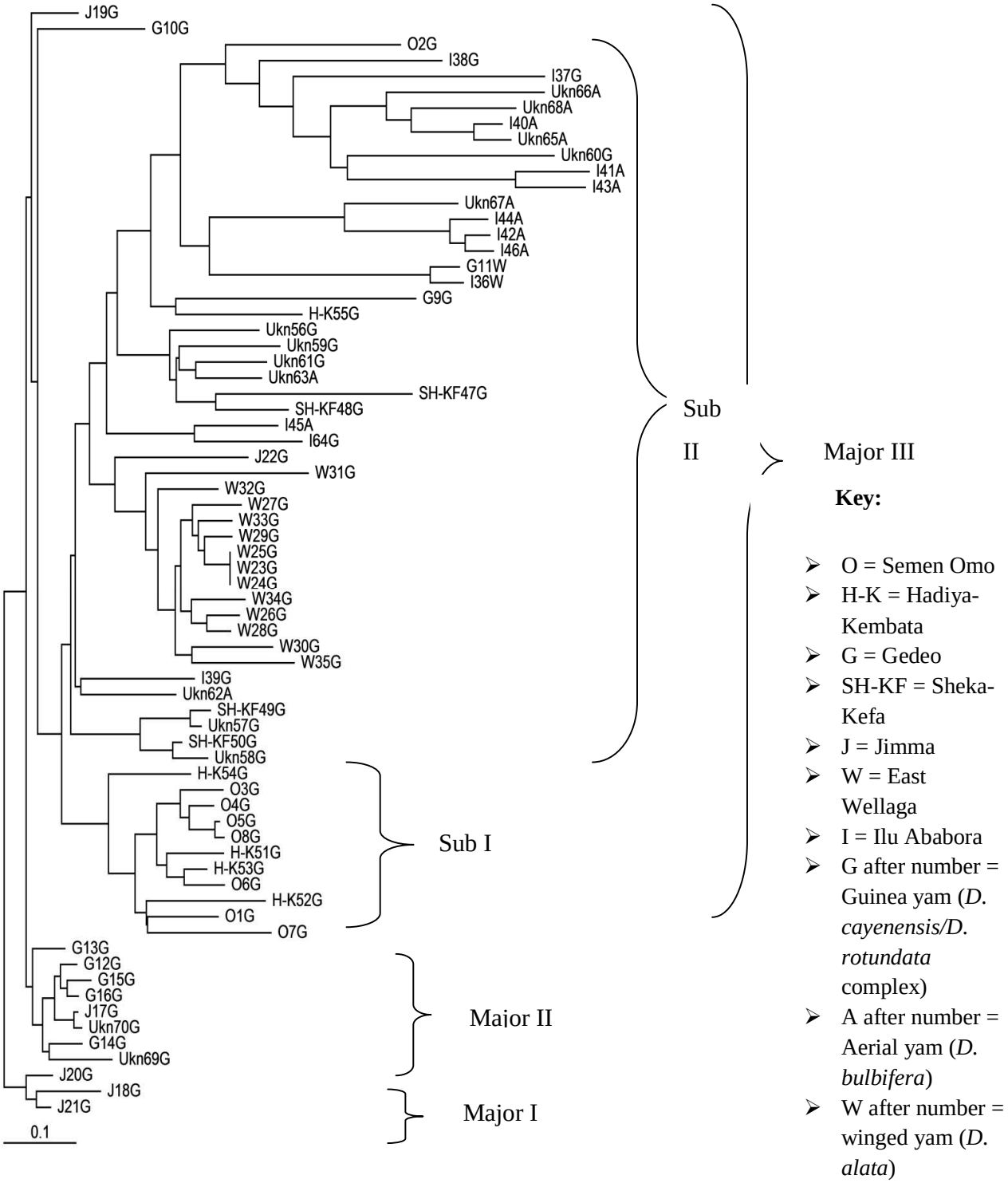

Figure 3. Neighbor-joining dendrogram for 55 *D. cayenensis/D. rotundata* complex, 13 *D. bulbifera* and 2 *D.alata* accessions using six ISSR-primers.

Key:

- O = Semen Omo
- H-K = Hadiya-Kembata
- G = Gedeo
- SH-KF= Sheka-Kefa
- J = Jimma
- W = East Wellega
- I = Ilu Ababora
- G after number = Guinea yam (*D. cayenensis/D. rotundata* complex)

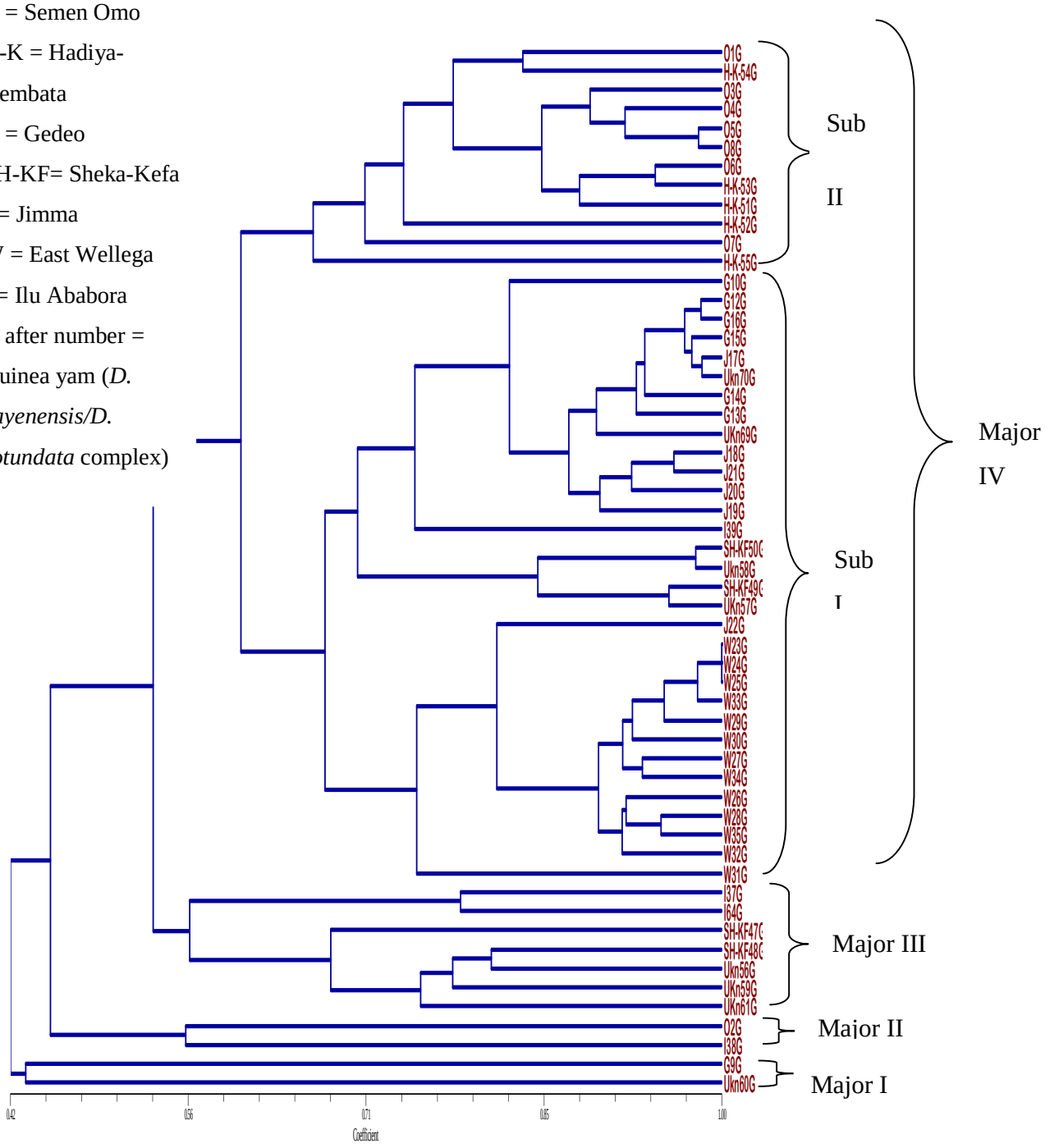

Figure 4. UPGMA dendrogram based on Jaccard's similarity coefficient among 55 *D. cayenensis/D. rotundata* complex accessions.

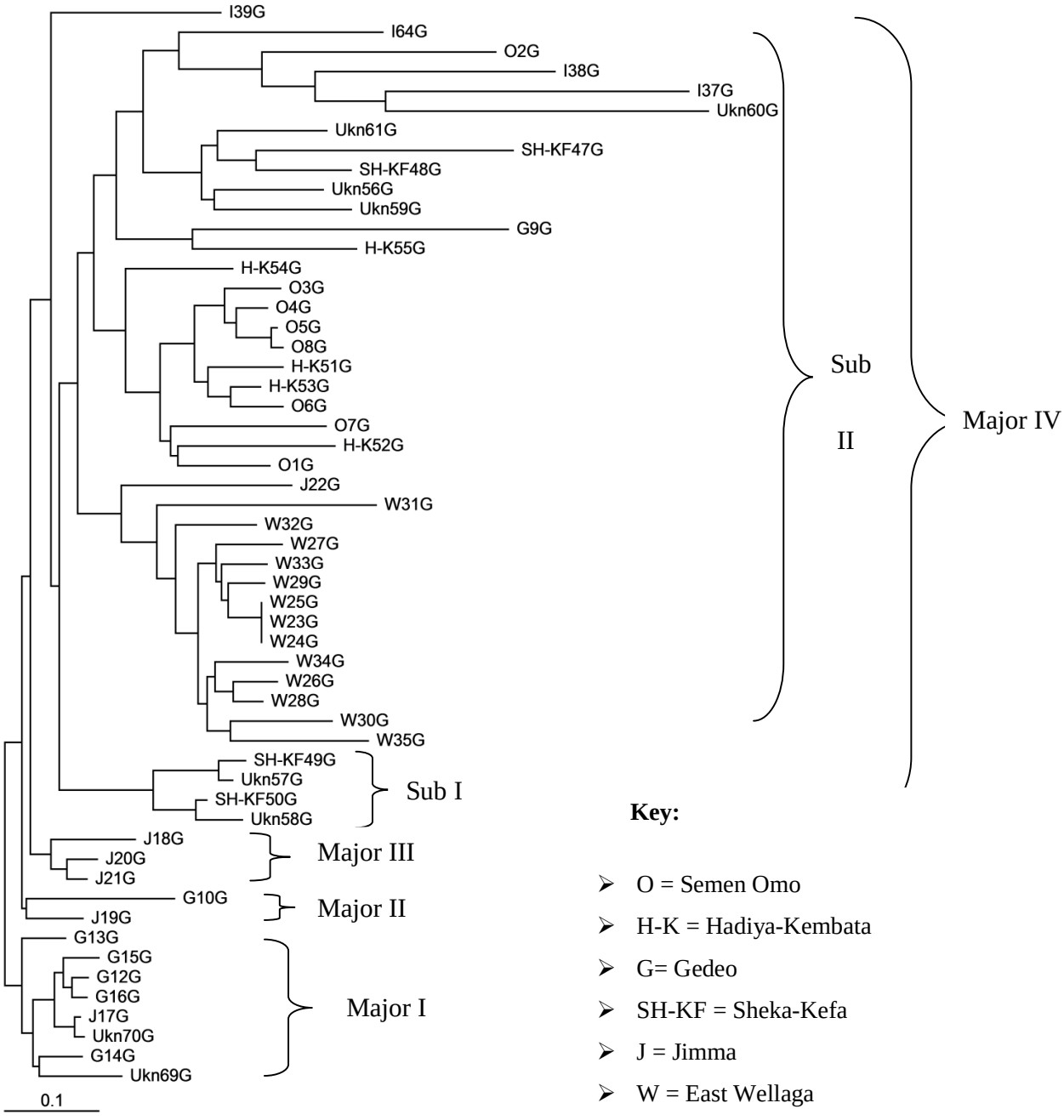

Figure 5. Neighbor-joining dendrogram constructed based on Jaccard's similarity coefficient for 55 accessions of *D. cayenensis/D. rotundata* complex.

Key:

- Omo = Semen Omo
- H-K = Hadiya-Kembata
- G = Gedeo
- SH-KF = Sheka-Kefa
- W = East Wellaga
- I = Ilu Ababora
- G after population name = Guinea yam (*D. cayenensis/D. rotundata* complex)
- A after population name = Aerial yam (*D. bulbifera*)

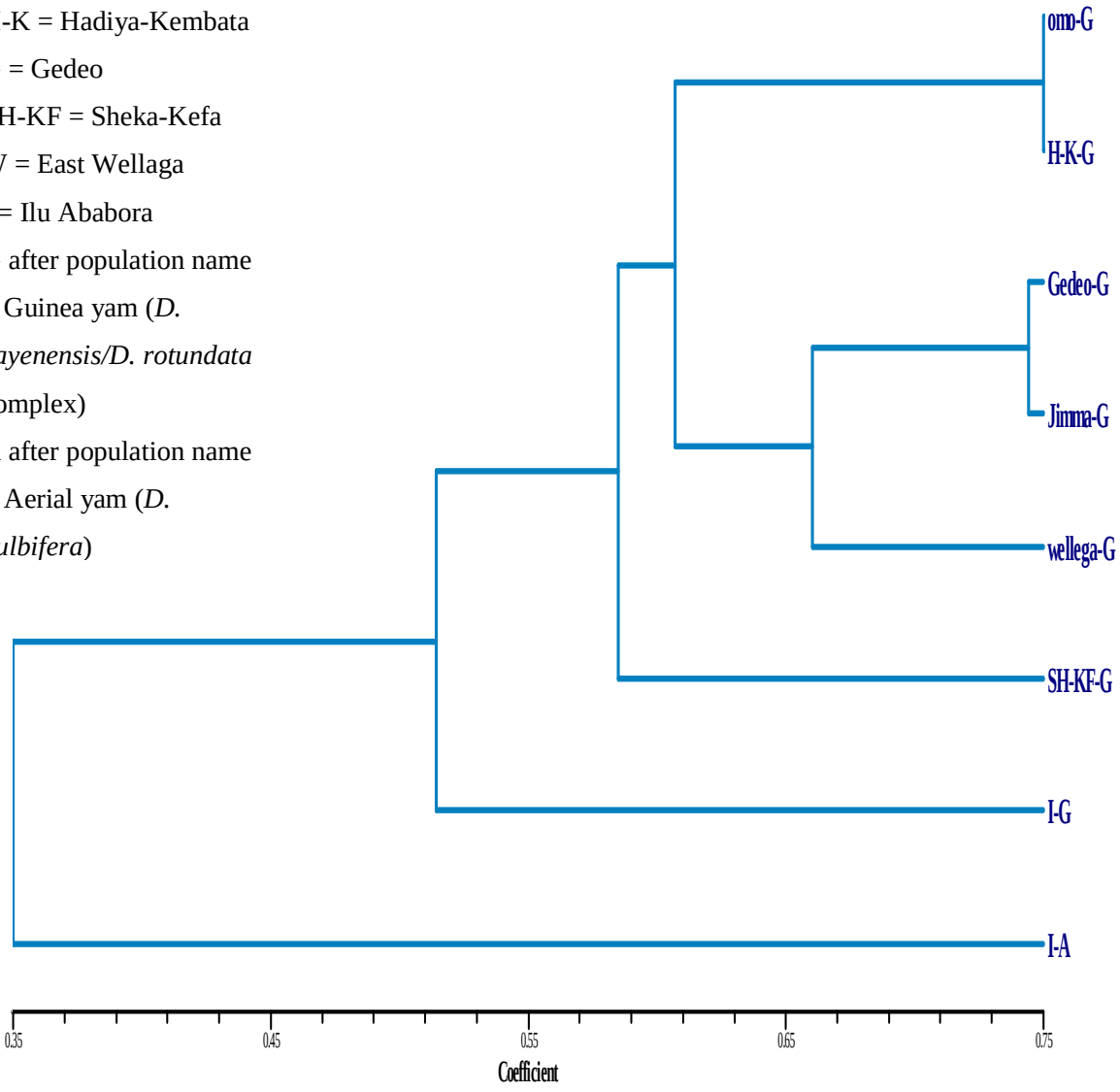

Figure 6. UPGMA based dendrogram for eight populations of *Dioscorea* spp. (seven *D. cayenensis/D. rotundata* complex; one *D. bulbifera*).

5.6. Principal coordinate (PCO) analysis

All the data obtained using six ISSR primers were used in PCO analysis using Jaccard's similarity coefficient to plot 2D and 3D coordinate planes for all 70 accessions (55 *D. cayenensis/D. rotundata c*omplex; 13 *D. bulbifera*; 2 *D. alata*) together and for *D. cayenensis/D. rotundata c*omplex alone. The first three coordinates of the PCO constructed for both species together had eigen-values of 7.1, 5.1 and 3.4 with percentage of 13.8%, 9.8% and 6.6%, respectively. The analysis with two coordinates showed that accessions belonging to *D. bulbifera* formed their own cluster (Figure 7), the two accessions of *Dioscorea alata* also clustered together while accessions of *D. cayenensis/D. rotundata* complex somehow spread throughout the graph with some of them forming sub-groups according to their geographic location. The result of three coordinate analyses also showed the same pattern (Figure 8).

The first three coordinates of the PCO constructed to show grouping of all accessions of *D. cayenensis/D. rotundata* complex had eigen-values of 5.1, 4.1 and 3.1 with percentage of 14.1%, 11.4% and 8.7%, respectively. On the 2D plot accessions from East Wellega formed their own group to the left side of the plot, accessions from Jimma and Gedeo grouped together to the right bottom of the plot and accessions from Semen Omo grouped with that of Hadiya-Kembata. Other as did not show clear grouping (Figure 9). This was further confirmed with three dimensional principal coordinate analyses (Figure 10).

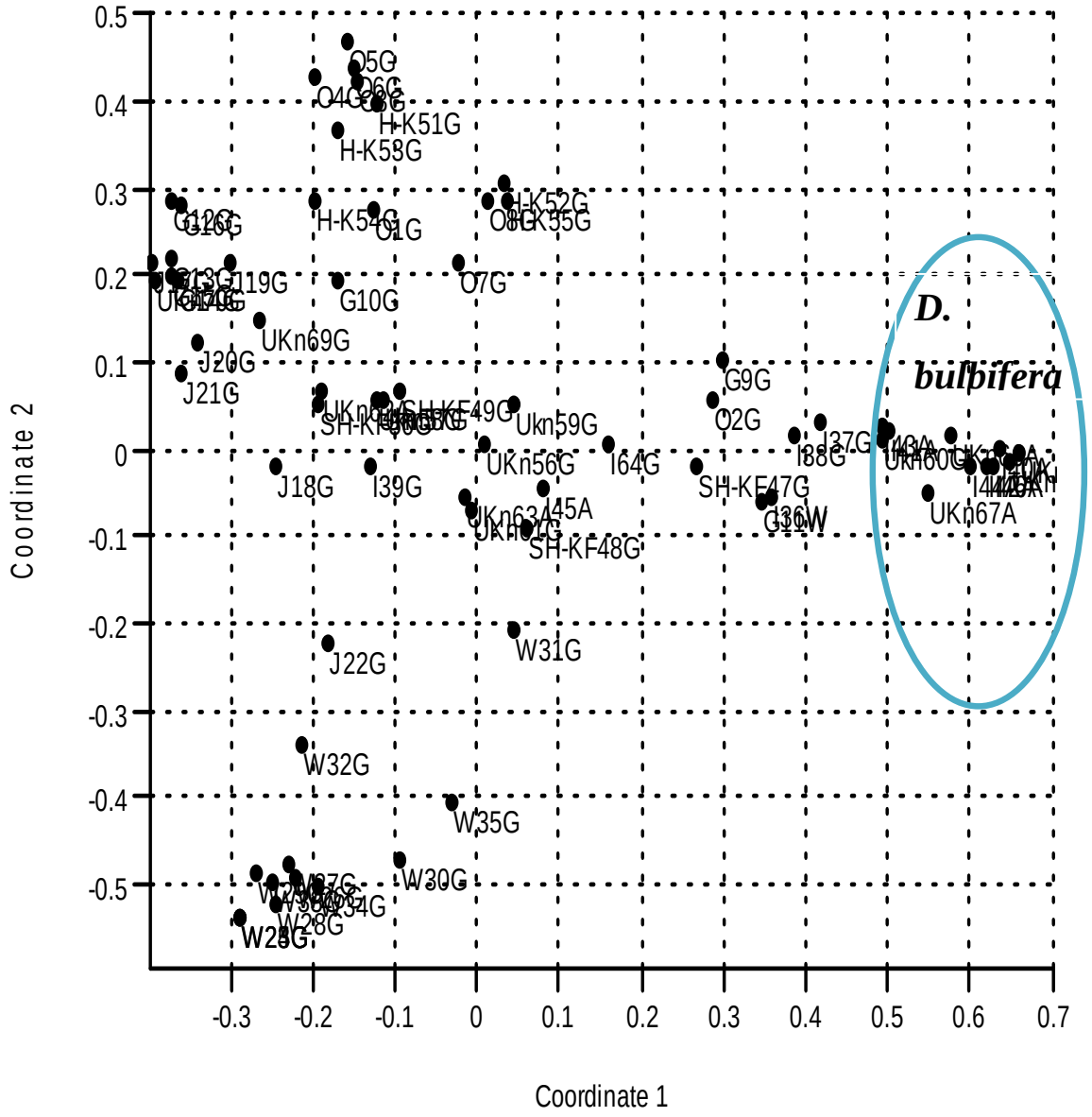

Figure 7. Two-dimensional plot obtained from principal coordinate analysis of 70 (55 *D. cayenensis/D. rotundata* complex, 13 *D. bulbifera*, 2 *D.alata*) accessions using Jaccard's similarity coefficient.

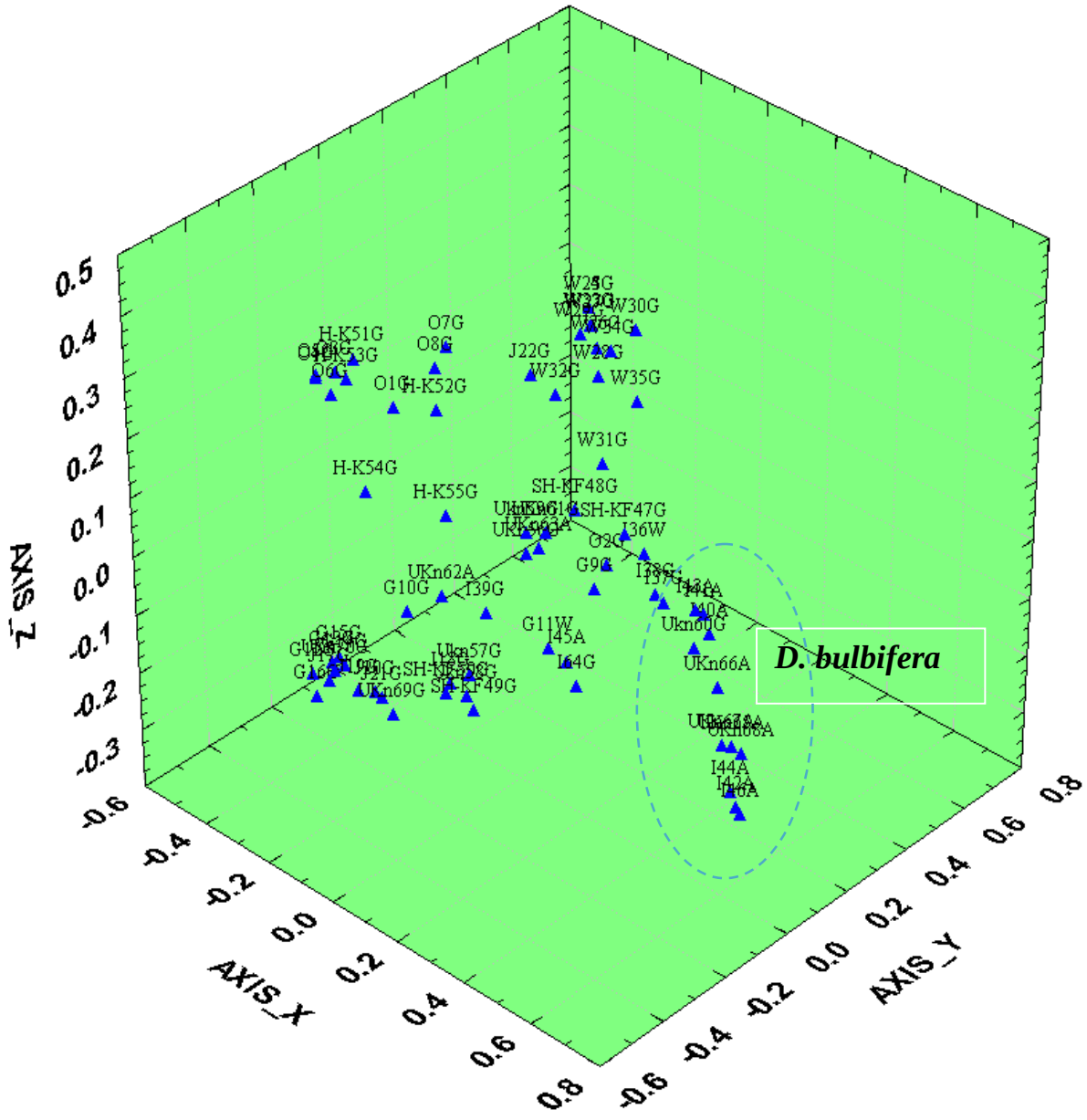

Figure 8. Three-dimensional plot obtained from principal coordinate analysis of 70 (55 *Dioscorea cayenensis/D. rotundata* complex; 13 *D. bulbifera*; 2 *D.alata*) accessions using Jaccard's similarity coefficient.

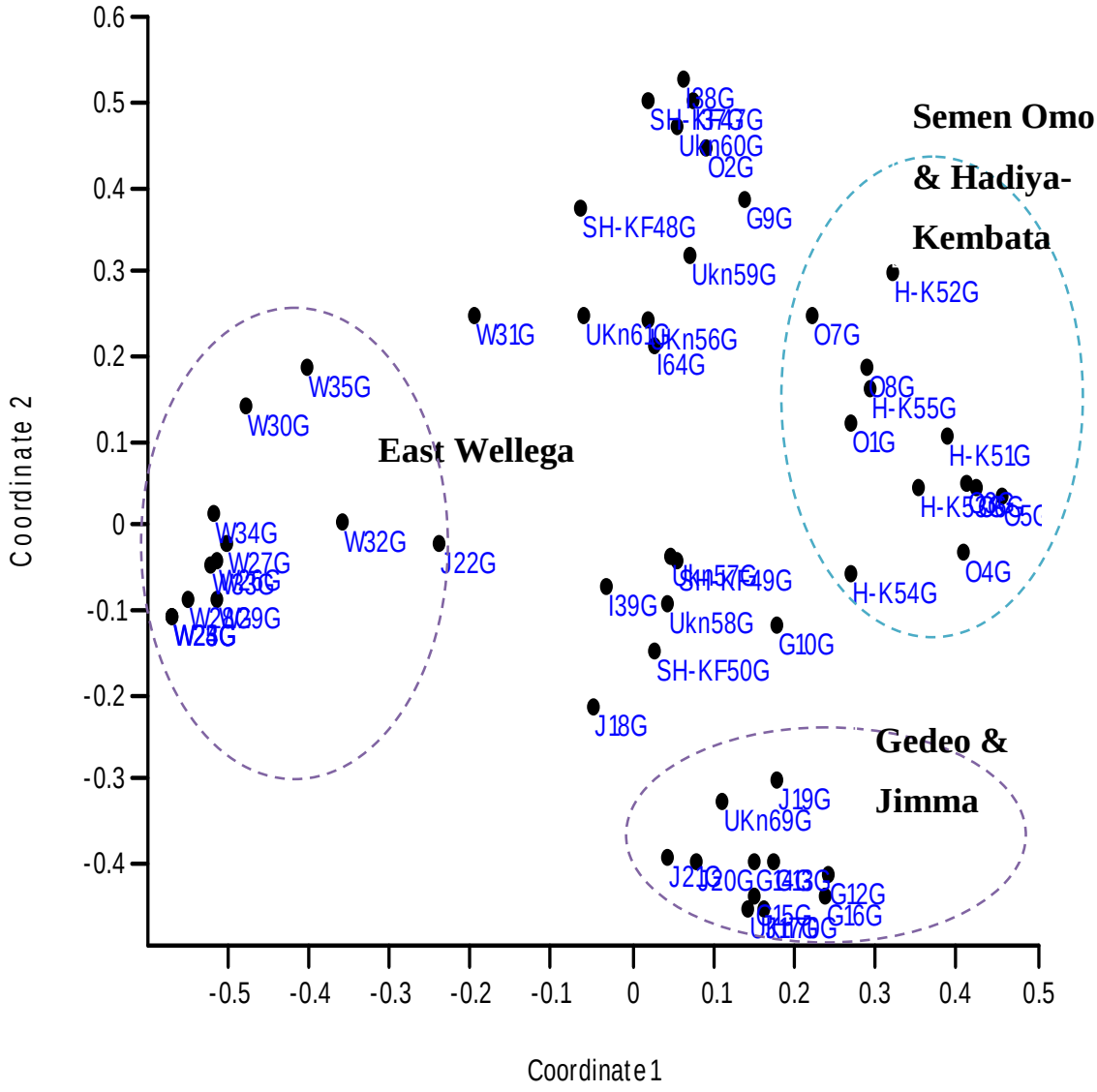

Figure 9. Two-dimensional plot obtained from principal coordinate analysis of 55 *D. cayenensis/D. rotundata* complex accessions using Jaccard's similarity coefficient.

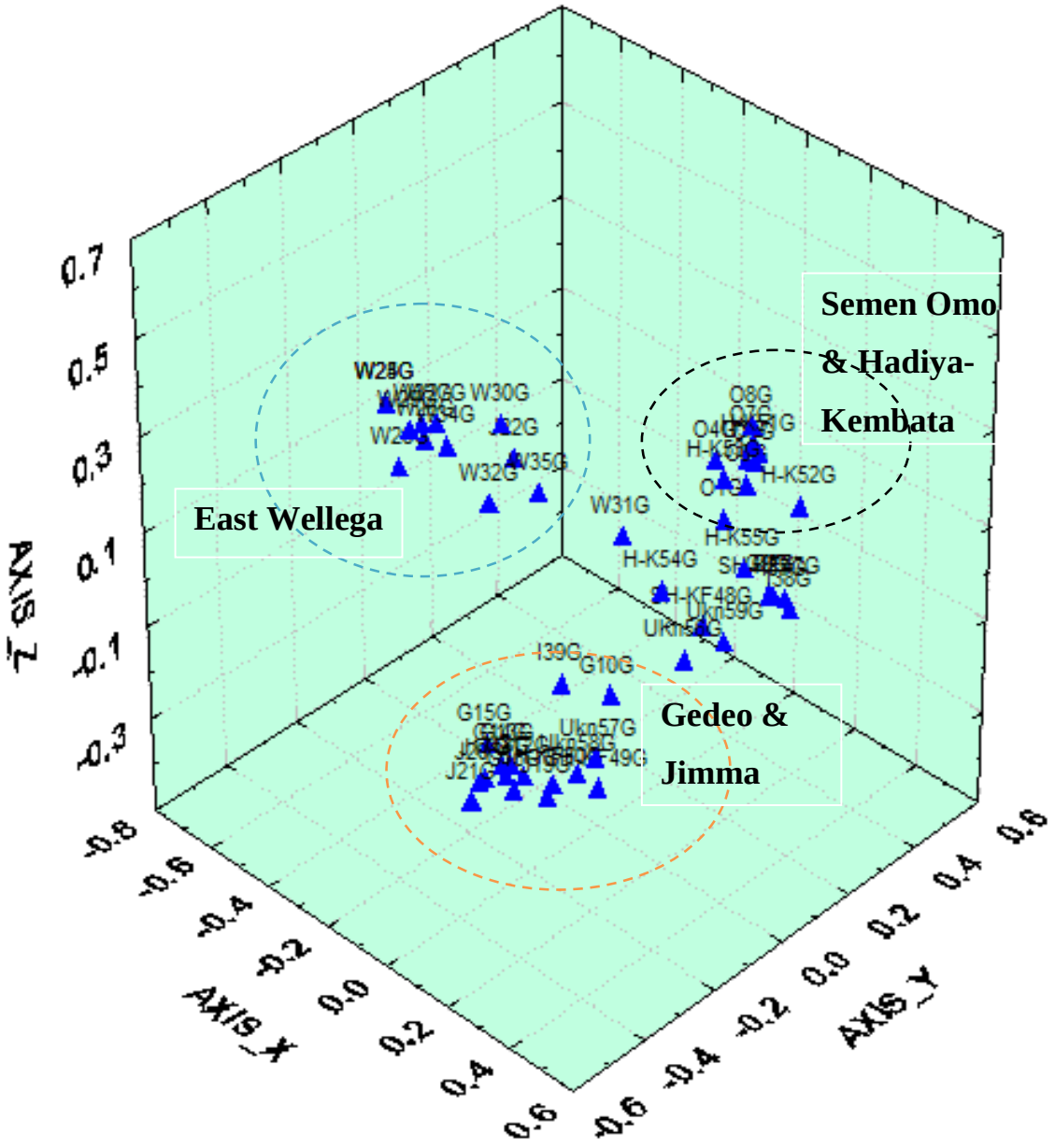

Figure 10. Three-dimensional plot obtained from principal coordinate analysis of 55 *D. cayenensis/D. rotundata* complex accessions using Jaccard's similarity coefficient.

6. DISCUSSION

6.1. Application of ISSR marker in *Dioscorea* species genetic diversity assessment

In the present study, ISSR markers have been used to assess the level and pattern of inter- and intra-species genetic diversity of *D. cayenensis/D. rotundata* complex and *D. bulbifera* accessions from Ethiopia. Six ISSR primers (four di-nucleotide, one tetra-nucleotide and one penta-nucleotide) were used. Out of which, four primers, namely 811, 844, 848 and 880 with GA, CT, CA and GGAGA repeats respectively, showed 100% polymorphism, and thus, suggests that the GA, CT, CA and GGAGA microsatellite repeats and their adjacent regions can be utilized for detecting wide range of genetic diversity throughout the genome of *Dioscorea* spp. The highest gene diversity (0.39) and Shannon's index (0.57) were shown by ISSR-880 as Shannon's information index and Nei's gene diversity are more reliable estimate of genetic diversity due to sensitivity of PPL to sample size (Freeland *et al.,* 2011). All primers used in this study revealed high level of genetic variation with overall polymorphism, Nei's gene diversity and Shannon's information index being 97.40%, 0.36 and 0.53, respectively, which indicates existence of high diversity within the two species. Among the two species *D. rotundata/D. cayenensis* complex was more diverse this might be due its larger sample, being a combination of various populations collected from different geographic areas and being representing two species complex. The larger the population size, the higher the variability (Ravikanth *et al*., 2008).

In *D. cayenensis/D. rotundata* complex, the overall percent polymorphic loci, Nei's gene diversity and Shannon's information index were 92.21%, 0.33 and 0.49, respectively. This indicates the existence of high genetic diversity within the complex. Although, there is no

report regarding the use of ISSR in diversity study of the complex, comparable result was reported by Wendawek Abebe *et al*. (2012) who used AFLP fingerprinting to evaluate and characterize 43 individuals belonging to different populations of wild and cultivated guinea yam (*D. cayenensis/D. rotundata* complex) using three primers combination and detected 78% polymorphism. Bressan *et al.* (2014) also evaluated 21 local varieties of *D. cayenensis* and two *D. rotundata* accessions using 7 isozymic loci and twenty four morphological markers and reported the existence of high genetic variability with 100% polymorphism using isozyme marker. Dansi *et al*. (2000a) and Mignougna *et al*. (2002) also studied genetic diversity of *D. cayenensis/D. rotundata* complex using isozyme markers in 7 and 6 isozyme systems, respectively, and reported the existence of high diversity (Polymorphism in all analyzed isozyme systems) which is in agreement with the present study.

The ISSR investigation of accessions of *D. bulbifera* using the six ISSR primers also indicated the existence of high genetic variation, with the overall percent polymorphism (PPL), Nei's gene diversity and Shannon's information index being 71.43%, 0.24 and 0.47, respectively. Despite its smaller sample size (13), *D. bulbifera* accessions still showed high genetic diversity but lower than *D. cayenensis/D. rotundata* complex. This shows that small populations or individuals are not always associated with a lack or low level of genetic variation (Yingjuan and Ting, 2009). Likewise, Tewodros Mulualem *et al*. (2013) studied the level of genetic diversity within *D. bulbifera* accessions collected from South and Southwestern Ethiopia based on key agronomic traits and reported the existence of high diversity in the region.

In terms of intra-species genetic variation of *D. cayenensis/D. rotundata* complex, the present study indicated the presence of high genetic diversity among seven populations collected from South, Southwest and West. From among the seven populations, Gedeo population was found to be the most polymorphic (PPL=49.35%; h=0.21; I=0.30), while East Wellega population was the least diverse (PPL=25.97%; h=0.103; I=0.15). Gedeo areas are known for their traditional agro-forestry system in which they grow a variety of crop plants including tuber crops and the area is considered as a candidate for Globally Important Agricultural Heritage System by Ministry of Forest and Agriculture (Wubalem Tadesse, 2014) which might be the reason for the highest diversity of yams in the area. In case of East Wellega, it is geographically a bit isolated with no/little incoming genes or tubers as a seed. Spatially separated populations may experience isolation-by-distance (Wright, 1943), in which geographical distances cause restricted gene flow. Therefore, it is more likely that the genetic diversity in this area might be lower due to presence of lower or no tuber exchange with other populations.

Both *D. cayenensis/D. rotundata* complex and *D. bulbifera* showed a higher genetic diversity at the species level than the average percentage (70%) observed in higher plants (Zou *et al.*, 2001). Shannon's information index of both species (0.49 *D. cayenensis/D. rotundata* complex; 0.47 *D. bulbifera*) were also higher than the average values for widespread species (0.202) as suggested by Hamrick and Godt (1989).

The high level of genetic variation found in this study might be accounted to functionality of sexual reproduction. Although, there is no report so far, on the use of seeds by farmers, there is a chance that the seeds falling off of the plant might germinate and result in

seedlings that could grow to a full plant and harvested by local farmers. Thus, incorporation of spontaneous seedlings could result in high genetic variability. Moreover, presence of wild relatives such *as D. abyssinica* and *D. praehensilis* (Miege and Sebsebe Demissew, 1997) in close proximity with cultivated species might also be the major source of higher variation observed in *D. cayenensis/D. rotundata* complex. The same holds true for *D. bulbifera* which is native to Ethiopia (Westphal, 1975).

6.2. Partitioning of genetic diversity in Yams (*Dioscorea* spp.)

The knowledge of how genetic variation is partitioned among populations may have important implications not only in evolutionary biology and ecology, but also in conservation biology. Hence, reliable estimates of population differentiation are crucial to understand the connectivity among populations and represent important tools to develop conservation strategies (Ballox and Moulin, 2002).

AMOVA result at genus level showed higher within species (63.87%) variation than among species (36.13%), which is in agreement with the molecular and agronomic trait diversity study on *Dioscorea* spp. from Ethiopia by Atnafua (2014). This might be accounted to the presence of several shared bands between these species, which indicates that they might have close evolutionary relationship and/or admixture on farmers field might have facilitated gene flow (Pollen flow) since they are most likely cross compatible due to their similar ploidy level (2n= 40, 60, 80 have been reported in both *D. cayenensis/D. rotundata* complex and *D. bulbifera*) (Coursey, 1967; Asiedu, 1997). The potential for gene exchange has long been recognized even between taxa with large differences in chromosome numbers (Stebbins, 1971).

AMOVA was also computed for *D. cayenensis/D. rotundata* complex alone. Most of the genetic variation in *D. cayenensis/D. rotundata* complex was found within population (53.56%) than among population (46.44%). Similarly, Muluneh Tamiru *et al*. (2007) also assessed genetic diversity of yam (*Dioscorea* spp.) germplasms from Ethiopia and their relatedness to the main *Dioscorea* spp. by AFLP markers and found 81% of the total genetic variation within populations and only 19% among populations. These suggests that there is gene flow between those areas in the form of tuber exchange for planting. Mixed type of propagation (vegetative; seed) might also be the reason.

6.3. Genetic relationships within and among species

Cluster analysis presents patterns of relationships between genotypes and hierarchical mutually exclusive grouping such that similar descriptions are mathematically gathered into same cluster (Aremu, 2005). Genetic relationship among and within breeding materials can also be identified and classified using multivariate grouping methods such as principal coordinate analysis (PCO) (Aremu, 2011). Thus, in order to visualize the genetic relationship among all accessions of the three species together and *D. cayenensis/D. rotundata* complex separately; UPGMA clustering, NJ clustering, and PCO analysis were used.

Accordingly, UPGMA constructed at genus level showed grouping of most of the accessions to their respective species. The same grouping was recovered from two dimensional and three dimensional PCO plots. Unlike UPGMA, dendogram constructed using neighbor joining analysis did not show grouping of the three species separately into

their own major clusters but accessions belonging to *D. bulbifera* and *D. alata* formed their own distinct groups as a sub-cluster. Two dimensional PCO also showed grouping of accessions according to their respective species. Same pattern were obtained from three dimensional PCO. Similar result was reported by Rivera-Jimenez *et al.* (2011) who carried out molecular characterization of twenty accessions of *Dioscorea* spp. using the AFLP molecular technique to determine the distribution of their intra and interspecific genetic variation. Mignouna *et al*. (2005) also used RAPD and double stringency PCR (DS-PCR) to investigate genetic relationship and the extent of redundancy among thirteen accessions of two cultivated and thirty five accessions of four wild yam (*Dioscore*a spp.) species collected from Nigeria and used UPGMA clustering which grouped most of the accessions according to the species. This report demonstrates the ability of ISSR markers to distinguish accessions of closely related species like other DNA markers.

The UPGMA dendrogram of *D. cayenensis/D. rotundata* complex accessions showed that some accessions of the same population formed their own cluster at about 70% similarity level, while others did not show clear group. Moreover, all accessions from East Wellega population were grouped and the genetic similarity values among accessions were relatively high. Accessions from Semen Omo were grouped together with that of Hadiya-Kembata population, while accessions from Gedeo were clustered together with that of Jimma but both populations somehow tend to form their own group within this cluster. The two dimensional plot (PCO) showed the same pattern. This was further confirmed by three dimensional PCO plot. Similarity between Semen Omo and Hadiya-Kembata population is expected due to geographical proximity of those areas. However, Genetic similarity was

also present between Gedeo and Jimma accessions inspite of their geographical distance. Hence, this study showed that there is no strong correlation between geographic distance and genetic diversity. This could be explained in terms of movement of the people carrying tubers with them and distribution of cultivars over great distance as clones in the course of human movement. Grouping of different population in the same cluster has been stated by Hollingsworth and Ennos (2004) as an evidence for gene flow between populations.

Based on cluster analysis, six accessions (I41A and I43A of *D. bulbifera*; G9G, Ukn60G, O2G and I38G of *D. cayenensis/D. rotundata* complex) were found to be distantly related to other accessions of their respective species. They can be used as parental material in future yam breeding program to exploit heterosis.

6.4. Relationship between *D. cayenensis/D. rotundata* complex accessions

Cluster analysis and principal coordinate analysis based on ISSR data failed to produce boundary between different Guinea yam (*D. cayenensis/D. rotundata* complex) types. One of the main characteristics previously used to distinguish *D. cayenensis* and *D. rotundata* was tuber flesh colour (white fleshed= *D. rotundata*; yellow fleshed = *D. cayenensis*) (Onyilagha and Lowe, 1985). However, in this study no clear boundary was found between different flesh coloured accessions as reveled by cluster analysis. Moreover, the tuber colour of the accessions analyzed was found to be variable. Very few accessions belonging to this complex showed clear white and yellow flesh colour, the other accessions showed purple, mixture of purple and white, purple and yellow, white and yellow and white, yellow and purple (Appendix 3). Purplish colour, a central white surrounded by purplish colour and a mixture of white and purplish colour are characteristics of *D. praehensilis* and

D. abyssinica (Wild Guinea yams). Therefore, the accessions with purplish as well as mixture of white and purplish colour in this study might belong to wild guinea yams (*D. praehensilis* and/*D. abyssinica*) (Appendix 3). The other accessions might be intermediates (inter-cluster hybrid). Dansi *et al*. (2000a) also found a third group intermediate between *D. cayenensis* and *D. rotundata* corresponding probably to an inter-cluster hybrids, when studying polymorphic enzyme systems using isozyme marker.

Generally, ISSR data failed to produce any clear boundary between different types of Guinea yams that showed domestication characteristics of different species (wild; cultivated; intermediate). Wendawek Abebe *et al*. (2012) used AFLP genetic finger printing to evaluate and characterize 43 individual plants belonging to different population of wild and cultivated guinea yams and reported that ordination and cluster analysis failed to produce any clear boundaries between either the guinea yam accessions or between them and their wild relatives which is in agreement with the present study. The same result was reported by Chaïr *et al*. (2005) who investigated changes in chloroplast DNA simple sequence repeats (cpSSR) in 148 yam accessions selected to cover the wider possible genetic diversity existing in Benin.

The finding of the present study based on cluster analysis supports the reports of Miege and Sebsebe Demissew (1997), which indicates that they are species complex with many intermediates as well as the suggestion that *D. abyssinica* and/or *D. praehensilis* are parents for several cultivars of *D. cayenensis*/*D. rotundata* complex.

7. CONCLUSION

ISSR markers used in this study revealed a significant genetic variation among and within *D. cayenensis/D. rotundata* complex and *D. bulbifera* populations as well as within and among populations and groups of *D. cayenensis/D. rotundata* complex accessions collected from major growing areas of Ethiopia as revealed by utilizing percent polymorphism, Nei's gene diversity, Shannon's information indices, AMOVA and cluster analysis.

Both *D. cayenensis/D. rotundata* complex and *D. bulbifera* showed high genetic variation. In *D. cayenensis/D. rotundata* complex, the highest genetic diversity was found within Gedeo population, which indicates that this population can be considered as a source of diverse individuals in future improvement of the crop. On the contrary, East Wellega population, which showed the least diversity needs special attention for conservation.

Variation within species seemed to be greater than that of among species. Similarly, AMOVA analysis of *D. cayenensis/D. rotundata* complex populations showed higher within population variation than among population variation which indicates existence of high level of gene flow (tuber exchange) resulting in low differentiation among populations.

UPGMA cluster analysis and PCO analysis showed clustering of most of the accessions to their respective species and in some cases to their geographic origin. UPGMA, NJ and PCO of *D. cayenensis/D. rotundata* complex accessions also showed grouping of some of

the accessions with respect to their population but it failed to differentiate between different guinea yam (*D. cayenensis/D. rotundata* complex) types.

Generally, the high level of genetic variation obtained in this study could be utilized for future improvement (breeding) of the crop for specific characteristics. It is interesting to note that the present study is the first report of its kind revealing genetic diversity of yams (*Dioscorea* spp.) from Ethiopia using ISSR markers.

8. RECOMMENDATIONS

Based on the analysis of 70 accessions representing seven populations using ISSR DNA marker, the following recommendations are forwarded:

- Gedeo populations which were found to be highly diverse should be considered for future improvement of the crop while, East Wellega populations that were observed to be least diverse needs special conservation attention.
- Ethiopian Institute of Biodiversity (EIB) and/or any stakeholder needs to collect additional accessions to better represent populations and regions. Moreover, both cultivated and wild relatives of *Dioscorea* spp. present in other regions of the country should also be collected to represent the country at large.
- *In situ* conservation and/or on-farm conservation should be given attention to prevent random genetic drift.
- Taxonomic ambiguity associated with Guinea yams (*D. cayenensis/D. rotundata* complex) should be clarified by sequence based analysis and barcoding genes.
- Population genetic analysis should be carried out with more samples and co-dominant markers such as microsatellites and SNPs.
- Introduction of this important crop to other parts of the country accompanied by awareness creation will be of great importance in ensuring food and nutrition security of growing Ethiopian population.

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10. APPENDICES

Appendix 1. List of accession of *Dioscorea* species used for genetic diversity analysis.

No	Accessions	Species	Zone	Altitude	Lab code
1	236017	Guinea Yam	Semen Omo	2000	O1G
2	236018	Guinea Yam	Semen Omo	-	O2G
3	236019	Guinea Yam	Semen Omo	-	O3G
4	236020	Guinea Yam	Semen Omo	1910	O4G
5	236021	Guinea Yam	Semen Omo	1940	O5G
6	236022	Guinea Yam	Semen Omo	1950	O6G
7	236026	Guinea Yam	Semen Omo	2130	O7G
8	236027	Guinea Yam	Semen Omo	1970	O8G
9	236034	Guinea Yam	Gedeo	1770	G9G
10	236036	Guinea Yam	Gedeo	1740	G10G
11	236037	Water yam	Gedeo	1610	G11W
12	236038	Guinea Yam	Gedeo	1610	G12G
13	236039	Guinea Yam	Gedeo	1580	G13G
14	236040	Guinea Yam	Gedeo	1590	G14G
15	236041	Guinea Yam	Gedeo	1970	G15G
16	236043	Guinea Yam	Gedeo	1870	G16G
17	128SDN(Rf#)	Guinea Yam	Jimma	-	J17G
18	158SDN(Rf#)	Guinea Yam	Jimma	-	J18G

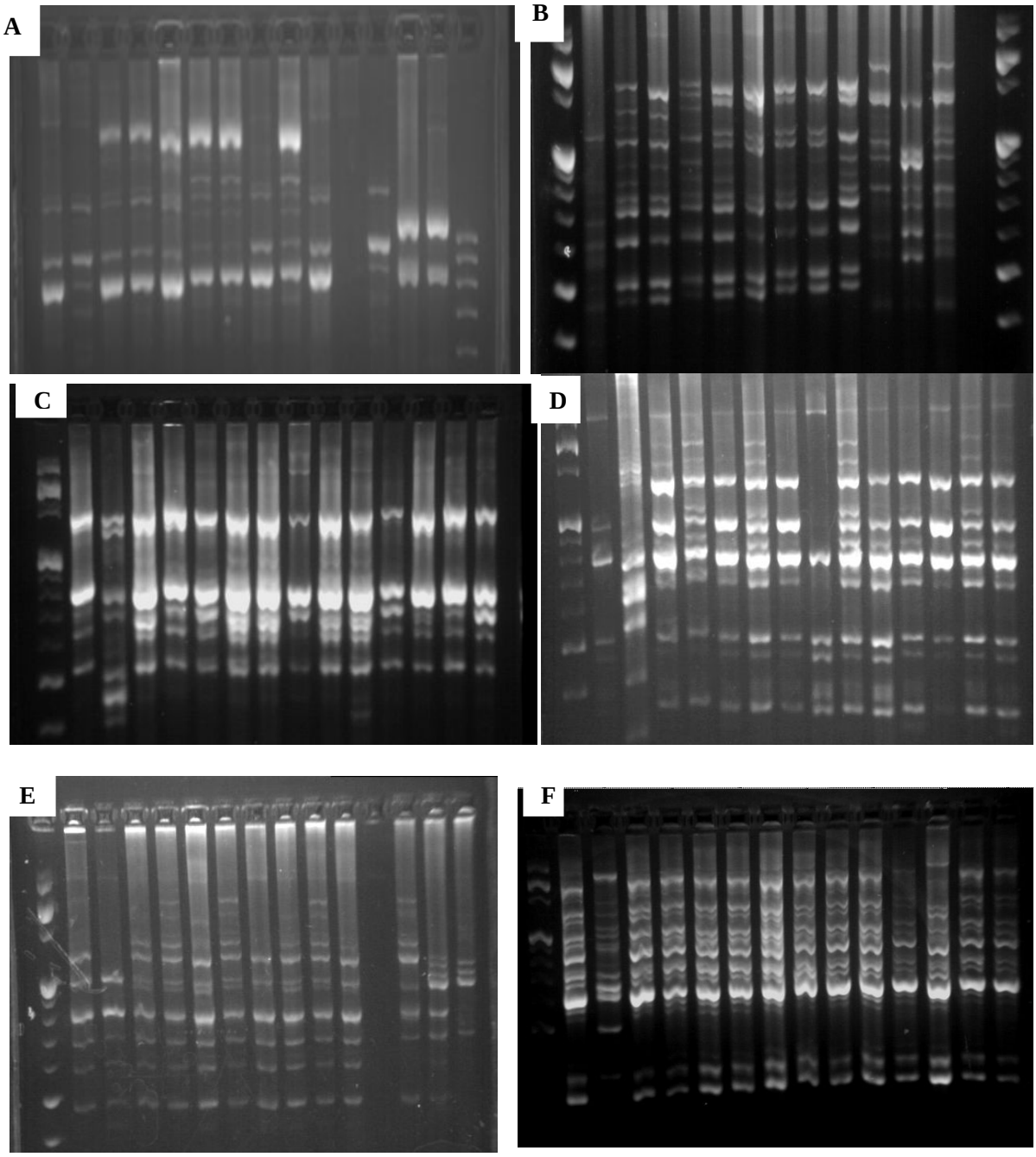
19	001SDN(Rf#)	Guinea Yam	Jimma	-	J19G
20	002SDN(Rf#)	Guinea Yam	Jimma	-	J20G
21	003SDN(Rf#)	Guinea Yam	Jimma	-	J21G
22	004SDN(Rf#)	Guinea Yam	Jimma	-	J22G
23	20986	Guinea Yam	East Wellega	1667	W23G
24	20987	Guinea Yam	East Wellega	1720	W24G
25	20988	Guinea Yam	East Wellega	1597	W25G
26	20989	Guinea Yam	East Wellega	1613	W26G
27	26YT(Rf#)	Guinea Yam	East Wellega	-	W27G
28	20991	Guinea Yam	East Wellega	1733	W28G
29	20992	Guinea Yam	East Wellega	1657	W29G
30	20993	Guinea Yam	East Wellega	1956	W30G
31	20994	Guinea Yam	East Wellega	1948	W31G
32	20995	Guinea Yam	East Wellega	2450	W32G
33	20996	Guinea Yam	East Wellega	1919	W33G
34	20997	Guinea Yam	East Wellega	1906	W34G
35	20998	Guinea Yam	East Wellega	2097	W35G
36	20999	Water yam	Ilu Ababora	2106	I36W
37	21000	Guinea Yam	Ilu Ababora	2106	I37G
38	130YT(Rf#)	Guinea Yam	Ilu Ababora	-	I38G
39	21387	Guinea Yam	Ilu Ababora	1758	I39G

40	21392	Aerial yam	Ilu Ababora	2106	I40A
41	21393	Aerial yam	Ilu Ababora	1680	I41A
42	21394	Aerial yam	Ilu Ababora	1758	I42A
43	21388	Aerial yam	Ilu Ababora	1807	I43A
44	21395	Aerial yam	Ilu Ababora	-	I44A
45	21396	Aerial yam	Ilu Ababora	1758	I45A
46	21397	Aerial yam	Ilu Ababora	1666	I46A
47	23982	Guinea Yam	Skeka	1299	SH-KF47G
48	23983	Guinea Yam	Skeka	1198	SH-KF48G
49	23984	Guinea Yam	Keffa	2008	SH-KF49G
50	23985	Guinea Yam	Keffa	1888	SH-KF50G
51	236023	Guinea Yam	Hadiya	2290	H-K51G
52	236024	Guinea Yam	Hadiya	2080	H-K52G
53	236025	Guinea Yam	Kembata	1970	H-K53G
54	236028	Guinea Yam	Kembata	1630	H-K54G
55	236030	Guinea Yam	Kembata	1630	H-K55G
56	003DESY(Rf#)	Guinea Yam	Uknown	-	UKn56G
57	005DESY(Rf#)	Guinea Yam	Uknown	-	UKn57G
58	017DESY(Rf#)	Guinea Yam	Uknown	-	UKn58G
59	020DESY(Rf#)	Guinea Yam	Uknown	-	UKn59G
60	059DESY(Rf#)	Guinea Yam	Uknown	-	UKn60G
61	068DESY(Rf#)	Guinea Yam	Uknown	-	UKn61G

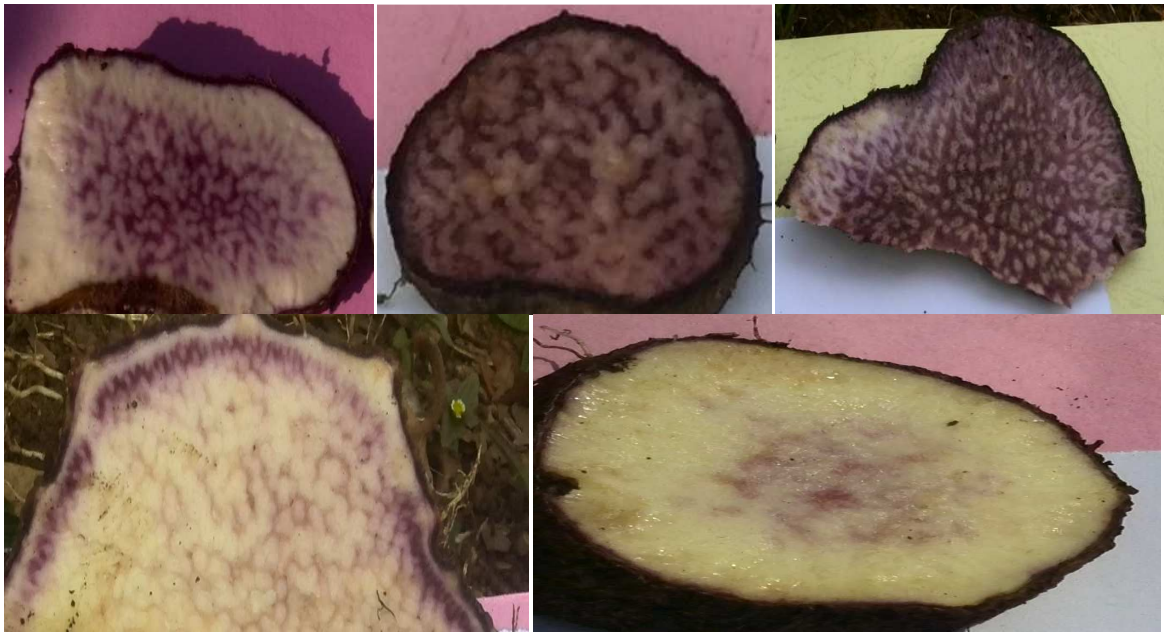
62	012H(Rf#)	Aerial yam	Uknown	-	UKn62A
63	015H(Rf#)	Aerial yam	Uknown	-	UKn63A
64	21391	Guinea Yam	Ilu Ababora	1666	I64G
65	105SDN(Rf#)	Aerial yam	Uknown	-	UKn65A
66	105SDN(2)(Rf#)	Aerial yam	Uknown	-	UKn66A
67	055KM(Rf#)	Aerial yam	Uknown	-	UKn67A
68	001KM(Rf#)	Aerial yam	Uknown	-	UKn68A
69	065SDN(Rf#)	Guinea Yam	Uknown	-	UKn69G
70	114SDN(Rf#)	Guinea Yam	Uknown	-	UKn70G

Rf# = Reference number; Guinea yam = D. cayenensis/D. rotundata *complex; Aerial yam* = D. bulbifera; *Water yam* = D. alata

Appendix 2. ISSR fingerprint generated for some individuals of *Dioscorea* species accession using six primers. Primer 811 (A), primer 818 (B), Primer 844 (C), primer 848 (D), primers 873 (E) and Prime 880 (F) respectively.



Appendix 3. Tuber flesh colour of *D. cayenensis/D. rotundata* complex



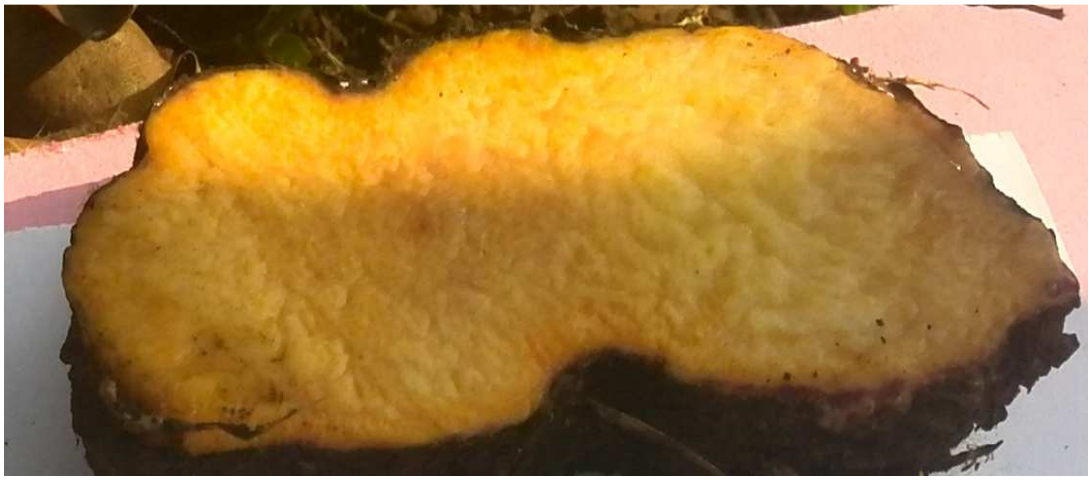
EIB (Ethiopian Institute of Biodiversity) description: *Dioscorea* spp.

Own observation: wild guinea yam (*D. abyssinica*, *D. praehensilis* or both)

For this study: *Dioscorea* spp. complex

EIB (Ethiopian Institute of Biodiversity) description: *Dioscorea* spp.

Own observation: Aerial yam (*D. bulbifera*)



EIB (Ethiopian Institute of Biodiversity) description: *Dioscorea* spp.

Own observation: Yellow Guinea yam (*D. cayenensis*)

For this study: *D. Cayenensis/D. Rotundata* Complex

EIB (Ethiopian Institute of Biodiversity) description: *Dioscorea* spp.

Own observation: White Guinea yam (*D. rotundata*)

For this study: *D. Cayenensis/D. Rotundata* Complex

EIB (Ethiopian Institute of Biodiversity) description: *Dioscorea* spp.

Own observation: Intermediate between wild and cultivated Guinea yam

For this study: *D. Cayenensis/D. Rotundata* Complex