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GENETIC DIVERSITY OF LACTUCA TARAXACIFOLIA AND LACTUCA SATIVA USING MORPHOLOGY, MOLECULAR MARKERS AND BIOCHEMICAL ANALYSIS

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Abstract

Lactuca taraxacifolia and Lactuca sativa are widely cultivated vegetables in Nigeria, with nutritional, economic and medicinal usefulness. The aim of study was to determine the genetic diversity among ten accessions of L. sativa and L. taraxacifolia using morphological analysis, biochemical analysis and molecular markers. In this study, saponin and phenol were extracted using double solvent extraction gravimetric method and Folin Ciocalteu reagent method respectively. The analysis revealed that L. taraxacifolia has a higher saponin content of 27.37mg/100g than L. sativa (17.04mg/100g) while the phenolic content was higher in L.sativa (138.0mg/100g) than in L. taraxacifolia (125.9mg/100g). Genetic diversity analysis was carried out using two SSR and two ISSR markers (SSR07, SSRA09, ISSR817 and ISSR866).The two SSR markers produced a total of five (5) alleles while the ISSR markers produced six (6) alleles; PIC values of 0.2859, 0.2772, 0.3648 and 0.605 for SSR07, SSRA09, ISSR817and ISSR866 respectively indicates a better resolving power of SSR marker over ISSR in this study. The cluster analysis grouped the accessions into three (3) major groups showing genetic relatedness

between the accessions: LTU09 and LTT10 are the closest varieties while ALI02was very distant from the other accessions. The factorial analysis categorized the ten accessions into four quadrants which has some similarity with the cluster analysis. This study revealed genetic diversity among the accessions evaluated and can be used to maximize the use of genetic resources as well as improve L. sativa and L. taraxacifolia varieties

CHAPTER ONE

INTRODUCTION

1.1 *LACTUCA* L.

Background information

Lactuca L. commonly known as lettuce, is a genus of flowering plants in the daisy family, Asteraceae. The name *Lactuca* means 'having milky sap' in latin. The genus includes approximately 100 species distributed worldwide, but mainly in temperate Eurasia. The distribution of the genus *Lactuca* L. worldwide includes 17 species in Europe, 51 in Asia, 43 in Africa, and 12 in the Americas (mostly the North American subcontinent). Species originating in Asia, Africa, and the Americas form approximately 83% of known *Lactuca* species richness (Lebeda *et al*., 2004). The genus *Lactuca* L. is composed of one cultivated species (*Lactuca sativa* L.) and about 100 wild species such as *Lactuca taraxacifolia* L. (Lebeda *et al*., 2007). *Lactuca* species are characterized by considerable morphological and genetic variation (Novotna *et al*., 2008).

Several species of the genus *Lactuca* L. are rich in a milky sap that flows freely from any wounds in the plant. This sap contains lactucarium which is used in medicine for its anodyne, antispasmodic, digestive, diuretic, narcotis, aphrodisiac, soporific and sedative properties (Kraujalyte *et al*., 2013).

1.2 CLASSIFICATION OF *LACTUCA* L.

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Asterales

Family: Asteraceae

Genus: *Lactuca* L.

1.3 *LACTUCA TARAXACIFOLIA*

Lactuca taraxacifolia (Efo yanrin) commonly referred to as the African lettuce belong to the family Astereceae (Compositae). It is a specie of the genus *Lactuca* L. It is an annual herb of Western Tropical Africa known as wild lettuce. *Lactuca taraxacifolia* occurs from Senegal east to Ethiopia and Tanzania. The Ethiopian highlands have been suggested as the place of origin, from where it was introduced elsewhere and spread as a weed (Adejuwon *et al*., 2014). It is called nne-noa in Ghana, bekuhoa-pomboe in Sierra Leone, valovalo in Senegal and latotue in Dahomey (Oladele *et al*., 2015). Apart from it being used as a common vegetable, it is also eaten by some people as a salad or cooked in soups sauces. *L. taraxacifolia* leaves are fed to lactating cows in Northern part of Nigeria to increase the milk yield and to sheep and goats to produce multiple births (Ololade *et al*., 2015). Medicinally, the leaves are rubbed on limbs to make children in Nigeria and Ghana walk, the leaves are also mixed with ashes to cure yaws (Adinorty *et al*., 2012).

The Yorubas of Southern Nigeria mix the leaves with local bathing black soap to prevent and/or cure skin diseases. Adegbite (1987) reported that the milky latex produced by the plant could be used to cure eye disease known as conjunctivitis/Apollo. Obi *et al* (2006) reported that consumption of *L. taraxacifolia* could, to a large extent, prevent infection, or even if infection has occurred, could prevent further replication of the

measles virus. In tests with animals in Ghana, leaves of *L. taraxacifolia* showed a cholesterol-lowering effect (Adebisi, 2004).

It is known as 'efo yanrin' among the Yorubas of the South-Western part of Nigeria, 'ugu' among the Ibos of the Eastern part of Nigeria and 'nonon barya' among the Hausas of the Northern part of Nigeria.

1.4 *LACTUCA SATIVA*

Lactuca sativa L. (Lettuce) is an annual plant of the daisy family, Asteraceae. It is most often grown as a leaf vegetable, but sometimes for its stem and seeds (Mampholo *et al*., 2015). Lettuce has a high nutritional value, eaten all over the world as a low-sugar low-fat "anticancer vegetable". It is native to Europe on the coast of the Mediterranean Sea, favored in European and American countries, and contains abundant active components such as vitamins, mineral elements, organic acids, dietary fiber, carotene, thiamine, nicotinic acid, mannitol and riboflavin (Kim *et al*., 2015).

Lactuca sativa L. (lettuce), which is the best representative of the genus is a well-known plant worldwide due to its use in the preparation of salad, soup, and vegetable curries. This plant also has excellent medicinal properties. Lettuce is cultivated worldwide, and is one of the most consumed green leafy vegetables in the raw form for its taste and high nutritive value. It is regarded as an important source of phytonutrients (Cheng *et al*., 2014).

Due to the impressive health benefits of both species, much attention has been drawn to the study of the genetic diversity between them, in order to ascertain the degree of variability or similarity among cultivars of both species. The numerous uses of *Lactuca taraxacifolia* and *Lactica sativa* have spurred the need to have a broaden knowledge of how diverse or similar both species are to each other. The study of genetic diversity between these two species is vital to plant breeders because of its importance for selecting germplasm included in a breeding program. The study of genetic variability is essential to receive information about propagation, domestication, which can be used in breeding programs and for conservation of genetic resources of *Lactuca* species.

1.5 MORPHOLOGY OF *L. TARAXACIFOLIA* AND *L. SATIVA*

Lactuca taraxacifolia is an erect perennial herb that is up to 150cm tall, with creeping root system and an erect stem that is often woody at the base. The leaves at the base of the plant are in a rosette but alternate on stem. The higher leaves are auriculate and toothed with the lower leaves tapering at the base. The fruit is a cylindrical to fusiform achene that is 3-5 mm long, slightly beaked, ribbed, and crowned by white pappus with hairs that are 5-8 mm long (Ayobola *et al*., 2011).

Lactuca sativa is an annual glabrous herb with a thin tap root and an erect stem 30-100cm tall, branched in the upper part. Leaves are spirally arranged, forming a dense rosette or head before bolting. Their shape is oblong to transverse elliptic, orbicular to triangular, undivided to pinnatisect. Stem leaves are oblong elliptic, with a cordate base. The inflorescence is composed of 7-15 yellow ligules. The heads form a corymbose, densely bracted panicle. The involucre is 10-15 mm long, cylindrical; involucral bracts are broadly to narrow lanceolate, light green, with white margins, erect at the stage of fruit maturity. The fruit has 5 to 7 setose ribs on each side, a beak and a white pappus. Its length (including beak) is 6-8 mm, and its colour is white, cream, gray, brown or black (Kristkova *et al*., 2008).

1.6 NUTRITIONAL VALUES OF *L. TARAXACIFOLIA* AND *L. SATIVA*

Lactuca species are a genus of healthy foods. *Lactuca sativa* is a good source of high dietary fiber (1.1 g/100g FW), vitamin A (166µg/100g, FW), vitamin C (4mg/100g), vitamin K (24 µg/100g), and phenolic compounds (FAOSTAT, 2012). Since *Lactuca taraxacifolia* is very low in calories, it is often prescribed for weight loss programs (Niederwieser, 2001).

1.7 ECONOMIC IMPORTANCE OF *L. TARAXACIFOLIA* AND *L. SATIVA*

The *Lactuca* genus is an economically important salad vegetable (FAOSTAT 2012). Medicinally, consumption of lettuce is reported to improve health benefits such as its higher dietary fiber content that aids in digestion. The higher b-carotene and lutein content in lettuce is associated with reducing risk of cancers, cataracts and heart disease and stroke (Mampholo *et al*., 2015). Phenolic compounds in lettuce are responsible for antioxidant scavenging properties. Carotenoids possess antioxidant capacity and vitamin C, the water soluble antioxidant, also shows antioxidant properties (Lopez *et al*., 2014).

Lactuca taraxacifolia is used as a remedy for prevention and treatment of diseases such as measles, yaws, conjunctivitis, hyperthesion, cancer etc. It is reported to possess hypolipidaemic, antihypertensive effects (Dairo *et al*., 2015). The leaves of *L. taraxacifolia* are used to stimulate lactation and also to induce multiple births in animals (Adinortey *et al*., 2012). The leaves are rubbed on limbs to aid walking in children. The milky latex of the plant is used to treat conjunctivitis (Sakpere and Aremu, 2008). The African lettuce is used as vegetable and used as salad or cooked as soups (Adinortey *et al*., 2012; Ruffina *et al*., 2015). It has been observed to be a good source of essential mineral elements (Soetan *et al*., 2010; Gbadamosi *et al*., 2012).

One of the oldest benefits of *Lactuca sativa*, which dates back to medieval times, is its property to induce sleep. This particular benefit has already been confirmed by studies (Kim *et al*., 2015). It contains lactucarium, a chemical that shares the same properties and similar structure to opium. *Lactuca sativa* extracts contain biochemical catalysts, lipoxygenase and carrageenan, which have shown significant control over inflammation (Araruna *et al*., 2010). *Lactuca sativa* possesses anti-fungal properties when digested. The latex, which is called lactucarium can eradicate various types of yeasts including *Candida albicans*. Moreover, it can help protect the body from harmful microbes. This property is due to its biochemical terpenes and cardenolides.

Lactuca sativa contains a significant amount of potassium, which plays a vital role in maintaining fluid balance in your body. The neurological benefits of *Lactuca sativa* have long been discovered since the ancient times to treat insomnia, improve memory retention, and preserve cognitive function well into old age. Further research carried out by Harsha *et al* (2012) has concluded that *Lactuca sativa* features not only sedative properties but also anxiolytic properties as well. Hence, lettuce eaten often can help to relieve stress and anxiety (Harsha *et al.*, 2012).

Lettuce is rich in Omega-3 fatty acids which improves cardiovascular health as it removes and prevents plaque that hardens the arteries; thus, promoting blood flow without forcing the heart to pump too hard. As a result, lettuce reduces the risk of stroke, hypertension, and cardiac arrest. It is abundant in vitamin K, an indispensable vitamin for pregnant women. Vitamin K helps prevent neural defects during gestation and hemorrhage during childbirth. Lettuce also contains folate that is essential for the DNA synthesis during pregnancy. Lettuce is among the best

foods for weight loss. It is rich in fiber and cellulose, but low in calories and has no fat. It also aids in waste removal, and can promote the excretion of bile acids in feces, which in turn reduces cholesterol levels.

1.8 GENETIC DIVERSITY STUDY

Genetic diversity refers to the variation of genes within species. It allows species to adjust to a changing environment, whether these changes are due to natural or human factors (Lebeda *et al*., 2013).

Genetic variation studies are vital for providing information for propagation, taxonomy, disease resistance, and breeding programs as well as conservation and utilization of *Lactuca* genetic resources. Genetic diversity can be evaluated based on morphological, cytogenetic, biochemical and molecular markers. However, the evaluation of genetic variation based on morphological and cytological traits has the disadvantages of being affected by both genetic and environmental factors and may not provide an accurate measure (El-Esawi *et al*., 2012). Hence, advanced molecular genetic technologies such as molecular markers have been developed to overcome those limitations of morphological and cytological traits.

1.9 MOLECULAR MARKERS

A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. The molecular markers are useful tools for assaying genetic variation and provide and efficient means to link phenotypic and genotypic variation (Varshney *et al.*, 2005). Molecular markers can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP) or a long one, like mini and micro satellites (Abdulkareem and Firas, 2015)

1.9.1 TYPES OF MOLECULAR MARKERS

- Simple Sequence Repeats (SSRs)
- Radom Amplified Polymorphic DNA (RAPD)
- Inter-Simple Sequence Repeats (ISSR)
- Amplified Fragment Length Polymorphism (AFLP)
- Restriction Fragment Length Polymorphism (RFLP)

- Expressed Sequence Tag markers (EST)
- Single strand conformation polymorphism (SSCP)
- Restriction landmark genomic scanning (RLGS)

1.9.2 SSR MARKERS/MICROSATELLITES

Microsatellites, also called Simple Sequence Repeats (SSRs), are short, tandemly repeated motifs of mono-, di-, tri-, tetra and penta-nucleotides generally distributed in all prokaryotic and eukaryotic genomes (Zane *et al.,* 2002). SSR molecular markers are frequently used to assess genetic variation within and between populations (Vigouroux *et al*., 2005) and there have been many studies describing genetic diversity in a wide range of species. SSR-based molecular markers are frequently used in plant genetics due to their high reproducibility, co-dominant inheritance, and high information content (Simko, 2009). The high polymorphism is due to different number of repeats in the microsatellite regions, therefore they can be easily and reproducibly detected by polymerase chain reaction (PCR) (Kalia *et al*., 2011).

1.9.3 ISSR MARKERS

Inter simple sequence repeat (ISSR) technique is a PCR based technique, reported by Ztetkiewicz *et al* (1994), which involves amplification of DNA segments between two identical microsatellite repeat regions ‘oriented in opposite direction using primers designed from microsatellite core regions. The technique uses microsatellite primers, usually 16 −25 bp long, of dinucleotide, tri nucleotide, tetra-nucleotide or penta nucleotide repeats to target multiple genomic loci (Reddy, 2002). Microsatellite sequencing and ISSR sequencing are mutually assisting, as one produces primers for the other (Pradeep *et al*., 2002). ISSR technique is simple, quick and less costly like the RAPD technique.

SSR and ISSR molecular markers are used in this study because they are abundant, distributed throughout the genome and are highly polymorphic compared with other genetic markers. They are also species-specific and co-dominant (Miah *et al*., 2013).

CHAPTER TWO

2.1 LITERATURE REVIEW

Lactuca taraxacifolia and *Lactuca sativa* are the most important species in the *Lactuca* L. genus due to their remarkable economic, medicinal and nutritional significance.

Oliya *et al*. (2015), synthesized 100 genic simple sequence repeat (SSR) primers with a 99-250 bp target amplicon from *Lactuca indica* transcriptomic sequences. These primers were examined in 8 diverse *L. indica* accessions, and 90 polymorphic SSRs were used to investigate transferability to another two *Lactuca* species, *Lactuca serriola* and *Lactuca sativa*. Genetic diversity was investigated in 77 *Lactuca* accessions, including 73 *L. indica* collected from across South Korea, 2 *L. serriola*, and 2 *L. sativa*. The genic-SSR markers were highly polymorphic with a mean polymorphic information content of 0.61 and, on average, 10.83 alleles per locus. The average of molecular variance revealed that most of the total variance in the population is attributable to genetic variation among accessions, rather than among provinces. Structure, unweighted neighbor-joining phylogenetic trees, and principal coordinate analyses resulted in three clusters. The *L. serriola* and L. sativa accessions did not produce a separate cluster due to a small sample size. These results showed that the genic SSR markers is useful in germplasm assessment and genetic studies of *L. indica* and other *Lactuca* species.

Yang *et al* (2015), used RAPD, Inter-SSR, and AFLP markers to assess the genetic diversity of lettuce cultivars and the phylogenetic relationships in *Lactuca* species. A total of 216 polymorphic bands from seven RAPD primers, four Inter-SSR primers, and five AFLP primer combinations were used to elucidate the genetic similarity

among lettuce cultivars. Forty-four lettuce accessions were subdivided into discrete branches according to plant type: crisphead, butterhead, and stem type, with some exceptions. The leafy- and cos-type accessions were intermingled in other groups with no discrete branch indicating that these are more diverse than others. Three accessions, including the Korean cultivar 'Cheongchima', the Korean local landrace 'Jinjam', and the German cultivar 'Lolla Rossa' were classified as the most diverse accessions. Twenty bands were unique in specific cultivars. Among these, three were specific in a plant type; one in Korean leafy type, one in crisphead type, and one in cos type lettuce. In the phylogenetic analysis among *Lactuca* species, *L. saligna*, *L. serriola*, and *L. georgica* clustered in a sister branch of the *L. sativa* complex. Two *L. virosa* accessions show the highest intra-specific relationships. *L. perennis* outlied from all the other *Lactuca* species at a genetic similarity of 0.53 and clustered with two *Cichorium* species, C. *intybus* and C. *endivia*, with genetic similarity of 0.67.

Sharma *et al* (2015) using RAPD markers assessed genetic diversity in lettuce germplasm. In the study, genetic variability and relationships among 25 *Lactuca sativa* L. genotypes were tested. A total of 45 random decamer oligonucleotide primers were examined to generate RAPD profiles, out of these reproducible patterns were obtained with 22 primers. A total of 87 amplicon were obtained, out of which all were polymorphic and 7 were unique bands. The level of polymorphism across genotypes was 100% as revealed by RAPD.

Ololade *et al* (2015) carried out a study on *Lactuca taraxacifolia* which was aimed at investigating the chemical composition, pH, TPC, TFC, TAA, carotenoid, antioxidant, anti-arthritic, anti-inflammatory and bactericidal activities of the plant. Using GC-MS, pH meter, Folin-Ciocalteu's, AlCl3, 2,4-DNPH, acetone-hexane, DPPH, PTAC, BSA and agar-well diffusion methods respectively, the most abundant components were found to be palmitic acid (8.5%), methyl-11-octadecenoate (7.7%), erythritol (7.5%), glycerol (6.5%), linolelaidic acid, methyl ester (6.2%) and phytol (5.5%). The plant was found to have high anti-arthritic/anti-inflammatory potentials. The extract was active against all the tested bacteria with high zones of inhibition (11.0-30.0 mm).

Rauscher and Simko in 2013 carried out a study on the development of genomic SSR markers for fingerprinting lettuce (*Lactuca sativa* L.) cultivars and mapping genes. Testing of newly developed markers on a set of 36 *Lactuca* accession (33 *L. sativa*, and one of each *L. serriola* L., *L. saligna* L., and *L. virosa* L.) revealed that both the genetic heterozygosity (*UHe* = 0.56) and the number of loci per SSR (*Na* = 5.50) are significantly higher for genomic SSR markers than for previously developed EST-based SSR markers (*UHe* = 0.32, *Na* = 3.56). Fifty-four genomic SSR markers were placed on the molecular linkage map of lettuce. Distribution of markers in the genome appeared to be random, with the exception of possible cluster on linkage group 6. Any combination of 32 genomic SSRs was able to distinguish genotypes of all 36 accessions. Fourteen of newly developed SSR markers originated from fragments with high sequence similarity to resistance gene candidates (RGCs) and RGC pseudogenes. Analysis of molecular variance (AMOVA) of *L. sativa* accessions showed that approximately 3% of genetic diversity was within accessions, 79% among accessions, and 18% among horticultural types.

Van de Wiel *et al* (1998) used SSRs in Southern hybridisation to assess the variation among different cultivars of *Lactuca sativa* as well as among the accessions of *Lactuca virosa*, *Lactuca serriola* and *Lactuca saligna*. The majority of this plant material has been characterized morphologically and analysed using ITS1 sequencing. Fourteen microsatellite and 3 minisatellite motifs were tested for diversity studies in *Lactuca* species. Van de Wiel *et al* (1991) isolated microsatellite-containing sequences from cultivated *Lactuca sativa* using enriched genomic libraries. Those isolated microsatellite loci will be useful for distinguishing lettuce cultivars and for assessing the genetic diversity of plant genetic resources (El-Esawi, 2015).

Kwon *et al* (2013) assessed the genetic variation, population structure, and genome-wide marker-trait association analyses in lettuce based on 322 high-quality SNP markers. Only 258 unique genotypes were revealed among the 298 lines. Nine significant marker-trait associations (SMTAs) were detected at $P < 0.0001$, with 5 SMTAs for seed coat color, 1 for leaf undulation, 2 for leaf anthocyanin, and 1 for stem anthocyanin.

Vicente *et al* (2008), carried out a molecular variability analysis on *Lactuca sativa* germaplasm which included, nine landrace accessions belonging to the cos type and three to the butterhead type. Six commercial varieties of crispshead, cos, leaf and butterhead were also included. Twenty ISSR markers were used, out of which thirteen ISSR primers amplified DNA fragments from all tested plants. One hundred and ten of 167 fragments scored

showed polymorphism, with an average of 8.5 polymorphic bands per primer. The number of bands per primer ranged from 6 to 19, with an average of 12.8. Based on these ISSR markers, genetic similarity coefficients were calculated and a dendogram was constructed. The eleven lettuce landraces and six commercial varieties were divided into three major groups at a similarity coefficient value of 0.81. All the lettuce landraces were grouped with the commercial varieties of cos and butterhead types. Two accessions were indistinguishable by ISSR primers.

The application of molecular markers such as Simple Sequence Repeats (SSR) and Inter-Simple Sequence Repeats (ISSR) markers in *Lactuca* germplasm would contribute to the better understanding of the molecular variability (Jansen *et al*., 2006).

2.2 AIM

The aim of this study is to determine the transferability of SSR and ISSR molecular markers present in the European lettuce to the African lettuce in order to analyse the molecular variation among cultivars of the African and European lettuce.

2.3 OBJECTIVES

1. To study the leaf morphology of *Lactuca taraxacifolia* L. and *Lactuca sativa* L.
2. To determine the transferability of SSRs and ISSR molecular markers from *Lactuca sativa* to *Lactuca taraxacifolia.*
3. To determine the total saponin and phenolic contents of wild and cultivated lettuce.

CHAPTER THREE

MATERIALS AND METHODS

3.1 MATERIALS

The following plant materials were used during the course of this study; varieties of *Lactuca sativa* and *Lactuca taraxacifolia*, plastic pots (nursery pots), loamy soil, compost, 70% ethanol, paper tape, scissors, Ziploc bags, cotton wool, iced cubes, hand gloves, permanent marker, insulated cooler.

3.2 PLANTS COLLECTION

A total of ten (10) accessions of *Lactuca taraxacifolia* and *Lactuca sativa* popularly known as the African lettuce and Cultivated lettuce respectively were obtained from different agro-geoecological regions in Nigeria, and Tanzania. The varieties obtained included imported lettuce (Plate 1) and African lettuce (Plate 2) collected from the south-western states in Nigeria (Lagos State, Ogun State, Ondo State, Osun State, Oyo State). These accessions were all used as source materials for this study (Table 1) showing locations of collection.

Table 1: **The description of all the lettuce samples collected**

S/N	Codes	Places of collection	Local names	Common names	Scientific names
1	ALI 01	Lagos		Imported lettuce	*Lactuca sativa*
2	ALI 02	Wilko iceberg lettuce		Imported lettuce	*Lactuca sativa*
3	ALL 03	Tanzania	Ladha ya barafu	Imported lettuce	*Lactuca sativa*
4	ALO 04	Akure	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
5	ALK 05	Iwo	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
6	ALU 06	Unilag	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
7	LTS 07	Lagos	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
8	LTU 08	Kwara	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
9	LTU 09	Ogbomosho	Efo yanrin	African lettuce	*Lactuca taraxacifolia*
10	LTT 10	Ibadan	Efo yanrin	African lettuce	*Lactuca taraxacifolia*

Plate 1: Picture showing varieties of *L. sativa*

Plate 2: Picture showing varieties of *L. taraxacifolia*

3.3 LEAF MORPHOLOGICAL ANALYSIS

The leaf morphological characteristics of each species was analysed and recorded. The quanlitative traits such as leaf width and length were determined using nine (9) representatives each from all the cultivars of *L. taraxacifolia* and *L. sativa* were analysed after which their mean values were determined.

TABLE 2: List of the qualitative trait descriptions used in this study

S/N	QUALITATIVE TRAIT	DESCRIPTION
1.	Leaf width	Measured in centimeter ranging from 7.3-3.7
2.	Leaf length	Measured in centimeter ranging from 16.6-12.2
3.	Leaf surface profile	Convex, concave, flat
4.	Shape of apex	Rounded, sub-acute, obtuse
5.	Leaf base shape	Uneven, tampering

Plate 3: Showing leaves of different varieties of *L. sativa* and *L. taraxacifolia*

3.4 PLANTING PROCESS

Planting was carried out in the University of Lagos, Akoka, on an open space to avoid intense heat from planting in a greenhouse with the motive of preventing the damage of plants during nursery period. The greenhouse was constructed using nylon (placed on the floor to prevent the growth of weed), bamboo sticks were placed into the ground to form a rectangular structure and construction net was used to cover the structure so as to form a shed. Ten (10) plastic pots were labelled and filled with a mixture of rich loamy soil and compost to about 2.5 kg which served as a growth medium for the plants. They were labelled appropriately according to location and

variety using a paper tape and a permanent marker. Ten (10) seeds each of the imported lettuce were inserted at least two (2) inches into the soil contained in their respective bowls while each of the different varieties of the African lettuce were transplanted into their respective pots. Watering and monitoring was done early in the morning for twice a week and records of growth was taken after a week of germination.

Figure 1: Greenhouse used for study just after construction

3.5 HARVESTING OF PLANT LEAVES

Harvesting was done four (4) weeks after planting, during the early hours of the day when leaves are still fresh and little water loss from transpiration. Hand gloves was worn and a scissors

disinfected with 70% alcohol was used to cut a good portion of the leaves which was then placed in a Ziploc bearing each sample's name. The scissors was sterilized with 70% ethanol after each cut, to avoid DNA mix among samples. The Ziploc was transferred to an insulated cooler containing ice pack. The essence of the Ziploc, insulated cooler and ice pack was to preserve the DNA and avoid degradation so it does not get denatured or compromised before getting to the laboratory for further analysis.

3.6 DNA EXTRACTION REAGENTS

β-Mercaptoethanol (BME), dissolution buffer, ethanol (70%, cold), isolation buffer, isopropanol (cold) potassium acetate (5M), SDS (10%, w/v), sodium acetate (3 M, Ph 5.2), TE-RNase, *Lactuca taraxacifolia* leaves and *Lactuca sativa* leaves (fresh).

3.7 DNA EXTRACTION MATERIALS

Fume hood, ice, liquid nitrogen, microcentrifuge maintained at 20^{o}C and microcentrifuge tubes (1.5 mL).

3.8 DNA EXTRACTION PROTOCOL

Total genomic DNA was extracted using the modified SDS extraction protocol.

The lettuce leaf tissue was disrupted in liquid nitrogen by placing 150mg of leaf tissue in a chilled mortar covered with liquid nitrogen. When the liquid nitrogen was almost evaporated, the leaf tissue were grounded with the chilled pestle until the tissue became a fine powder. 80-130mg of tissue was carefully transferred into a microcentrifuge tube, afterwards 800ulof isolation buffer was added, mixed well and vortexed. 100ul of 10% SDS and then 14ul of BME were added to the tissue samples. Contents of the tubes were mixed vigorously and incubated for 15 minutes at 65oC. 350ul of 5M potassium acetate was added, mixed vigorously, cooled on ice for 5 minutes and

centrifuged at 12000 rpm for 15 minutes at 20oC. The supernatant was decanted into a clean microcentrifuge tube, then 535µl of cold isopropanol was added and mixed gently to precipitate the DNA. Tubes were incubated for 5-10 minutes on ice, then centrifuged at 12000 rpm for 10-20 minutes at 20oC, afterwards the supernatant was carefully decanted. The DNA pellet was rinsed with 500ul of cold 70% ethanol afterwards the ethanol was drained completely and the pellet dried for several hours (overnight) in a fume hood until all traces of ethanol were completely removed. After drying, 60ul of TE was added to re-suspend the DNA. After DNA re-suspension, 2ul of RNase was added to remove RNA and the solution was incubated in 37oC for 40 minutes. The DNA was loaded in 0.8% agarose gel for checking DNA quality, and the gel picture was taken and saved.

3.9 DNA QUALITY AND QUANTITY

The Nanodrop spectrophotometer was used to analyse the quantity and purity of the DNA. The concentration of DNA per microliter (ng/ul) against the A260/A280 and results obtained were compared with the standard purity value of DNA. Pictures taken during the agarose gel electrophoresis were compared with the nanogram reading to derive its conclusion.

3.10 PCR AMPLIFICATION METHOD

A. ISSR PCR Amplification

The amplification reactions were made for a final volume of 25 µL, containing 20 ng of DNA, a unit of Taq DNA polymerase (Invitrogen), 10mM of Tris-HCl (pH 8.0), 2 mM of $MgCl_2$, 0.25 µM of each deoxyribonucleotide triphosphate (DNTPs) and 0.2 µM of oligonucleotide. The DNA amplifications were done using a MJ Research, Inc. PTC100 Programmable Thermal Controller (Watetown, USA) thermocycler, under the following conditions: 15 minutes at 95°C (initial denaturation), followed by 35 cycles of 30 seconds at 94°C (denaturation), 45 seconds

at 50 or 55°C (ringing) and 2 minutes at 72°C (extension), with final extension for 7 minutes at 72°C. The products from the amplifications were separated in 2% agarose gel, crowned with Syber gold (Invitrogen), using the 100 bp Ladder 50 µg (1.0 µg/µL) (Invitrogen) marker and observed under an ultra-violet light and registered in a digital Vilber Lourmat photodocumentor.

B. SSR PCR AMPLIFICATION

Using the primer pairs of an SSR marker, the genomic DNA of the accessions of each the three species of *Lactuca* was amplified in 25 µL reaction mixtures each containing 100ng genomic DNA, 1 × PCR buffer, 15 mM $MgCl_2$, 200 µM dNTP, 0.2 µM each of the forward and reverse primers and 1 U Taq polymerase (Bangalore Genei) using the following PCR profile in an Eppendorf Mastercycler. Initial denaturation at 94°C for 3 min; followed by 39 cycles of 94 °C for 30 s, 54 to 62°C for 1 min, 72 °C for 2 min and a final extension at 72°C for 10 min. The amplified products were resolved on 10 % polyacrylamide gels and visualized following ethidium staining.

TABLE 3: List of SSR primers and their sequences used in this study

PRIMERS	FORWARD PRIMER (5'-3')	REVERSE PRIMER (3'-5')
SSR07	CAA GTC TCC CAA TGT TGT TTCA	GTT ACG CTC TCA AAA CCC TCA T
SSRA09	ACA TAC CAT CAC ATG CCC CTA C	GCC CAA GAC CTT AAC AAA CCT C

Table 4: List of ISSR markers and their sequences used in this study.

ISSR MARKERS	SEQUENCE
ISSR 817	CACACACACACACACAA
ISSR 866	CTCCTCCTCCTCCTCCTC

3.11 SDS PAGE PROTOCOL

Polyacrylamide gel also known as PAGE was prepared following standard procedure. Samples were loaded into the gel and the gel was ran at 25mA in 1x SDS Running Buffer. The gel was placed in a plastic container, covered with isopropanol fixing solution and agitated at room temperature. Fixing solution was poured off then gel was covered with ethidium bromide staining solution and agitated room temperature for two (2) hours. Staining solution was poured off. Then the gel was washed with 10% acetic acid to de-stain, agitating it at room temperature.

3.12 GENETIC DIVERSITY ANALYSIS

Polymorphic SSR amplified product was considered to be a unit character and the populations were manually scored using binary data with presence as "1" and absence as "0" (Appendix). The numbers of monomorphic and polymorphic bands were derived from the agarose gel electrophoresis diagram. Genetic distances were measured based on shared allele frequencies. A dendrogram was constructed from a UPGMA (Unweighted Pair Group Method with Arithemetic mean). Data obtained from SSR allelic diversity among *L. sativa* and *L. taraxacifolia* varieties were used to compute the Jaccard's dissimilarity matrix using Darwin 5.0 software. The dissimilarity matrix was also used to construct a factorial analysis to reveal their molecular diversity. Polymorphic Information Content (PIC) and cross-species transferability was calculated as the presence of target loci to the total number of amplified loci for each SSR marker.

3.13 BIOCHEMICAL ANALYSIS

Determination of Saponins

This was done by the double solvent extraction gravimetric method (Harborne, 1973). Five grams (ppt) (5g) of the sample was mixed with 50ml of 20% aqueous ethanol solution and incubated for 12 hours at a temperature of 55oC with constant agitation. After that, the mixture was filtered through Whatman No 42 grades of filter paper. The residue was re-extracted with 50ml of the ethanol solution for 30 minutes and the extracts weighed together. The combined extract was reduced to about 40ml by evaporation and then transferred to a separating funnel and equal volume (40ml) of diethyl ether was added to it. After mixing well, there was a partition and the other layer was discarded while the aqueous layer was reserved. This aqueous layer was re-extracted with the ether after which its pH was reduced to 45 with drop-wise addition of dilute NaOH solution.

Saponin in the extract was taken up in successive extraction with 60ml and 30ml portion of normal butanol. The combine extract was washed with 5% NaCl solution and evaporated to dryness in a previously weighted evaporating dish. The Saponin was then dried in the oven at 60^{o}C (to remove any residual solvent) cooled in a desicator and re-weighted. The Saponin was determined and calculated as a percentage of the original samples.

$$\% \text{ Saponin} = \frac{W2 - W1}{W1} \times 100$$

Where, W = weight of sample used

W1 = weight of empty evaporation dish

W2 = weight of dish + Saponin extract

Determination of phenolic content

This was determined by Folin Ciocalteu reagent method, with some modifications. Plant extract (1mL) was mixed with Ciocalteu reagent (0.1mL, 1N) and allowed to stand for 15 minutes. Then 5 mL of saturated Na_2CO_3 was added. The mixtures were allowed to stand for 30 minutes at room temperature and the total phenols were determined spectrophotometrically at 760nm. Gallic acid was used as a standard and the total polyphenol content in the extract was expressed in mg of gallic acid equivalents per gram of dry matter.

CHAPTER FOUR

RESULTS

4.1 MORPHOLOGICAL ANALYSIS

Table 4 shows the morphological assessment of qualitative leaf traits among ten (10) varieties of *Lactuca taraxacifolia* and *Lactuca sativa*. The leaf width among the varieties ranged from 7.1cm to 3.7cm, with ALI01 having the highest mean value (7.1cm) and ALO04 having the least mean value, while the leaf length ranged from 16.7cm to 12.2cm with LTT10 having the highest mean value and ALO04 having the least mean value. The dominant leaf surface profile among the varieties was flat, which was exhibited by seven (7) samples (ALO04, ALK05, ALU06, LTS07, LTU08, LTU09 and LTT10) accommodating up to 70% of the entry, while two (2) samples (ALI02 and ALL03) exhibit the concave leaf surface profile trait and one (1) sample (ALI01) exhibit the convex leaf surface profile trait, accounting for 20% and 10% of the entry respectively.

For the shape of apex trait, (ALU06, LTS07, LTU09 and LTT10) four (4) in total are obtuse, (ALI01, ALI02 and ALL03) totalled three (3) are all rounded and three (3) samples (ALO04, ALK05 and LTU08) are sub-acute.

For the leaf base shape trait, (ALL03, ALO04, ALK05, ALU06, LTS07, LTU08, LTU09 and LTT10) seven (7) in total are tampering, while three (3) samples (ALI01, ALI02 and ALL03) are uneven. From this morphological assessment, it can be said that the leaf width, leaf length, leaf surface profile, shape of apex and leaf base shape traits successfully grouped these samples into three (3) subgroups which entails there is genetic diversity among the varieties of *L. sativa* and *L. taraxacifolia*.

Table 5: Qualitative leaf traits of 10 *L. sativa* and *L. taraxacifolia*

Accession No	Leaf width (cm)	Leaf length (cm)	Leaf surface profile	Shape of apex	Leaf base shape
ALI01	7.1	13.1	Convex	Rounded	Uneven
ALI02	6.2	15.3	Concave	Rounded	Uneven
ALL03	7.3	13.0	Concave	Rounded	tampering
ALO04	3.7	12.2	Flat	Sub-acute	tampering
ALK05	4.1	13.5	Flat	Sub-acute	tampering
ALU06	6.5	14.2	Flat	Obtuse	tampering
LTS07	7.1	16.6	Flat	Obtuse	tampering
LTU08	5.7	13.8	Flat	Sub-acute	tampering
LTU09	5.7	12.9	Flat	Obtuse	tampering
LTT10	6.2	16.7	Flat	Obtuse	tampering

4.2 BIOCHEMICAL ANALYSIS

Table 4 shows the Saponin and phenolic content of one variety of *L. taraxacifolia* and one variety of *L. sativa* cultivars. Saponin content was found to be higher (27.37mg/100g) in the African lettuce (ALO 04) than in the European cultivars (ALI 02) with a mean value of 17.04mg/100g. However, the phenolic content was found to be higher in the European lettuce than in the African lettuce.

Table 6: Biochemical analysis of two (2) cultivars of European lettuce and African lettuce

SAMPLES	SAPONIN (Mg/100g)	PHENOL (Mg/100g)	MEAN VALUE (Mg/100g) SAPONIN	PHENOL
ALI 02	17.35	138.02		
	16.56	138.17		
	17.21	137.86	17.04	138.0
ALO 04	27.46	126.00		
	27.73	125.84		
	26.94	126.07	27.37	125.9

4.3 MARKER POLYMORPHISM

Reproducible, well resolved, unambiguous fragments from the gels (Plates 5 and 6) were scored using Gel analyzer. Each scorable band was scored as presence (1) or absence (0). The profiles generated in different *L. sativa and L. taraxacifolia* accessions were analyzed to compute polymorphic information content (PIC). Three ISSR markers and three SSR markers were screened of which just two ISSR and two SSR marker amplified. A total of eleven (11) alleles were revealed among the 10 accessions from different locations using two (2) SSR and two (2) ISSR markers. SSR07 and SSRA09 had 2 and 3 alleles respectively, while ISSR817 and ISSR866 had 2 and 4 alleles respectively. All four markers showed different percentage polymorphism of 100%, 66.67%, 100% and 100% respectively. Each marker had PIC (Polymorphism Information Content) of 0.2859, 0.27723, 0.3648 and 0.605 respectively (Table 5). Each also had an expected heterozygousity of 0.3457, 0.2917, 0.48 and 0.6563 respectively.

Table 7: Number of alleles per locus and PIC gotten from SSR and ISSR markers

	SSR07	SSRA09	ISSR817	ISSR866
No. of alleles	2	3	2	4
No. of monomorphic allele	0	1	0	0
No. of polymorphic allele	2	2	2	4
Percentage polymorphism (%)	100	66.67	100	100
PIC	0.2859	0.27723	0.3648	0.605
Expected Heterozygousity	0.3457	0.2917	0.48	0.6563

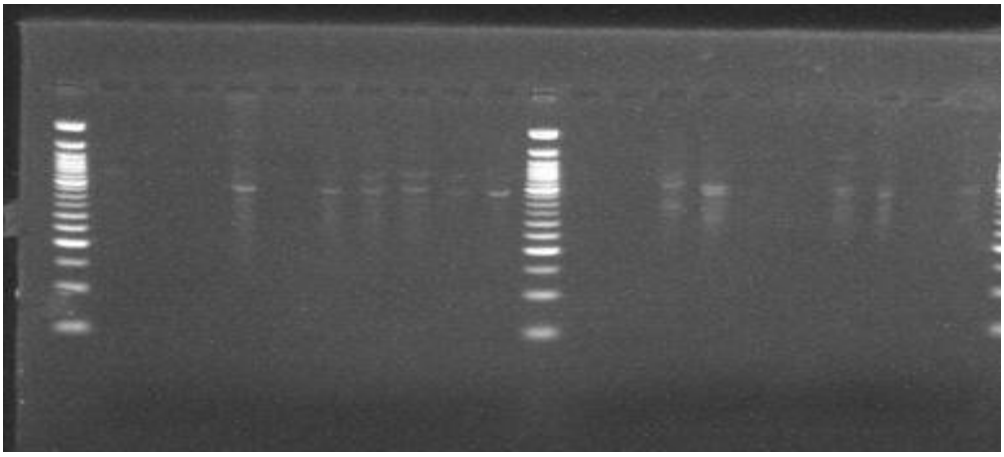

Figure 2: ISSR primer ISSR817, ISSR866 amplified in 10 varieties of *L. sativa* and *L. taraxacifolia*

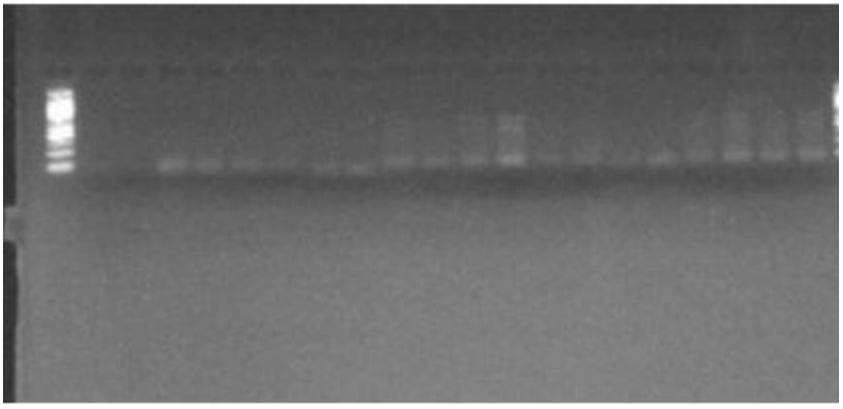

Figure 3: SSR primer SSR07, SSRA09 amplified in 10 varieties of *L. sativa* and *L. taraxacifolia.*

4.4 CLUSTER ANALYSIS

The UPGMA dendrogram showed that the 10 varieties of *L. sativa* and *L. taraxacifolia* grouped into three (3) main groups (I, II, III) (FIGURE 1). Group 1 included 7 accessions (LTU 08, LTS 07, ALO 04, ALI 01, ALU 06, ALK 05 and ALL 03), which is further subdivided into three (3)

subgroups with (LTU 08, LTS 07 and ALO 04) and (ALI01) having no genetic distance between them, showing a very close relatedness between them and with ALL 03 observed to be more divergent in the group. Group II included two (2) accessions (LTT 10 and LTU 09) showing a close phylogenic relationship between them. Group III included only ALI 02.

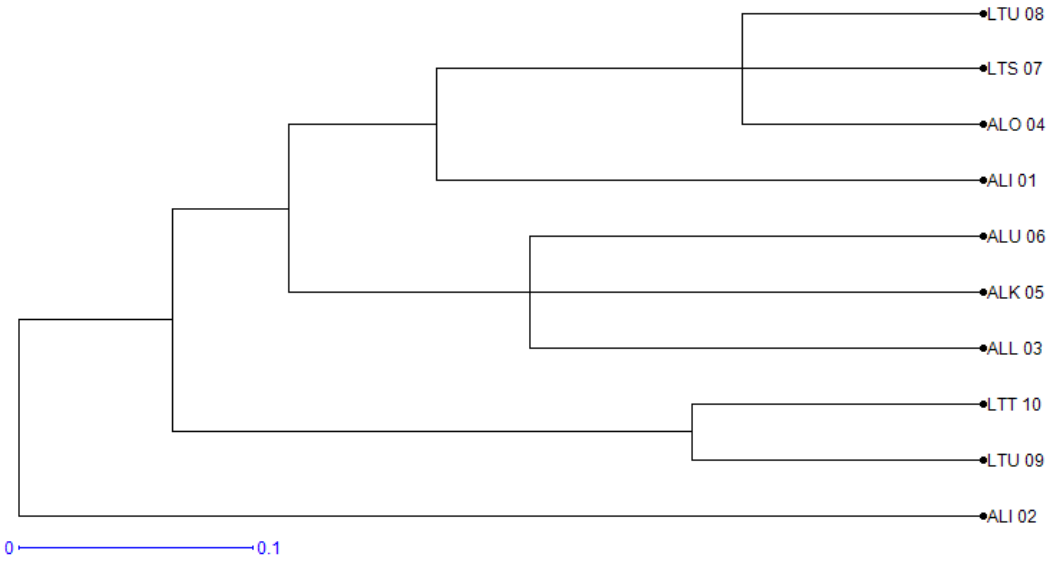

FIGURE 4: UPGMA dendrogram showing the genetic distance among the 10 *L. sativa* and *L. taraxacifolia* accessions.

4.5 FACTORIAL ANALYSIS

Based on the factorial analysis, the ten (10) accessions were categorized into four (4) quadrants. According to the analysis, four (4) samples (ALI02, ALL03, ALK05 and ALU06) formed the

first quadrant with ALL03 and ALK05 observed to be overlapping, which shows a very close relationship. Also, ALI02 and ALU06 looked to be distant from each other. In the second quadrant, it is observed that LTU09 and LTT10 are quite apart from each other showing low genetic relatedness, even though both formed a cluster. From the analysis, three (3) samples (LTS07, LTU08 and ALO04) formed the third cluster, which are closely related with LTS07 and LTU08 overlapping showing a high genetic relatedness. The fourth quadrant, ALI01 seems to be an outlier as it was further apart from others.

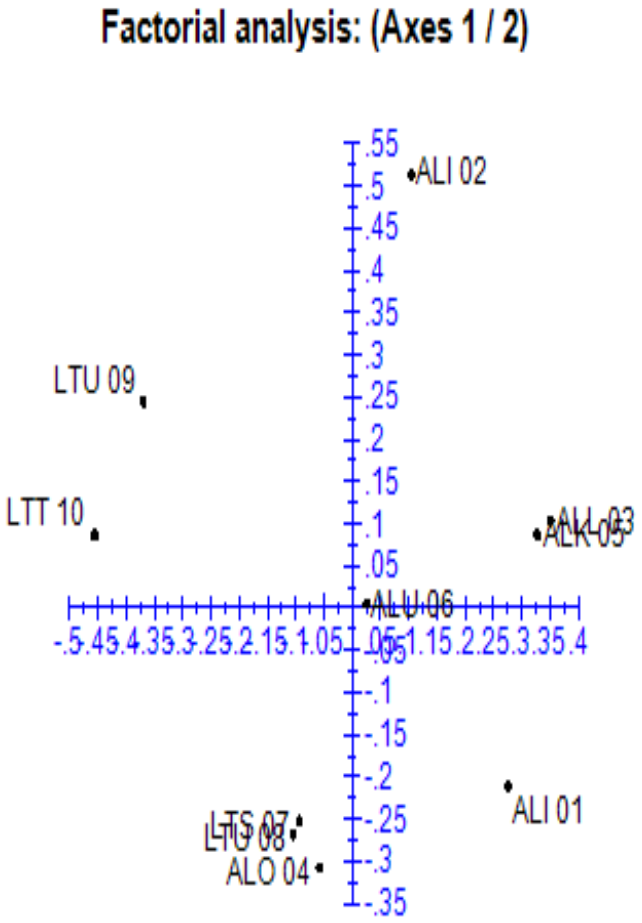

FIGURE 5: A factorial analysis showing relationship among 10 varieties of *L. sativa* and *L. taraxacifolia*

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

In this study, leaf qualitative traits, biochemical analysis (saponin and phenolic content) and two (2) polymorphic simple sequence repeats (SSR) markers (SSR07 and SSRA09) and inter simple sequence repeats (ISSR) markers (ISSR817 and ISSR866) were used to assess the genetic diversity of ten (10) varieties of *Lactuca sativa* and *Lactuca taraxacifolia* (ALI01, ALI02, ALL03, ALO04, ALK05, ALU06, LTS07, LTU08, LTU09 and LTT10) gotten from 8 different regions in Nigeria and a region in Tanzania. The variable leaf qualitative traits such as leaf width, leaf length, leaf surface profile, shape of apex and leaf base shape were observed among the 10 accessions. The leaf surface profile observed includes convex, concave and flat which is similar to the findings of (Kristkova *et al*., 2015) who also reported differences in the leaf surface profile of *Lactuca sativa* L. The leaf base shape observed includes uneven and tampering which indicates a morphological difference in the leaf margin of the studied accessions.

DNA- based molecular markers can demonstrate differences between accessions even when a morphological description is severely limited. In this study, two simple sequence repeats (SSR) markers (SSR07 and SSRA09) designed by Rauscher and Simko (2013) and two Inter simple sequence repeats (ISSR) marker (ISSR817 and ISSR866) designed by Melo *et al*. (2011) were used to determine the genetic diversity among ten accessions of *L. sativa* and *L. taraxacifolia.*

In this study, these markers have shown to be efficient in being informative and useful in analyzing genetic diversity in 10 varieties of *L. sativa* and *L. taraxacifolia.* It was observed that SSR07 generated 2 alleles, SSR09 generated 3 alleles, ISSR817 generated 2 alleles and ISSR866 generated 4 alleles. The mean percentage polymorphism of the SSR marker is 83.33% while that of ISSR marker is 100%. The observed high proportion of polymorphic alleles suggests that there is a profound genetic heterogeneity in the species. Comparison of PIC values for marker systems (a parameter associated with the discriminating power of markers) (Sangwan *et al*., 2001) indicated that the PIC values for SSR07 primers was 0.2859 and SSR09 was 0.2723, while ISSR817 was 0.3648 and ISSR866 was 0.605 indicating a better resolving power of ISSR marker over SSR.

The ability to resolve genetic variation among different genotype may be more directly related to the number of polymorphisms detected with each marker technique rather than a function of which technique is employed (Bhattacharya *et al.,* 2010). With this study we can conclude that the molecular analysis of different geographically distributed population of *L. sativa and L. taraxacifolia* across Nigeria using ISSR and SSR markers provides a powerful tool for the generation of potential diagnostic markers for cultivar analysis.

The dendrogram constructed to estimate genetic diversity among ten accessions grouped the accessions into four (4) cluster groups according to their genetic relatedness and diversity. In this present study, the four (4) cluster groups formed in the dendrogram demonstrated the existence of genetic diversity among the accessions used for this study. The factorial analysis also shows some correlation with the dendrogram analysis, distributing the varieties of *L. sativa and L. taraxacifolia* around four (4) quadrants. This study has analyzed that ISSR and SSR markers as well as leaf qualitative traits and saponin content were useful in the assessment of genetic diversity of *L. sativa* and *L. taraxacifolia.*

5.2 CONCLUSION

In conclusion, the composition of saponin in *L. taraxacifolia* had a high mean value which is responsible for the antioxidant property of the varieties analyzed. The SSR and ISSR markers used in this study were found to be useful in the assessment of genetic diversity among selected varieties of *L. sativa* and *L. taraxacifolia* indicating that the markers are efficient for genetic diversity analysis as they provide valuable information about the diversity among varieties of *L.sativa* and *L. tarxacifolia.*

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APPENDIX

APPENDIX 1: Gel electrophoresis pictures showing total genomic DNA extracted from 10 varieties of *L. sativa* and *L. taraxacifolia* genotypes and amplified by 2 SSR and ISSR based primers

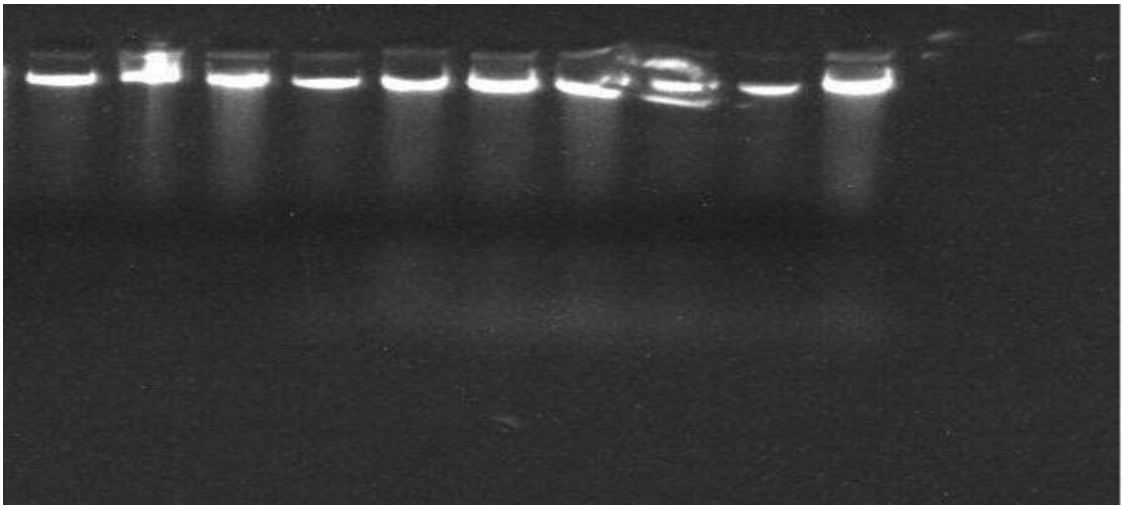

Plate 1: Showing total genomic DNA extracted from each of the 10 varieties of *L. sativa* and *L. taraxacifolia* genotypes.

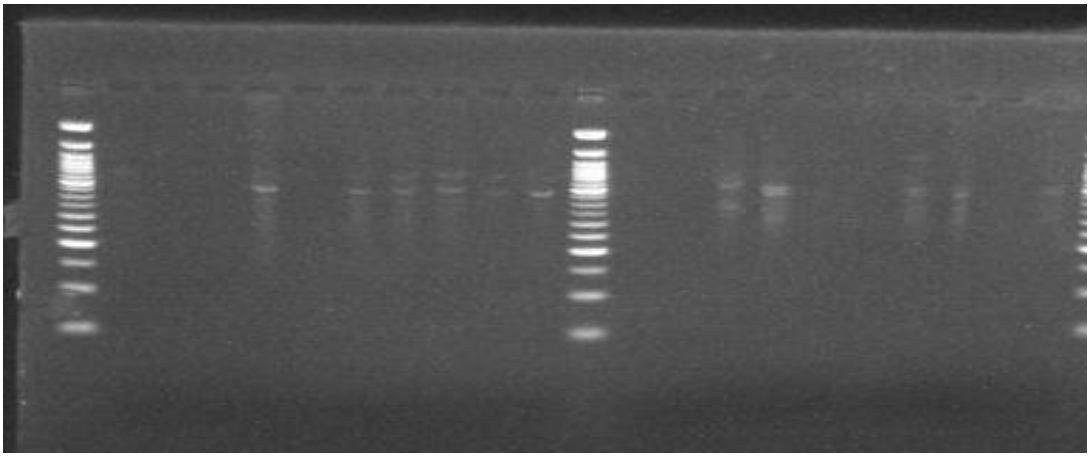

Plate 2: ISSR primer ISSR817, ISSR866 amplified in 10 varieties of *L. sativa* and *L. taraxacifolia*

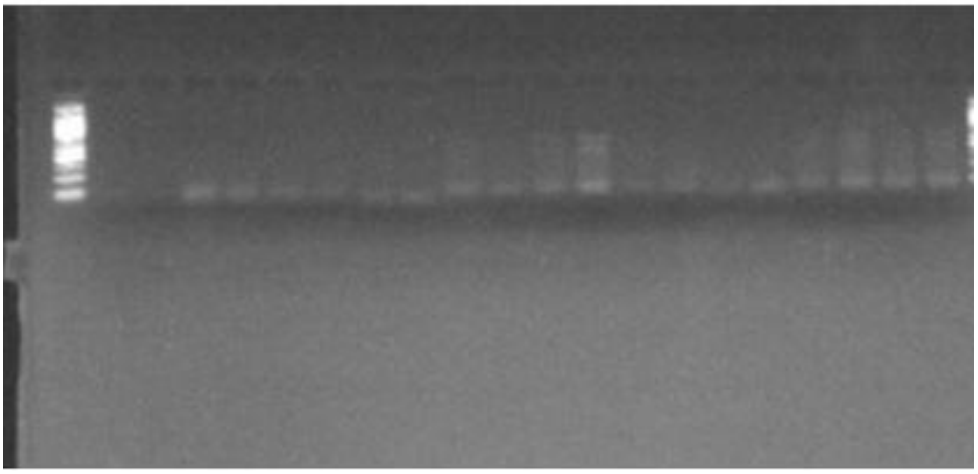

Plate 3: SSR primer SSR07, SSRA09 amplified in 10 varieties of *L. sativa* and *L. taraxacifolia*

APPENDIX 3: Table showing the mean width and length of 10 varieties of *L. sativa* and *L. taraxacifolia*

CODE	WIDTH (cm)	LENGTH (cm)	MEAN	
			Width	Length
ALI 01	6.2 6.3 7.0 6.1 7.7 10.2 6.8 6.0 7.9	10.1 12.4 12.3 10.9 11.5 15.4 11.5 16.9 16.5	7.1	13.1
ALI 02	7.2 6.8 7.0 7.4	11.2 15.0 15.2 13.5	6.2	15.3

	6.0 6.2 6.0 6.0 7.9	10.8 13.2 12.5 11.8 10.5		
ALL 03	6.3 7.7 6.2 10.4 6.8 6.0 7.9 6.7 7.5	10.8 12.5 11.5 17.0 13.3 11.6 15.4 12.4 12.2	7.3	13.0
ALO 04	5.6 4.3 3.7 3.3 2.7 4.6 3.5 2.8 3.1	15.3 13.1 12.0 12.2 12.2 11.8 11.5 10.7 11.0	3.7	12.2
ALK 05	4.5 3.1 3.9	14.4 11.5 13.5	4.1	13.5

	3.2 3.7 4.6 5.2 5.3 3.6	10.9 14.4 16.0 15.3 13.0 12.4		
ALU 06	6.0 6.7 8.1 4.5 6.7 5.8 7.0 6.3 7.8	14.9 14.3 15.8 11.6 12.4 14.4 15.8 13.1 15.4	6.5	14.2
LTS 07	6.6 7.8 5.2 8.1 7.0 6.0 8.0 7.9	16.5 19.6 13.5 17.6 15.5 12.9 18.9 18.4	7.1	16.6
LTU 08	6.5 6.5	16.0 16.0	5.7	13.8

	6.2	15.6		
	6.4	14.4		
	5.8	13.8		
	4.5	11.4		
	4.4	10.4		
	3.9	12.2		
	7.0	14.3		
LTU 09	5.0	13.5	5.7	12.9
	4.8	11.6		
	4.8	13.4		
	5.8	13.4		
	7.2	16.2		
	5.1	13.3		
	4.4	15.0		
	7.0	16.9		
	7.1	16.4		
LTT 10	4.2	15.9	6.2	16.7
	6.7	18.0		
	8.0	16.0		
	8.1	18.1		
	6.6	15.4		
	4.8	14.5		
	6.5	19.4		
	4.6	16.5		
	6.0	16.3		

APPENDIX 4: Table showing the scored data acquired from the 4 Primer amplification of 10 *L. sativa* and *L. taraxacifolia*

Scored data for primer SSR07

	Alleles	
Lines	**1**	**2**
ALI 01	1	0
ALI 02	0	0
ALL 03	1	0
ALO 04	1	0
ALK 05	1	0
ALU 06	1	0
LTS 07	1	0
LTU 08	1	0
LTU 09	0	1
LTT10	0	1

Scored data for Primer SSRA09

	Alleles		
Lines	**1**	**2**	**3**
ALI 01	1	0	0
ALI 02	1	1	1
ALL 03	1	0	0
ALO 04	1	0	0
ALK 05	1	0	0

ALU 06	1	0	0
LTS 07	1	0	0
LTU 08	1	0	0
LTU 09	1	0	0
LTT10	1	0	0

Scored data for ISSR817

	Alleles	
Lines	**1**	**2**
ALI 01	1	0
ALI 02	0	0
ALL 03	0	0
ALO 04	1	1
ALK 05	0	0
ALU 06	0	1
LTS 07	1	1
LTU 08	1	1
LTU 09	0	1
LTT10	0	1

Scored data for ISSR866

	Alleles			
Lines	**1**	**2**	**3**	**4**
ALI 01	1	0	0	0
ALI 02	0	0	0	0
ALL 03	0	0	1	0
ALO 04	1	0	0	1
ALK 05	0	0	0	0
ALU 06	0	0	0	0
LTS 07	0	0	0	1
LTU 08	0	1	0	1
LTU 09	0	0	0	0
LTT10	0	0	0	1

APPENDIX 5: Plates showing varieties of *L. sativa* and *L. taraxacifolia* used for this study

Plate 4: Showing leaves of different varieties of *L. sativa* and *L. taraxacifolia*

Plate 5: Picture showing varieties of *L. sativa*

Plate 6: Picture showing varieties of *L. taraxacifolia*