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GENETIC DIVERSITY AND RELATIONSHIP AMONG ETHIOPIA, EAST AFRICAN ACCESSIONS AND IITA INBRED LINES OF COWPEA (*VIGNA UNGUICULATA* (L.) WALP) AS REVEALED BY SSR AND SNP MARKERS

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Abstract

Genetic Diversity and Relationship Study among Ethiopian, East African accessions and IITA Inbred Lines of Cowpea (*Vigna unguiculata* (L.) Walp) as revealed by SSR and SNP markers

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PhD Thesis

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Cowpea (Vigna unguiculata L. Walp) is an important grain legume in East Africa and is mainly grown by small-scale farmers. Drought tolerance, early maturity, nitrogen fixation, and low fertility requirement are important characteristics for adaptation to the dry regions of Sub-Saharan Africa. Cowpea is a multifunctional crop providing food to both humans and animals. Because it is rich in protein, cowpea is a cheap source of protein for resource-limited families in Sub-Saharan Africa. Understanding the genetic diversity in cowpea is a crucial initial step towards planning a comprehensive conservation and cowpea improvement strategy. Little or no study has been conducted to understand the diversity and relatedness of cowpea germplasm in East Africa, particularly in Ethiopia. This PhD research first investigated patterns of molecular diversity among 210 accessions of Ethiopian local cowpea of cowpea *using 23 microsatellite markers (SSR). Additionally, the levels of genetic diversity of 95 cowpea accessions from East Africa and inbred lines from IITA were assessed using a set of 13 microsatellites (SSR) and 151 single nucleotide polymorphisms (SNPs). The average genetic diversity (D), as quantified by the expected heterozygosity, was 0.47. A total of 75 alleles with the average number of 3 alleles per locus were recorded and two rare alleles were registered when screened with SSR1 marker. The mean polymorphic information content was 0.4. The accessions were not grouped according to their geographical origins. Three main cluster groups were identified, and the sub-groups identified by neighbor joining were in accordance to the clusters revealed by the structure analysis. The AMOVA result showed moderate differentiation among populations (Fst = 0.075) and high gene flow (Nm = 3.176) between and among regions; indicating that there was significant germplasm exchange between the regions considered. The accessions in this study possess wide diversity within both individuals and populations. The study also detected five promising Ethiopian accessions from 'Amhara', 'Gambella' and SNNP regions, tightly clustered and sharing a common allele with the multi-race striga resistant accession B301from Botswana, which could help in identification of appropriate parental lines among Ethiopian accessions of cowpea for Striga gesnerioides resistance. The rare alleles identified were linked to desirable trait of striga resistance against Striga gesnerioides, SG3, RACE. The SSR markers tested in this study can thus be used for analyzing cowpea diversity reliably The polymorphic information content (PIC) value was more than that of some African countries, Ghana, Senegal and Kenya; however, considerably lower than that of the wild cowpea types;*

therefore, the results obtained in the present study reinforce the need to protect and conserve valuable genetic resources and the finding also showed that the genetic base of Ethiopian cowpea is narrow. Regarding the comparative study of accessions from some of East African countries and IITA inbred lines of cowpea, the result showed that the average genetic diversity (D), as quantified by the expected heterozygosity, was higher for SSR loci (0.52) than for SNPs (0.34). The average number of alleles per locus was higher for the SSR markers (6) than for the SNP (2) markers. The PIC was 0.48 for SSRs and 0.28 for SNPs while the fixation index was 0.095 for SSRs and 0.15 for SNPs showing moderate differentiation and high gene flow among East African countries; and thus the existence of germplasm exchange among the East African countries. The cluster analysis showed a similar pattern for both SSR and SNP markers detecting a substantial degree of association between origin and genotype. However, some accessions from different regions clustered together. The geographic distance and the genetic background of the accessions were not clearly reflected in the accession clustering of genetic tree constriction and principal component analysis. The results of fixation index, neighbor joining and genotype clustering agreed in both SNP and SSR markers. Thus for the assignment of genotypes to subgroups both neighbor joining and PCoA clustering methods are equally appropriate. The polymorphic information content (PIC) value of all of the east African countries were more than some of west African countries, Ghana and Senegal but noticeably lower than the wild cowpea types. Therefore, the results obtained in the present study showed that the cowpea genetic base of Ethiopian and other East African countries is narrow. It is recommended that the narrow genetic base can be augmented through introduction of accessions with desirable alleles. Since the study showed that most of the accessions from the gene bank are a mixture of sub-accessions Ethiopian Biodiversity Institute (EBI) should consider reorganizing their seed stock by taking the subtypes identified in this study into account.

Keywords: Cowpea, Genetic diversity, SSR, SNP, Ethiopia, Kenya, Somalia, Sudan, inbred line, East Africa, germplasm

Dedication

I dedicate this work to:

- My beloved wife Mimi M. Abera and my daughter Michaela Belayneh;
- My parents Ayalew Desalegne and Etenesh Alayu;
- and to my siblings: Tariku Ayalew, Abayneh Taye, Aynadis Ayalew, Haimanot Ayalew, and Netsanet Ayalew

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About the author

Belayneh Ayalew Desalegne was born on April 22, 1976 in Addis Ababa, Ethiopia. He grew up in ''Ferensay Legasion'' area where he did his elementary education at Tigel Lenestanet and high school education at Higher 12 Comprehensive Secondary (Addis Ababa). He studied both his first and second degrees at Alemaya University in 2000 and 2004 respectively. His first degree was in Plant Sciences/Agriculture and the second was in plant breeding and genetics. The Gambella Agriculture Research Institute recruited him in 2001 soon after he concluded his undergraduate study; during his stay at the Research Institute, he had the opportunity to participate in research, management, and leadership activities; he has served the Institute as a Deputy Director General and Crop Research Director for 5 years.

As a breeder he was also involved in planning, execution, and coordination of different research projects, which have been very crucial to the betterment of living standard of farmers, and realizing the national aim of ensuring food-security in Ethiopia; he served the Institute until he joined Addis Ababa University in 2009/2010, which is based in Addis Ababa, Ethiopia, to pursue his PhD study in Applied Genetics Stream of the Cellular, Molecular and Microbial department in the College of Natural Science; his PhD research work was supported by RCBP and Bean Project at Bioscience Lab of International Institute of Tropical Agriculture (IITA), Ibadan Nigeria, and a partial scholarship from KirkHouse Trust at Biology Department of University of Virgina and Timko Lab.

Acronyms and abbreviations

AFLP:	Amplified Fragment Length Polymorphism;
AMoVA:	Analysis of Molecular Variance;
IITA:	International Institute of Tropical Agriculture
CTAB:	Cetyltriethylammonium bromide;
D:	Gene diversity;
EIAR:	Ethiopian Institute of Agricultural Research;
GARI:	Gambella Agricultural Research Institute
EBI :	Ethiopian Institute of Biodiversity
IBPGR:	International Board for Plant Genetic Resources;
ISSR:	Inter Simple Sequence Repeats;
LG:	Linkage Group
MAS:	Marker Assisted Selection;
PC:	Principal Component;
PCA:	Principal Component Analysis
PCoA:	Principal Coordinate Analysis
PIC:	Polymorphic Information Content;
PVP:	PolyVinyl PolyPyrrolidone
RAPD:	Random Amplified Polymorphic DNA;
RCBP:	Rural Capacity Building Project
RFLP:	Restricted Fragment Length Polymorphism;
SNNP:	Southern Nations Nationalities and peoples;
SSR:	Simple Sequence Repeats
SNP:	Single Nucleotide Polymorphism

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1. Introduction

1.1 Background and Justification

Cowpea, *[Vigna unguiculata* (L.) Walp.*]*, is one of the most important food and forage legumes in the semi-arid tropics that includes parts of Asia, Africa, Southern Europe, Southern United States, Centeral and South America (Singh 2005; Timko et al.,. 2007a). Cowpea grows in a wide range of environments covering 40 ^{0}N to 30 ^{0}S (Richie 1985), and it has considerable ability to adapt to high temperatures and drought compared to most crop species (Ehlers and Hall 1997). Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, where it is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops (Langyintuo et al.,., 2003). Like other grain legumes, the protein found in cowpeas is rich in the essential amino acids, lysine and tryptophan (Timko and Singh 2008). Cowpea serves as both human food and animal feed. The crop is a source of income to both small-scale farmers (especially women farmers) and larger scale grain traders (Singh 2005; Timko and Singh 2008).

Cowpea was introduced from Africa to the Indian sub-continent approximately 2000 to 3500 years ago (Allen 1983). Cowpeas had reached Europe from Asia and have been cultivated in southern Europe at least since the 8th century BC and perhaps since prehistoric times (Tosti and Negri 2002). From the West Indies, cowpea was taken to the USA in about 1700 BC (Pursglove 1968). The slave trade from West Africa resulted in the crop reaching the southern USA early in the 18th century however, many US cultivars appear more closely related to germplasm from Asia or southern Europe than West Africa (Fang et al.,., 2007). Although cowpea is the second most important food grain legume of tropical Africa, next to *Phaseolus vulgaris*, it is the least cultivated and scarcely distributed pulse crop in Ethiopia (Gemechu et al.,., 2003). However, it is significantly important in the Gamogofa zone and especially in Konso, Derashe, and Hamerbako areas of Southern Nations, Nationalities, and Peoples (SNNP) Regional State. It is also fairly distributed in the northern part of Ethiopia bordering Eritrea, pockets of Shoa, Gojam, Wellega, and Harareghe (Gemechu et al.,., 2003). Cowpea is one of the most important pulse crops in low moister stressed areas in Babile, Guresum, and Jijiga, Eastern Ethiopia (AU, 1996).

Based on the distribution of diverse wild cowpeas along the entire length of Eastern Africa, from Ethiopia to Southern Africa, (Baudoin, and Maréchal 1985) proposed Eeast and Southern Africa to be the primary region of diversity, and west and central Africa to be the secondary centre of diversity. These researchers also proposed Asia as a third center of diversity. More recent studies strongly indicate that the highest genetic diversity of primitive wild forms of cowpea can be found in the region of the African continent currently encompassed by Namibia, Botswana, Zambia, Zimbabwe, Mozambique, Swaziland, and South Africa, with among the most primitive species observed in the Transvaal, Cape Town, and Swaziland (Padulosi 1987, 1993; Padulosi et al.,., 1990, 1991). Based on this latter observation, Padulosi and Ng (1997) suggested that southern Africa may be the site of origin of cowpea with subsequent radiations of the primitive forms to other parts of southern and Eastern Africa, and subsequently to West Africa and Asia.

Cowpea breeding and genetic improvement programs around the world are mainly focused on combining desirable agronomic characteristics, e.g., time to maturity, photo-period sensitivity, plant type, and seed quality with resistance to the major diseases, insect pests or parasites, which, agronomically afflict adapted cowpea cultivars (Timko et al.,., 2007; Timko and Singh, 2008). The conventional methods for estimating genetic diversity have been based on the use of morphological markers. However, the low availability of morphological markers, the lack of knowledge about how genes are controlled, and the environmental influence on phenotypic expression at different stages of growth have been the major limitations for using these markers as reliable tools in diversity studies (Dikshitet et al.,., 2007). Cultivated cowpea germplasm diversity studies based on isozyme diversity and other proteins have shown very low genetic diversity (D'Urzo et al.,., 1990; Pedalino et al.,., 1990; Panella et al.,., 1993; Vaillancourt et al.,., 1993) and cultivar group sesquidalis could not be distinguished from the cultivar group unguiculata (Vaillancourt et al.,., 1993). Molecular markers based on differences in DNA sequences between individuals generally detect more polymorphisms than morphological and protein-based markers and constitute a new generation of genetic markers (Botstein et al.,., 1980; Tanksley et al.,., 1989).

The development and use of molecular markers technologies, such as Restriction Fragment Length Polymorphisms (RFLP) (Lambrides et al.,., 2000), Random Amplified Polymorphic DNAs (RAPD) (Betal et al.,., 2004; Lakhanpaul et al.,., 2000; Santalla et al.,., 1998), Amplified Fragment Length Polymorphisms (AFLPs) (Zong et al.,., 2003) and microsatellites or Simple Sequence Repeats (SSR) (Li et al.,., 2001; Wang et al.,., 2004), have greatly facilitated the analysis of the structure of plant genomes and their evolution including the genetic structure and variations among cowpeas accessions (cultivated and wild). An analysis of *Vigna* species done by Fatokun et al., (1993) using RFLP markers revealed the existence of a high level of genetic variations within the genus from African origin relative to those from Asian origin.

In a study of the structure of 23 accessions of five species within the subgenus *Ceratotropics* using RAPD markers, Kaga et al., (1996) reported the existence of two main groups differing by 70% at molecular level. A study conducted by Ajibade et al.,. (2000) using Inter Simple Sequence Repeat (ISSR) DNA polymorphism for analysis of genetic relationships among 18 *Vigna* species found that closely related species within each sub-general clustered together, and cultivated cowpea grouped closely with the wild sub-species of *Vigna unguiculata*. Ba et al.,. (2004) studied the characterization of genetic variation in domesticated cowpea and its wild progenitor, and their relationship using RAPD (Sariah et al.,., 2010).

The advent of new sequencing technologies has dramatically changed the landscape for detecting and monitoring genome-wide polymorphism (Craig et al.,., 2008; Metzker, 2005; Schuster 2008). Today, single nucleotide polymorphisms (SNPs) are rapidly replacing simple sequence repeats (SSRs) as the DNA marker of choice for applications in plant breeding and genetics because they are more abundant, stable, amenable to automation, efficient, and increasingly cost-effective (Duran et al.,., 2009; Edwards and Batley, 2010; Rafalski, 2002). However, the review made by Huaqiang *et al.,* (2012), indicated that SSR was the most frequently used molecular marker, whereas the use of SNP markers for genetic diversity study of cowpea was not common. Very large numbers of SNP markers are now available for detailed analysis of genome structure, genome-wide association studies, and precision breeding, especially for those animals and plants for which high-density genotyping arrays are commercially produced (Ramos *et al.,.,* 2009; Ganal *et al.,* 2011). However, this activity has largely bypassed ''orphan crops'' such as cowpea which are crops of relevance to food security and income for subsistence farmers in developing countries (Delmer, 2005). Nowadays, SNP markers are available with genotyping service providers to quickly and affordably assay lines for diversity analysis.

The importance of local genetic resources and informal seed systems for adaptation of smallholder agriculture to climate change is starting to receive some attention and research (Bellon et al., 2011). The vulnerability of agriculture-based livelihoods to climate change is directly connected with the accessible genetic resources and characterizing these resources at the seed system level is an important contribution to understand local adaptive capacity (Ola, 2012). Assessment of genetic diversity in cowpea genotypes would facilitate development of cultivars for specific production constraints by providing an index of parental lines to be used in breeding programmes. Little information is available about the extent of genetic diversity among cowpea landraces in Ethiopia for long-term conservation and improvement. Genetic diversity is essential to decrease crop vulnerability to abiotic and biotic stress, ensure long-term selection gain in genetic improvement, and promote rational use of genetic resources (Barrett and Kidwell, 1998).

Knowledge of the genetic diversity available within the local and regional germplasm collection of cowpea can enhance the utilization of these germplasm in effective cowpea improvement programs (Hegde and Mishra, 2009). Nonetheless, no efforts have been made to address this untouched, albeit, important area of research in cowpea germplasm of Ethiopia; whereas, some studies were made on local cowpea accessions of East African countries such as Kenya (Kuruma et al., 2008) and Tanzania using SSR markers (Sariah et al., 2010), but the diversity and relatedness of cowpea germplasm between Ethiopian and other East African countries i.e. Kenya, Somalia and Sudan are poorly understood. Hence, this study was undertaken to help bridge the knowledge gap on the level of genetic diversity of Ethiopian cowpea accession and East African cowpea genotypes using highly informative DNA markers.

1.2 The major objective

To study genetic diversity of cowpea landraces collected from Ethiopia using SSR markers and to determine the genetic diversity and relationships among some local East African (Ethiopia, Kenya, Somalia and Sudan) cowpea accessions and inbred lines obtained from IITA Nigeria, based on fluorescent SSR and SNP markers.

1.3 Specific objectives

1. To determine the level of genetic diversity of cowpea germplasm collected from Ethiopia using SSR marker.
2. To support the subtype developed based on within accession morphological variability by the molecular variability.
3. To identify landraces that is tolerant and/or resistant to *Striga gesnerioides* from the Ethiopia cowpea collections.

4. To determine and compare the level of genetic diversity of cowpea in Ethiopia, Kenya, Somalia, Sudan accession, and IITA inbred lines using SSR markers.

5. To determine and compare the level of genetic diversity existing in Ethiopia, Kenya, Somalia, Sudan accessions and IITA Inbred lines using SNP markers.

6. To compare the information generated by SSR makers with SNP markers.

1.4 Research Questions

- How much genetic diversity is there in cowpea germplasm collected from Ethiopia, Kenya, Somalia, Sudan, and inbred lines of IITA?
- How much genetic diversity exists in Ethiopian cowpea accessions?
- Is there *Striga gesnerioides* resistance within Ethiopian cowpea accessions?
- Can subtypes developed from the admixture having morphological diversity (within accession morphological variability i.e. seed colour and size) be supported by molecular diversity?
- Can SNP markers give the same genetic information as SSR markers?

1.5 Research Hypotheses

- The level of genetic diversity of cowpea genotypes in Ethiopia is low.
- The subtype developed based on within accession morphological variability is not supported by molecular variability.
- There is no *Striga gesnerioides* resistant genotype within Ethiopian cowpea accessions.
- The level of genetic diversity of cowpea genotypes in Ethiopia, Kenya, and Somalia, Sudan, and Inbred lines is low.
- There is no genetic diversity within and among Ethiopia, Kenya, Somalia, Sudan, and IITA Inbred lines cowpea genotypes.
- SSR maker provide better genetic information than SNP markers

2. Literature review

2.1 Origin, Domestication, Diversity and Distribution

Cowpea [*Vigna unguiculata* (L.) Walp.], ($2n = 2x = 22$), is one of the most ancient human food sources and has probably been used as a crop plant since Neolithic times (Summerfield et al.,., 1974). Cowpea is commonly referred to as "niébé," "wake," and "ewa" in many of West African countries, and "caupi" in Brazil. In the United States, other names include "southern peas," "black-eyed peas," "field peas," "pink eyes," and "crowders". These names reflect traditional seed and market classes that developed over time in the southern United States. The name cowpea probably originated from the fact that the plant was an important source of hay for cows in the South-Eastern United States and in other parts of the world (Timko et al.,. 2007). In Ethiopia, it has different names in different regions: "Gafole" in Oromiffa, "Degera'' or ''Yelam Ater'' in Amharic, "Ngouri" in Anuak and "Ngoar" in Nuer. The determination of the origin and domestication of cowpea has been based on morphological and cytological evidence, information on its geographical distribution and cultural practices (Ng, 1995; Ng and Maréchal, 1985). Padulosi and Ng (1997) suggested Southern Africa to be the centre of origin, while domestication occurred in West Africa.

The precise origin of cultivated cowpea has been a matter of speculation and discussion for many years. Early observations showed that the cowpeas present in Asia are very diverse and morphologically different from those growing in Africa, suggesting that both Asia and Africa could be independent centers of origins for the crop. However, the absence of wild cowpeas in Asia as possible progenitors has led some investigators to question whether the Asian center of origin is valid. All of the current evidence suggests that cowpea originated in southern Africa, although, it should be noted that it is difficult to ascertain where on the continent the crop was first domesticated (Sariah et al., 2010).

According to Timko et al. (2007), interestingly, while West Africa appears to be the major center of diversity of cultivated forms of cowpea (Ng and Padulosi, 1988) and was probably domesticated by farmers in this region (Ba et al.,., 2004), the center of diversity of wild *Vigna* species is South-Eastern Africa (Padulosi and Ng, 1997). Cowpea is considered to have been domesticated in Africa from its wild ancestral form, *V. unguiculata* subsp. *dekindtiana* (Harms) Verdc. (Ng and Marechal, 1985). The cultivated cowpea (*V. unguiculata*) evolved through domestication and selection from the annual wild cowpea (ssp. *dekindtiana*), and during domestication process plant lost seed dormancy and pod dehiscence (Ng, 1995).

Cowpea was introduced from Africa to the Indian sub-continent approximately 2000 to 3500 years ago (Allen, 1983). Cowpeas reached Europe from Asia and has been cultivated in southern Europe at least since the 8th century BC and perhaps since prehistoric times (Tosti and Negri, 2002). From the West Indies, cowpea was taken to the USA in about 1700 BC (Pursglove, 1968). The slave trade from West Africa resulted in the crop reaching the southern USA early in the 18th century. However, many US cultivars appear more closely related to germplasm from Asia or southern Europe than West African (Fang et al.,., 2007). At present, cowpea is grown throughout the tropic and subtropics of the world (Agbicodo et al., 2009). The small seed size of wild cowpeas likely facilitated their dispersal by birds throughout East and West Africa contributing to the diversity and development of secondary wild forms (Sariah et al., 2010). Human selection for larger seeds and better growth habits from natural variants in wild cowpeas likely led to diverse cultigroups and their domestication in Asia and in Africa (Steele, 1976; Ng and Padulosi, 1988; Ng 1995; Ba et al., 2004).

2.2 Cowpea Taxonomy

Cowpea [*Vigna unguiculata* (L) Walp.] is a dicotyledonous crop in the family Fabaceae, subfamily Faboideae (Syn. Papillionoideae), tribe Phaseoleae, subtribe Phaseolinae, genus *Vigna*, and section Catiang (Verdcourt, 1970; Maréchal et al., 1978). It contains 22 chromosomes ($2n = 2x = 22$) (Timko and Singh 2008) and its nuclear genome size is estimated to contain about 620 Mbp (Timko et al.,. 2008).

The genus *Vigna* is pantropical and highly variable genus. In addition to cowpea, other members of the genus include mungbean (*V. radiata*), adzuki bean *(V. angularis*), blackgram (*V. mugo*), and the bambara groundnut (*V. subterranea*). The genus was initially divided into several subgenera based upon morphological characteristics, extent of genetic hybridization/reproductive isolation, and geographic distribution of species (Maréchal et al., 1978; Timko and Singh, 2008). The major groupings consist of the African subgenera *Vigna* and *Haydonia*, the Asian subgenus *Ceratotropis*, and the American subgenera *Sigmoidotropis* and *Lasiopron*. Under the scheme proposed by Maréchal et al. (1978) cultivated cowpea was placed in the subgenus *Vigna*, whereas mungbean and blackgram were placed in the Asian subgenera. *V. unguiculata* subspecies *unguiculata* which includes four cultigroups: *unguiculata*, *biflora* (or *cylindrica*), *sesquipedalis*, and *textilis* (Ng and Maréchal, 1985). *V. unguiculata* subspecies *dekindiana*, *stenophylla*, and *tenuis* are the immediate wild progenitors of cultivated cowpea and form the major portion of the primary sub-groups of cowpea. Members of subspecies *dekindiana*, *stenophylla*, and *tenuis* are also considered part of these sub-groups. Secondary subgroups are constituted by other wild subspecies, like *pubescence* that do not readily hybridize and show some degree of pollen sterility and require embryo rescue (Fatokun and Singh, 1987). Observations recent attempts to cross *V. vexillata* and *V. radiata* with *V. unguiculata* (Barone et al., 1992; Gomathinayagam et al., 1998) indicate that these species may constitute a tertiary sub-groups for cowpea.

2.3 Morphological and phenological characteristics

Cowpea is a herbaceous warm-season annual that is similar in appearance to common bean except that leaves are generally darker green, shinier, and less pubescent. Cowpeas also are generally more robust in appearance than common beans with better-developed root systems and thicker stems and branches. Plant growth habit can be erect, semi-erect, prostrate (trailing), or climbing depending mostly on genotype, although photoperiod and growing conditions can also affect plant stature. Most cowpea accessions have indeterminate stem and branch apices. Early flowering cowpea genotypes can produce a crop of dry grain in 60 days, while longer season genotypes may require more than 150 days to mature; depending on photoperiod. Flowers are borne on racemes on 15- to 40 mm peduncles that arise from the leaf axils. Two or three pods per peduncle are common, and often four or more pods are carried on a single peduncle if growing conditions are very favorable. The presence of these long peduncles is a distinguishing feature of cowpea, and this characteristic facilitates hand harvesting according to (Timko and Singh, 2008).

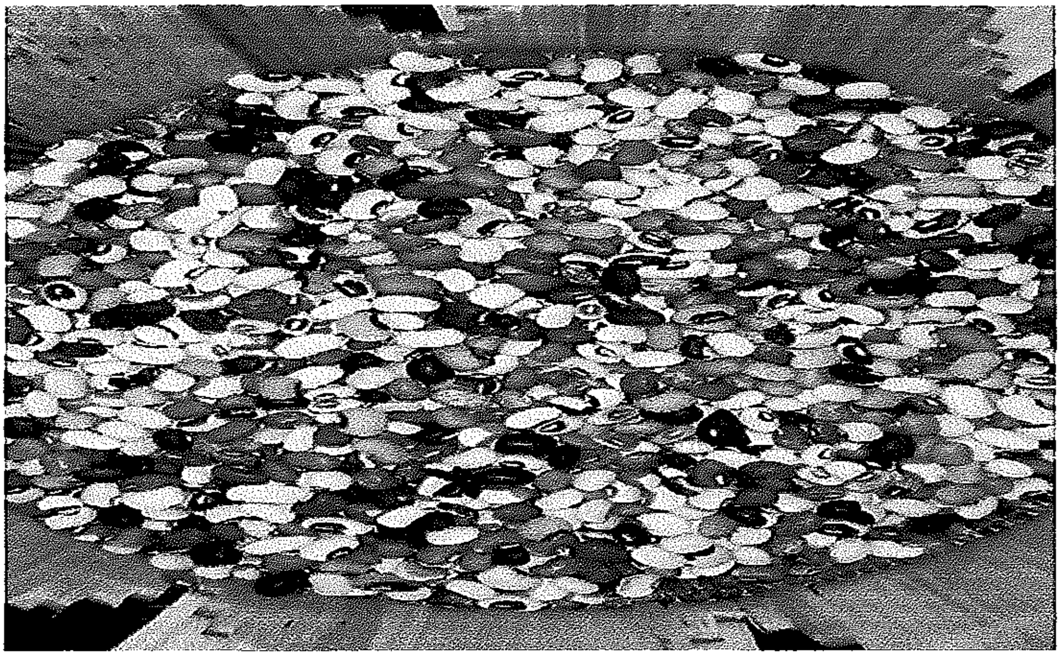


Figure 1 : Diversity of seed types in cowpea. Shown in photograph is variation in seed shape, color, and texture observed in cowpea from around the world (picture courtesy of J.D. Ehlers) from Timko etal, (2007)

Cultivated cowpea seed weighs between 8 and 32 mg and ranges from round to kidney shaped. Pods are cylindrical and may be curved or straight, with between 8 and 15 seeds per pod. The seed coat can be either smooth or wrinkled and of various colors including white, cream, green, buff, red, brown, and black (Fig. 1) (Agbicodo et al., 2009). Seed may also be speckled or patterned. Seeds of well-known cowpea types, such as "black-eyed pea" and "pinkeye," are white with a round irregular-shaped black or red pigmented area encircling the hilum, giving the seed the appearance of an eye. Emergence is epigeal (similar to common bean and lupin), where the cotyledons emerge from the ground during germination. This type of emergence makes cowpea more susceptible to seedling injury, since the plant does not regenerate buds below the cotyledonary node (Agbicodo et al., 2009). The open display of flowers in and above the canopy and the presence of extrafloral nectaries contribute to the attraction of insects. Cowpea primarily is self-pollinating, but outcrossing rates as high as 5% have been recorded and care needs to be taken to avoid outcrossing during the production of breeder and foundation seed, or unacceptable levels of "off-types" will result. Cowpea is a short day plant, and many cowpea accessions exhibit photoperiod sensitivity with respect to floral bud initiation and development, while others are day neutral (Ehlers and Hall, 1996; Craufurd et al., 1997). For some genotypes, the degree of sensitivity to photoperiod (extent of delay in flowering) is modified by temperature (Wein and Summerfield, 1980; Ehlers and Hall, 1996). In West Africa, selection for differing degrees of photosensitivity or differences in juvenility has occurred in different climatic zones such that pod ripening coincides with the end of the rainy season in a given locale, regardless of planting date, which is often variable due to the variable onset of wet seasons (Steele and Mehra, 1980). This attribute allows pods to escape damage from excessive moisture and pathogens. Photoperiod sensitivity, when appropriately deployed in a breeding program, can be valuable to ensure crop maturity after wet seasons or before drought or cold weather limits crop growth. However, it may constrain the direct usefulness of an otherwise desirable cultivar to a small area of adaptation or even to a specific season within this restricted area.

Cultivated cowpeas have been divided into five cultivar groups based mainly on pod and seed characteristics (Pursglove, 1968; Pasquet, 1999). Cultivar group *unguiculata* is the largest and includes most medium- and large-seeded African grain and forage-type cowpeas. Cultivar group Melanophthalmus includes "black-eyed pea"-type cowpea with large, somewhat elongated seeds with wrinkled seed coats and fragile pods (Pasquet, 1998). Members of cultivar group Biflora (also known as "catjang") are common in India and characterized by their relatively small smooth seeds borne in short pods that are held erect until maturity. Cultivar group Textilis is a rather rare form of cowpea with very long peduncles that were used in Africa as a source of fiber. Cultivar group Sesquipedialis (known as "yard-long bean," "long bean," "Asparagus bean," or "snake bean") is widely grown in Asia for production of its very long (40 to 100 cm) green pods that are used as "snap" beans. Despite the striking differences in morphological characteristics among the cultivar groups, there are no practical barriers to hybridization or recombination between members of the different groups (Timko and Singh, 2008).

2.4 Production system and Socio-economic Importance of Cowpea

Different source reported different data of cowpea production and production area; cowpea is grown worldwide with an estimated cultivation area of about 12.5 million hectares annually and an annual worldwide production of over 3 million metric tons (Li et al., 2001). About 70% of the cowpea production occurs in marginal areas of West, Central, East and Southern Africa. Nigeria is the largest producer and consumer of cowpea at estimated annual yields of 2 million metric tons (Singh et al., 2002; Timko et al., 2007). In Tanzania, cowpea is regarded as a 'women's crop, because, contrary to other crops, the production process to marketing is often handled by women. Thus, it is among the crops that are generating income to female farmers and traders (Sariah et al., 2010). Production of cultivar group Sesquipedialis (yard-long) bean is widespread throughout Asia and is thought to be grown on about 300,000 ha. Dry grain production is the only commodity of cowpea formerly estimated on a worldwide basis (Tmko et al., 2007). The United Nations Food and Agricultural Organization (FAO) estimates that nearly 4 million metric tons (mT) of dry cowpea grain is produced annually on about 10 million ha worldwide (www.faostat.fao. org/faostat). Worldwide cowpea grain production has gone from an annual average of about 1.2million mT in the 1970s to approximately 3.6mmt per annum (during the five-year period spanning 1998 to 2003). Cowpea grain production estimates by Singh et al. (2002) are slightly higher than FAO estimates, with worldwide production of 4.5 million mT on 12 to 14 million ha. About 70% of this production occurs in the drier Savanna and Sahelian zones of West and Central Africa, where the crop is usually grown as an intercrop with pearl millet [*Pennisetum glaucum* (L.) R.Br.] or sorghum [*Sorghum bicolour* (L.) Moench] and, less frequently, as a sole crop or intercropped with maize (*Zea mays* L.), cassava (*Manihot esculenta* Crantz), or cotton (*Gossypium* sp.) (Langyintuo et al., 2003) (Fig. 2). Other important production areas include lower elevation areas of eastern and southern Africa and in South America (particularly in northeastern Brazil and in Peru), parts of India, and the southeastern and southwestern regions of North America (Timko et al., 2007). Nigeria is the largest producer and consumer of cowpea grain, with about 5 million ha and over 2 million mT production annually, followed by Niger (650,000 mT) and Brazil (490,000 mT) (Singh et al., 2002). The United States produces about 80,000 mT, in several southern states (Alabama, Arkansas, Georgia, Louisiana, Missouri, Tennessee) and in Texas and California (Fery, 2002).

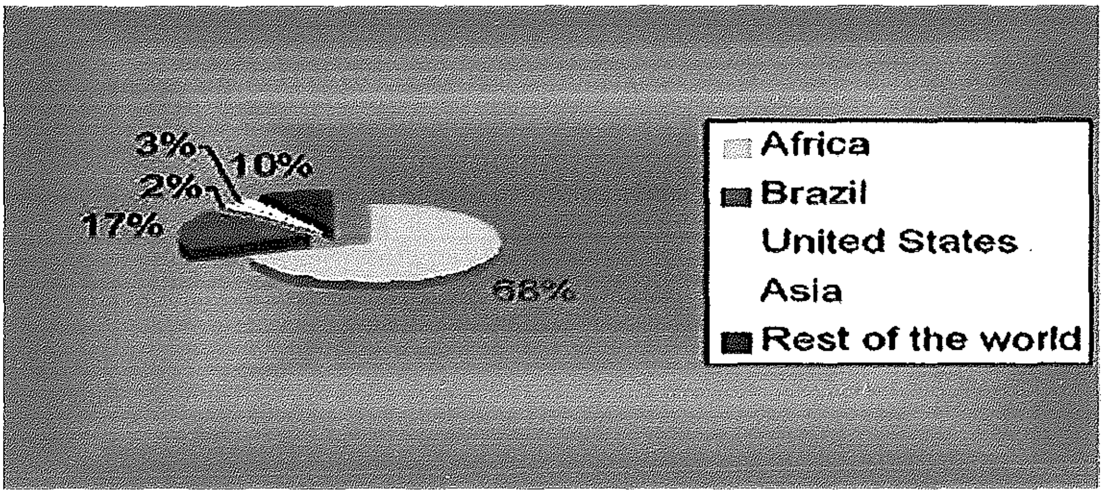


Figure 2. Percentage of production of Cowpea (dry grain) by continent and countries (FAO) http://www.fao.org/inpho/content/compend/text/ch32/ch32.htm)

Cowpea is a multipurpose crop, providing food for human and feed for livestock and it is a cash generating commodity for farmers, small and medium-size entrepreneurs (Sariah et al., 2010). It can also be used as cover crop (Langyintuo et al.,. 2003; Singh 2002; Timko et al.,. 2008). The very early maturity characteristics of some cowpea varieties provide the first harvest earlier than most other crops during production period. This is an important component in hunger fighting strategy, especially in the Sub-Saharan Africa where the peasant farmers can experience food shortage a few months before the maturity of the new crop (Sariah et al., 2010). Its drought tolerance, relatively early maturity and nitrogen fixation characteristics fit very well to the tropical soils where moisture and low soil fertility is the major limiting factor in crop production (Hall, 2004; Hall et al., 2002).

It is usually the first crop harvested before the cereal crops are ready and, therefore, is referred to as "hungry-season crop" (Agbicodo et al., 2009). With more than 25% protein in dry seeds as well as in young leaves (dry weight basis), cowpea is a major source of protein, minerals and vitamins in daily diets and is equally important as nutritious fodder for livestock (Singh et al.,. 2003). The high protein content of cowpea grain represents a major advantage for use in infant and children's food (Lambot, 2002). The mature pods are harvested and the haulms are cut while still green and rolled into small bundles containing the leaves and vines. These bundles are stored on rooftops for uses as feed supplement in the dry season, making cowpea a key component of crop-livestock systems. Cowpea haulms fetch 50% or more of the grain price (dry weight basis). Therefore, cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, and is a valuable and dependable commodity that produces income for farmers and traders (Singh, 2002; Langyintuo et al., 2003). Additionally, cowpea is a valuable component of farming systems in many areas because of its ability to restore soil fertility for succeeding cereal crops grown in rotation with it (Carsky et al., 2002; Tarawali et al., 2002; Sanginga et al., 2003).

2.5 Nutritional composition of cowpea

The protein found in cowpea, similar to the one from other legumes, rich in the essential amino acids lysine and tryptophan (Timko and Singh 2008). However, the protein nutritive value of these legumes is lower than that of animal proteins because they are deficient in sulfur amino acids and contain a non-nutritional factors (phytates and polyphenols), enzymes inhibitors (against trypsin, chymotrypsin and R-amylase) and hemagglutinins (Jackson 2009). The nutritional content of cowpea grain is important because millions of people who otherwise have diets lacking in protein, minerals, and vitamins eat it in quantity. The nutritional profile of cowpea grain is similar to that of other pulses, with a relatively low fat content and a total protein content that is two to four times greater than cereal and tuber crops. Like other pulses, the protein in cowpea grain is rich in the amino acids lysine and tryptophan, compared to cereal grains (Timko et al., 2007). However, it is deficient in methionine and cystine when compared to animal proteins. In a study of 100 cowpea breeding lines in the IITA collection, seed protein content ranged from 23 to 32% of dry seed weight (Nielson et al.,. 1993). Similarly, protein content of 12 West African and US cultivars ranged from 22 to 29%, with most accessions having protein content values between 22 and 24% (Hall et al., 2003). These results suggest that sufficient genetic variation exists to develop new cowpea cultivars with protein content of at least 30%. Cowpea grain is also a rich source of minerals and vitamins (Hall et al., 2003) and it has one of the highest levels of any food of folic acid, a crucial B vitamin that helps prevent spinal tube defects in unborn children.

Cowpea can be used at all stages of growth as a vegetable crop, and the leaves contain significant nutritional value (Nielson et al., 1993; Ahenkora et al., 1998). The tender green leaves are an important food source in Africa and are prepared as a pot herb, like spinach. Immature green pods are used in the same way as snap beans, often being mixed with cooked dry cowpeas or with other foods. Nearly mature "fresh-shelled" cowpea grains are boiled as a fresh vegetable or may be canned or frozen. Dry mature seeds are also suitable for boiling and canning. In many areas of the world, cowpea foliage is an important source of high-quality hay for livestock feed (Tarawali et al., 2002). In developed countries, cowpea is expected to become increasingly important as consumers seek interesting and healthy "new" foods and rediscover "traditional" foods that are low in fat, high in fiber, and that have other health benefits. Fat contents of 100 advanced breeding lines from IITA showed a range in fat contents from 1.4 to 2.7% (Nielson et al., 1993), while fiber content is about 6% (Bressani, 1985). Besides being low in fat and high in fiber, the protein in grain legumes like cowpea has been shown to reduce low-density lipoproteins that are implicated in heart disease (Phillips et al., 2003). In addition, because grain legume starch is digested more slowly than starch from cereals and tubers, their consumption produces fewer abrupt changes in blood glucose levels following consumption (Phillips et al., 2003). Innovative and appealing processed- food products using dry cowpea grain, such as cowpea-fortified baked goods, extruded snack foods, and weaning foods, have been developed (Phillips et al., 2003). Protein isolates from cowpea grains have good functional properties, including solubility emulsifying and foaming activities (Rangel et al., 2004), and could be a substitute for soy protein isolates for persons (especially infants) with soy protein allergies.

Varieties of cowpea with a "persistent-green" grain have been developed by breeding programs in the USA that are a versatile product for frozen vegetable applications (Ehlers et al., 2002 a). Persistent- green cowpea grains are green-colored when dry but when soaked in water for several hours closely resemble fresh-shelled cowpea that can be used in frozen vegetable products to add color and variety. Because persistent-green cowpea grain can be harvested and stored dry until rehydration and freezing, it is a quite convenient and economical frozen vegetable compared to other frozen vegetable crops that require highly coordinated harvesting and processing operations and expensive long term frozen storage (Timko et al., 2007).

2.6 Production constraints

Both abiotic and biotic stresses can result in a significant yield reduction in cowpea. Despite cowpea being more drought tolerant than many other crops, still moisture availability is the major constraints to growth and development, especially during germination and flower setting. Erratic rainfall affects adversely both plant population and flowering ability, resulting tremendous reduction of grain yield and total biomass in general (Timko and Singh, 2008).

2.7.2 Flower thrips

Cowpea crop has been reported to be infested with two species of thrips, *Sericothrips occipitalis* and *Megalurothrips sjostedti* (*Thripidae*) (Ezueh ,1981). Thrips, (*Megalurothrips sjostedti*),.are small; opportunistic; and ubiquitous insects, of often only a few millimeters length and generally yellow, brown or black in color (Morse and Hoddle 2006). Singh and Taylor (1978) pointed out that plant parts mainly attacked by thrips are flower buds and later the flower themselves. Flower abortion is of normal magnitude in plants that are infested with thrips. Apart from the direct damage caused by thrips, it has been reported that they are vector for a number of pathogens that they transmit mechanically from plant to plant (Ullman et al., 1997).

2.7.3 Storage Weevil

Messina (1984) reported high mortality of larvae in the field due to failure of larvae to penetrate the seed after drilling through the pod wall. The adult emergence occurs after harvest (Booker, 1967) in the store where real destruction happens due to re-infestations and easiness of larvae penetration into the seed because usually the seeds are stored after shelling.

2.7.4 Striga gesneriodes

The genus *Striga* (family Scrophulariaceae) is composed of ≈50 species, most of which are obligate parasites of tropical cereals and legumes (Butler, 1995). The most agronomically important species of the genus are *S. hermonthica* (Del.) Benth, *S. asiatica* (L.) Kuntz, and *S. gesnerioides* (Botanga, 2005;Pieterse, A. H. 1985). All the three species associate intricately with their hosts and constitute a serious threat to food crop production in Africa (Lagoke et al., 1991) by parasitizing the roots of crop plants and serving as water and nutrient sinks (Rogers and Nelson, 1962). *S. hermonthica* and *S. asiatica* are most devastating on cereal crops. In West Africa, they cause serious yield losses to staple food crops such as corn (*Zea mays*), sorghum (*Sorghum bicolour*), upland rice (*Oryza sativa*), and pearl millet (*Pennisetum glaucum*) (Aggarwal, 1985) and (Table 1).

Cowpea production is limited by several abiotic and biotic factors, including parasitic weeds, among which *Alectra vogelii* Benth. and *S. gesnerioides* are the most important. *Alectra vogelii* is restricted to Africa while *S. gesnerioides* is found in Africa, and in parts of Asia and the USA (Musselman et al., 1991; Parker and Riches, 1993). The parasitic angiosperm, *Striga gesnerioides,* infects the roots of cowpeas and can cause grain yield losses of up to 50%

(Aggarwal and Oue'draogo, 1989). Aggarwal and Oue'draogo (1989) recorded 30% yield losses while farmers in Northern Nigeria experienced 100% losses, because of *Striga* (Emechebe et al., 1991). *S. gesnerioides*, on the other hand, attacks cowpea (*Vigna unguiculata*), and results in 100% yield loss in some instances (Emechebe, et al., 1991). It also is reported to attack tobacco (*Nicotiana tabacum*) in South Africa (Musselman, and Parker, 1981; Visser, 1981) (Table 1). In West Africa, *S. gesnerioides* is an increasingly serious problem and when there is drought; its impact becomes even more significant (Obilana, 1987). Furthermore, its rapid spread to new regions constitutes a severe threat to cowpea production (Agbobli, 1999)

Table 1: Geographic distribution and host range of *Striga* species of major economic importance.

Striga species	Distribution	Susceptible crops
S. asiatica	West, East and south Africa; Indian sub-continent; Near East; Far East; USA	Maize, finger and pearl millets, sorghum, sugar cane, upland rice
S. hermontica	West and East Africa	Maize, finger and pearl millets, sorghum, sugar cane
S. gesnerioides	West and south Africa; Indian sub-continent; Near East; USA	Cowpea and tobacco in southern Africa

Source: J. A. Lane and J. A. Bailey. 1992

It has been estimated that the loss of carbon from the host by export to the parasite is more important than reduced photosynthetic capacity of the host imposed by the parasite in accounting for observed reduction in host growth in the cowpea *S. gesnerioides* interaction (Graves, et al., 1992). The damage done to the crop host is greater than can be explained by nutrient diversion alone. It is reported that the exchange of chemicals is bi-directional and that the parasite possibly releases some toxins that inhibit the growth and development of the host (Efron, etal 1988; Graves, et al., 1992 and Graves et al.,., 1989). This argument is supported by the fact that a small portion of 14C-labeled CO_2 taken up by photosynthetically active. *Striga* plants eventually could be traced in the host plant (Rogers and Nelson, 1962).

2.8 Abiotic Stresses of Cowpea

Over 35% of the world's land surface is considered arid and semi-arid, experiencing inadequate rain fall for agricultural crop production (Le Houerou, 1996). In such areas, the occurrence of recurrent and extended period of drought is not uncommon. The problem is wide spread in areas where agriculture has been extended in to marginal rainfall areas and where subsistence agriculture is the main stay of the economy (Mc Williams, 1989) as a consequence, in the developing countries such as in part of sub-Saharan Africa, much of Asia and north America, during the past several years a reduction in agricultural crop production has been a frequent occurrence. The global atmosphere CO_2 has been observed to be rising steadily and is expected to double its current level before the end of this century, because of human activities (Jarrvis, 1993; Keeling et al., 1995; Kimball et al., 2001). The rise in atmospheric Co_2 concentration is likely to affect global climate and to cause regional changes in air temperature and humidity, length of growing season, rainfall pattern and evaporation all of which will have considerable impact on plant water relations (Daie, 1988; Jarvis, 1993). Global climate change is now generally considered to be underway (Hillel and Rosenzweig, 2002), and is expected to result in a long-term trend towards higher temperatures, greater evapotranspiration, and an increased incidence of drought in specific regions.

William (1989), reported that about 26% of the world's cultivated land falls in arid and semi-arid areas, where water is the major limiting factor to crop production. For instance, in Ethiopia the drought stressed areas covers about half (46%) of the total arable land (Reddy and Kidane, 1993).

Agriculture currently uses 75% of the total global consumption of water (Molden, 2007). North, Eastern and Southern Africa as well as West, South and Far East Asia will be among the most water-vulnerable regions of the world in 2025 (Rijsberman,2006). Global agriculture in general and African agriculture in particular is at a cross roads due to a biotic stresses, particularly drought (Sinha, 1987). Food security is unsustainable without yield increases in marginal environments, especially drought-prone areas as 80% of agriculture worldwide is rain fed (Lorieux, 2005). Population growth and climate change present crop researchers and plant breeders with one of the great grand challenges of the 21st century to productively grow nutritious crops in water-scarce environments (Pimentel et al., 2004). The growing world population together with the lack of expansion, or even reduction of available arable lands needed to maintain agricultural sustainability (Cassman, 2003), implies that the relative importance of plant breeding to raise crop yield potential and adaptiveness is now greater than in the past (Slafer et al., 1999; Araus et al., 2002). The situation is rather worsened in Africa where most of the major famines, even if aggravated by civil war, were due to drought (Ceccarelli et al., 2004).

Traditionally, drought tolerance is defined as the ability of plants to live, grows, and yields satisfactorily with limited soil water supply or under periodic water deficiencies (Ashley, 1993). According to Mitra (2001), the mechanisms that plants use to cope with drought stress can be grouped into three categories viz. drought escape, drought avoidance, and drought tolerance. However, crop plants use more than one mechanism at a time to cope with drought. Plants respond and adapt to drought at molecular, cellular and whole plant levels by activating a range of physiological and biochemical responses controlled by a network of genes (Bartels and Sunkar, 2005). Changes, such as ABA accumulation, osmotic stress adjustment, and root morphology, are known to be controlled by multiple genes (Champoux et al., 1995; Lebreton et al., 1995; Lilley et al., 1996; Tuberosa et al., 1998).

Drought tolerance is associated with many different morphological and physiological traits or responses including stomatal regulation, variation in leaf cuticle thickness, root morphology and depth, osmotic adjustment, antioxidant capacity, desiccation tolerance (membrane and protein stability), maintenance of photosynthesis and the timing of events during reproduction (Bohnert et al., 1995; Bray 1997; Nguyen et al., 1997; Klueva et al., 1998). In cowpea, morphological, biochemical and physiological traits affecting responses to drought have been identified (Turk et al., 1980; Kulkarni et al., 2000; Ogbonnaya et al., 2003; Matsui and Singh, 2003; Slabbert et al., 2004; Anyia and Herzog, 2004a; Souza et al., 2004; Hamidou et al., 2007).

The increased incidence of drought in some cowpea growing areas has caused a shift to early maturing varieties (Mortimore et al., 1997). Early maturity of cowpea cultivars is desirable and has proven to be useful in some dry environments and years because of their ability to escape drought (Hall and Patel, 1985; Singh 1987; 1994). Such early cultivars can reach maturity in as few as 60 to 70 days in many of the cowpea production zones of Africa. Earliness is important in Africa as early cultivars can provide the first food and marketable product available from the current growing season, and they can be grown in a diverse array of cropping systems. In addition to escaping drought, early maturing cultivars can escape some insect infestations (Ehlers and Hall, 1997).

Although cowpea has considerable adaptation to high temperatures and drought when compared to other crop species (Hall et al., 2002; Hall, 2004), it still suffers important yield reduction due to erratic rainfall. Efforts to develop cowpea varieties with enhanced drought tolerance have focused on mid- and terminal-season drought stress because of the negative effects on yield (Hall, 2004; Dadson et al., 2005). However, due to the increased frequency of drought stress over the last 30 years (Hall et al., 2003) and the irregular rainfall pattern especially at the beginning of the cropping season, tolerance to drought at seedling stage has become more important.

Cowpea is considered a drought-avoiding plant with stomata that are extremely sensitive to water stress (Shackel and Hall, 1979; Hamidou et al., 2007). Because of its negative effect on yield, complete stomata closure is not useful in breeding for drought tolerance (Mitra, 2001). Cruz de Carvalho et al. (1998) compared cowpea and common bean cultivars and found that cowpea genotypes kept their stomata partially opened and had a lower decrease in their net photosynthetic rates than the common bean genotypes. Cowpea also changes the position of leaflets under drought (a drought avoidance mechanism). They become paraheliotropic and orientated parallel to the sun's rays when subjected to soil drought, causing them to be cooler and thus transpire less (Shackel and Hall, 1979), which helps to minimize water loss and maintain water potential.

Given that these trends will likely continue there is increasing need to identify and develop drought tolerant crop cultivars for these agro-ecological zones. Despite its inherent drought tolerance, significant differences in response to drought stress exist among cowpea genotypes (Hall et al., 2003; Hall, 2004b; Muchero et al., 2008). In general, cowpeas are very sensitive to drought during pod set and pod filling (Turk et al., 1980). A delayed-leaf-senescence (DLS) trait has been discovered in cowpea that conferred some resistance to reproductive-stage drought in erect cowpea cultivars. In California, the Delayed-Leaf-Senescence (DLS) trait had been shown to enhance the ability of early flowering cowpea to recover after an early drought and produce a compensatory second flush of pods in some field conditions (Gwathmey and Hall, 1992). The DLS trait enabled them to recover after the drought and produce a larger second flush of pods that compensated for the low yield by the first flush of pods (Gwathmey and Hall, 1992).The DLS trait also had been shown to enhance the second flush of pods in one tropical location where lines were tested in the wetter part of the Sahelian zone (Hall et al., 1997b). The combination of DLS with early flowering, which allow plants to produce a second flush of pods, offers potential to circumvent adverse effects due to both mid- and terminal-season drought conditions (Gwathmey et al., 1992).

Most studies in the past have dealt with screening for drought tolerance as a whole and not individual components involved in drought tolerance (Lawan, 1983, Watanabe et al., 1997). Since several factors and mechanisms (in shoots and roots) operate independently or jointly to enable plants to cope with drought stress, drought tolerance appears as a complex trait (Krishnamurthy et al., 1996). Singh et al., (1999a) described a simple wooden box screening method showing good correlation with drought tolerance at vegetative and reproductive stages to select drought-tolerant plants or progenies in cowpea at the seedling stage. The method has proven to be efficient in screening for drought tolerance in different crop species (Singh et al., 1999b; Tomar and Kumar, 2004; Slabbert et al., 2004; Ewansiha and Singh, 2006).

The identification of the genetic components of drought resistance is a necessary requirement to ensure further progress in plant breeding for drought resistant crops. Bohnert et al. (1995) rightly pointed out that "one promising genetic avenue is the mapping of quantitative trait loci that relate performance and yield to drought, low-temperature, or salinity tolerance. Candidate gene analysis starts with selection of some target genes based on biological pathway or genome location relative to a known QTL identified for the target trait (Byrne and McMullen, 1996; Rothschild and Soller, 1997).

Cowpea is a particularly valuable component of low-input farming systems of resource-poor farmers because of its productivity and yield stability in the face of abiotic stress (drought, heat, low soil fertility), and the ability of the crop to enhance soil fertility for succeeding cereal or tuber crops grown in rotation (Sanginga et al., 2003).With its greater tolerance to heat, drought, and low soil fertility (Hall, 2004) and yet close evolutionary relatedness to other economically important grain legumes such as common bean (*Phaseolus vulgaris*) and soybean (*Glycine max*), cowpea can serve as a model species for crop adaptation to these stresses. The ability of cowpea to tolerate severe drought conditions and its relatively small nuclear genome size (estimated at ~620 Mbp) (Arumuganathan and Earle, 1991) makes it model plant to study the molecular mechanisms of drought tolerance in crops. Several approaches can be utilized to identify genes that underlie drought tolerance in cowpea, one of the approaches would be to identify candidate genes that are known to be relevant to drought tolerance from previous studies in cowpea and other related crops and test its functionality in cowpea (Agbicodo et al., 2009). Another and often-used approach is to identify differential expression of mRNAs in drought stressed vs. control plants. Contrary to the candidate genes approach, differential expression of mRNA has been used in cowpea to identify genes that are involved in the drought response (Agbicodo et al., 2009). About 19 genes in 19 different accessions have been identified as being involved in drought tolerance in Cowpea (Iuchi et al., 1996a; Iuchi et al.,1996b; El-Maarouf et al.,1999; Iuchi et al., 2000; Marcel et al., 2000; Matos et al., 2001; Diop et al., 2004; Contour-Ansel et al., 2006 ;D'Arcy-Lameta et al., 2006)..

2.9 Cowpea in Ethiopia

In Ethiopia, pulses are among the major important crops next to cereals, are a cheap source of protein, and play a modest role in export market. They also significantly contribute to enhancing soil fertility. However, their production and productivity is, by far, below their expected potential (Tesema and Eshetayehu, 2003). Although cowpea is the second most important food grain legume of tropical Africa, next to *Phaseolus vulgaris*, it is the least cultivated and scarcely distributed pulse crop through different geographical and growing regions of Ethiopia. However, it is significantly important in the Gamogofa zone and especially in 'woredas' (districts) of Konso, Derashe, and Hamerbako of SNNP (Gemechu et al., 2003). It is also fairly distributed in the northern part of Ethiopia bordering Eritrea, pockets of Shoa, Gojjam, Wellega, and Hararge. Recently cowpea cultivation and production has been increasing especially in Rift Valley areas Miesso; it is being intercropped with Sorghum. Despite the remarkable potential of the crop in drought-prone areas, less attention was given to it during the past collection missions, and as a result only 64 accessions were collected from different cowpea growing areas of Ethiopia (altitude 1360-1900m asl), these materials are conserved ex-situ in Ethiopia Gene Bank(Tesema and Eshetayehu, 2003). (Table 2).

Table 2 Variation in Agro-morphological trait (mean±SD) for cowpea across the former provinces

Former province	Days to flowering	Days to maturity	Pod length (cm)	Pod width (cm)	Beak length (cm)	Seed length (cm)	Seed width (cm)	Number of seeds per pod
Gamogofa	(10)68±9	(10)107±4	(6)14±.8	(6)6.9±0.8	(6)8.8±4.7	(6)7.3±1.1	(6)5.3±0.6	(6)12.3±2.8
Gojam	(2)74±3	(2)103	(2)11	(2)5	(2)4.5±0.7	(2)6	(2)4	(2)13.4±0.9
Hararge	(1)74	(1)120	(1)15	(1)8	(1)6	(1)7.5	(1)6.54	(1)11.2
Shoa	(3)79±4	(3)111±13	(3)10.7±1.2	(3)5.2±0.8	(3)5.7±1.2	(3)6.3±0.6	(3)4.2±0.3	(3)11.5±1
Wellega	(1)60	(1)109	(1)12	(1)6	(1)7	(1)6	(1)4	(1)13.4

Source: Tesema and Eshetayehu (2003); The number in brackets indicate the number of accessions the are characterized for each agronomic traits

Food legumes constitute an important component of crop production systems in Eastern Ethiopia (Storck et al., 1991; AU, 2000). The region comprises eastern and Western Hararge Zone of Oromia, Somalia and Harari Regional State and Dire Dawa Administrative Council. More than 21150 ha of land is under cultivation of six type of food legumes in the region covering 2.3% of land used for pulses cultivation in Ethiopia. A total of 23404 tons were produced from these legumes in 2001 cropping season (CACC, 2001). Common bean and cowpea are the major lowland pulses cultivated in the region. These crops, grown in altitude ranges of 1500-2000 m asl, are important components in various intercropping system in Hararghe Eastern Ethiopia. Cowpea is one of the most important pulse crops in low moister areas in eastern Ethiopia, and its grains are rich and cheap source of protein. Its leaves and stems are also useful as supplemental animal feed. The crop has a potential for moister stressed areas in Babile, Gursum, Jijiga of Eastern Ethiopia (AU, 1996). However, the scale of its cultivation and production is not well developed owing to poor performance of available local cultivars and unavailability of improved varieties for the region.

About 13 species of lowland pulses are grown in Ethiopia. However, the most commonly grown across the country are haricot bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), soybean (*Glycine max*), mung bean (*Vigna radiata*) and pigeon pea (*Cajanus cajan*) (Imru, 1980; Kyamanywa and Ampofo, 1988; Abate, 1990a, b; Ferede, 1994). Six cowpea varieties have been released for different agroecologies of Ethiopia (Table 7). These lowland pulses are grown for food, market, and soil conservation. The national average for most of lowland pulses is below 1 t/ha (Abate et al., 1985a, b). Several factors contribute to low yield among which entomological problems are the most visible ones (Imru, 1980).

Table 3: Important arthropod pets and total number of pests recorded on Cowpea

Crop	Important pest(s)	Number of pest recorded
Cowpea	Aphids, Bruchids, Cotton leaf worm, Flower trips, Pod bugs and Pod-borer	35
Haricot bean	Bruchids, African ballworm, Red spider mite, Pod bug and Aphids	36
Soybean	Green stink bug, Banded stink bug, pod bug, Bruchids and Aphids	54
Mungbean	Black pod-weevil, Green stink bug, Pod bug and Bruchids	13
Pigeonpea	Pigeon pea pod-borer, African bollworm, Pod bug and Cotton cushion scale	16

Source: Abate (1995)

Data on losses due to major insect-pests on the two major lowland pulse crops are shown in (Table 4). In general, losses in the two pulse crops ranged from 9 to 100%. However, BSM caused the maximum yield loss that ranged from 11-100% followed by African ball-worm (12-16%). In cowpea, cotton leaf worm caused the maximum damage (range of 27-39%) (Abate, 1995).

Legumes are consumed daily in Ethiopia in one way or another by large part of the population. They are eaten in the form of sauce to supplement the cereal based staple diet (Mulugeta et al., 2003).

Table 4: Data on losses due to major insect-pests on the two major lowland pulse crops

Crop	Pest	Loss (%)
Haricot bean	Bean stem maggots	11-100
	African bollworm	12-16
Cowpea	Cotton leaf worm	27-39
	General	9-12

Source: Abate (1995)

Lowland pulses are reasonably adequate to the amino acid requirements. It is exceptionally rich in lysine and can compensate for the deficiency of lysine in animal protein (Mulugeta et al., 2003). Utilization of more lowland pulses for human consumption should be a nutritional advantage as it helps to prevent anaemia and protein energy malnutrition (PEM) diseases (Graspeck et al., 1982). In order to determine proximate analysis and to select the best varieties by comparing their nutrient composition 3 Cowpea varieties were studied by the MIARC in collaboration with the Quality and Standard Authority of Ethiopia (QSAE) (MIARC 2001). Generally, cowpea varieties had better crud protein than haricot bean varieties (Table 5 and Table 6).

Table 5: Mean value of nutritional composition of different varieties of cowpea

No	Variety	Crud protein (% by mass on wet basis)	Crud fat (% by mass on dry basis)	Crud fibre (% by mass on dry basis)	Carbohydrate (% by mass)	Energy value (Kcal/100g)
1	Black eye bean	23.08	1.67	2.4	59.22	329.43
2	TUV-1977	21.65	3.46	3.21	57.84	334.64
3	WWT	23.18	1.50	3.90	58.29	324.81
	LSD (P=0.05)	0.772	0.658	0.75	0.29	0.88
	CV(%)	5.23	42.08	24.84	0.13	2.15

Source: MIARC (2001)

Table 6: Mean values of minerals contents in different varieties of cowpea

No	Variety	Phosphorous (mg/100g)	Calcium (mg/100g)	Iron (mg/100g)	Zinc (mg/100g)
1	Black eye bean	16.18	65.72	5.28	1.68
2	TUV-1977	7.81	66.45	6.38	2.05
3	WWT	14.26	64.21	5.26	1.77
	LSD (P=0.05)	0.32	0.92	0.73	0.62
	CV(%)	2.92	1.04	16.57	46.15

Source: MIARC (2001)

To date, more than six varieties of cowpea have been released by different federal and regional research centers namely: Melkassa Agricultural Research Centre (MARC), Awassa Agricultural Research Centre (AWARC), and South Region Agricultural Research Centre (SRARC). The varieties were released for different agroecologies of Ethiopia (Table 7).

Table 7: List of Cowpea released varieties

No	Variety	Year of release	Yield (qt/ha) (under research and farmer field)	Adaptation area	Altitude (masl)	Rain fall (mm)	Seed color	Disease reaction	Breeder/maintainer
1	Asrat (IT 92KD-273-3	2001	16.6 and 20-22	Eastern Wollo area	1450-1850	660-1025	cream	-	SRARC*
2	Bekur (838 689 4)	2001	19.6 and 19 -21	Eastern Wollo area	1450-1850	660-1025	Red brown	-	SRARC*
3	82D-889	2008	17-20 and 18-26	Central rift valley, Miesso, Goffa, Kobo, Babile, Pawe and similar agro ecologies	1300-1650	350-750	pink	-	MARC*
4	Bole (85D-3517-2)	2006	17 and 19	Central rift valley, Goffa, Kobo, Pawe and similar agro ecologies	1300-1850	350-1100	white	Tolerant to virus and bacterial disease	MARC*
5	IT-98K-131-2	2006	14 and 17.9	Awassa, Amaro, Gofa and similar areas in southern regions/ southern rift valley	1100-1750	> 500 during the growing season	Creamy	Moderate resistance to aschochyta blight and septorial leaf spot; susceptible to aphid	AWARC*
6	Keti(IT99K-1122)	2012	-	-	-	-	-	-	MARC*

* MARC: Melkassa Agricultural Research Centre, AWARC: Awassa Agricultural Research Centre, SRARC: South Region Agricultural Research Centre

2.10. Germplasm Collections and Molecular Approaches in Genetic Diversity Studies of Cowpea

2.10.1. Germplasm Collections

Cowpea germplasm is maintained in collections around the world with varying levels of accessibility, and documentation. The largest collections are held by the IITA with more than 14,000 accessions. The collection can be accessed via an electronic database maintained through the CGIAR-SINGER system (http://singer.cgiar.org). The United States Department of Agriculture (USDA) maintains a collection with ca. 8,000 accessions. Access to this collection is through the USDA Germplasm Resources Information Network or GRIN system (www.ars-grin-gov). The University of California-Riverside has a collection with ca. 5000 accessions accessible on a Microsoft Access database. There is also a large collection of Mediterranean and African landraces (ca. 600 accessions) held at the Istituto di Genetica Vegetale at Bari, Italy (www.ba.cnr.it). Other centers maintaining seed of wild and cultivated cowpeas include the following: Agricultural University-Wageningen (Wageningen, The Netherlands), Botanical Research Institute (Pretoria, South Africa), Le Jardin Botanique National de Belgique (Meise, Belgium), International Plant Genetic Resources Institute (IPGRI) in Harare (Zimbabwe), Institut Français de la Recherché Scientifique pour le Développementen Coopération (ORSTOM; now IRD) in Montpellier (France), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) in Goiana (Brazil), Zentralinstitut für Genetik und Kulturpflanzenforschung (GAT) in Gatersleben (Germany), and the National Bureau of Plant Genetic Resources in New Delhi (India). In addition to the centers and facilities mentioned above, many national cowpea-breeding programs in Africa (including programs in Botswana, Burkina Faso, Ghana, Kenya, Nigeria, and Senegal) also have substantial germplasm collections. Likewise, in Ethiopia Biodiversity Institute (EBI) of and Conservation (IBC) maintains more than 64 germplasm collection. The condition of some of these collections, which are important reserves of local diversity, could be improved with funding for germplasm maintenance and facility repair (Timko et al., 2007).

2.10.2 Molecular Approaches in Genetic Diversity Studies of Cowpea

Molecular genetics techniques using DNA polymorphism have been increasingly used to characterize and identify novel germplasm within the available collections for uses in the crop breeding process (O'Neill et al., 2003). Diversity studies in wild and cultivated cowpea germplasms employ a variety of approaches, such as analyzing morphological and observed between molecular and morphological features was observed. Chen et al. (2008) analysed 40 yard long beans collected from Jianghan University by RAPD makers. A total of 30 primers generated 140 polymorphic RAPD bands. The various numbers of bands amplified by RAPD among the varieties were noticed.

2.10.2.2 The Application of SSR

SSR is the most frequently used marker in the genetic diversity analysis of cowpea (Huaqiang et al., 2012). The earliest cowpea SSR research was conducted by Li et al. (2007) and 27 SSR primers were developed. After that, SSR research on cowpea from different areas, mainly Africa and Asia, has been carried out. Africa is the diversity center of wild cowpea, which was proved by Ogunkanmi et al. (2008) with SSR analysis. Asare et al. (2010) utilized SSR molecular markers to evaluate genetic diversity and phylogenetic relationships among 141 cowpea accessions collected throughout the nine geographical regions of Ghana. PIC (the polymorphism information content) varied from 0.07 to 0.66 with an average of 0.38. The Ghanaian cowpea accessions clustered into five main branches, each of which was loosely associated with the geographical regions from which samples were obtained. Badiane et al. (2012) assessed the genetic diversity and phylogenetic relationships among 22 local cowpea varieties and inbred lines collected throughout Senegal by SSR markers, and developed a set of 44 polymorphic primer combinations from cowpea genomic or expressed sequence tags, the PIC value ranging from 0.08 to 0.33. Sawadogo et al., (2010) evaluated the genetic diversity and phylogenetic relationships among cowpea genotypes used in breeding for resistance to *Striga gesnerioides* in Burkina Faso using simple SSR molecular markers. Very few primer combinations showed polymorphic bands capable of discriminating *Striga*-resistant from susceptible cultivars, which revealed a high efficiency of SSR markers. Although Asia is one of the major cowpea growing areas, genetic diversity researches on cowpea in Asia are still very little. Lee et al., (2009) estimated the genetic diversity of 492 Korean cowpea landrace accessions using six SSR markers. The mean of Weir's gene diversity was 0.665 from all the accessions. Cowpea gene diversity of six local provinces in Korea ranged from 0.370 in accessions of Gangwon to 0.680 in Jeonra provinces. Xu et al. (2007) extracted the DNA of a total of 316 cultivated cowpea resources from China, Africa and other Asian countries, which were amplified by SSR to study their genetic diversity.

2.10.2.3 The Application of AFLP

AFLP is recognized as one of the most efficient molecular markers (Huaqiang et al., 2012). Coulibaly et al., (2003) employed AFLP to evaluate genetic relationships within a total of 117 cowpea accessions to assess the organization of their genetic diversity. The study showed that, wild annual cowpea (var. *spontanea*) was more diverse than domesticated cowpea. Wild cowpea in eastern Africa was more diverse than in western Africa, suggesting an eastern African origin for the wild taxon. Fang et al., (2007) examined genetic relationships among 60 advanced breeding lines from six breeding programs in West Africa and USA, and 27 landrace accessions from Africa, Asia, and South America. AFLP markers with six near infrared fluorescence labeled *EcoR*I + 3/1bases/*Mse*I + 3/1bases primer sets were used in the study. Principal coordinates analysis showed clustering of breeding lines by program origin, indicating lack of genetic diversity compared to potential diversity.

Huaqiang et al. (2012) indicated that many researchers have come to the same conclusion; i.e the genetic diversity of cultivated cowpea is very low (Vaillancourt, 1993; Pasquet, 2000; Li et al., 2001; Ba et al., 2002; Coulibaly et al.,.2002; Xu et al., 2007). The narrow genetic base is one of the major limiting factors for today's cowpea breeding, and the consequences are decline in vitality and range of variation. Cowpea improvement should partly rely on the diversity of large wild sub-groups, Pasquet et al. (2002). In order to improve the potential for high yield, adaptability, disease and insect resistance, a large number of excellent wild germplasm should be collected and applied in cowpea breeding program (Huaqiang et al., 2012).

2.10.2.4 The Applications of SNP

The advent of new sequencing technologies has dramatically changed the landscape for detecting and monitoring genome-wide polymorphism (Metzker, 2005; Craig et al., 2008; Schuster, 2008). Today, single nucleotide polymorphisms (SNPs) are rapidly replacing simple sequence repeats (SSRs) as the DNA marker of choice for applications in plant breeding and genetics because they are more abundant, stable, amenable to automation, efficient, and increasingly cost-effective (Rafalski, 2002; Duran et al., 2009; Edwards and Batley, 2010). However, this activity has largely bypassed ''orphan crops'' such as cowpea which are crops of relevance to food security and income for subsistence farmers in developing countries (Delmer, 2005). Very large numbers of SNP markers are now available for detailed analysis of genome structure, genome-wide association studies, and precision breeding, especially for those animals and plants for which high-density genotyping arrays are commercially produced (Ramos et al., 2009; Ganal et al., 2011). Nowadays, SNP markers for cowpea are available with genotyping service providers to quickly and affordably assay lines for diversity analysis.

2.10.2.5 The Application of Combinative Markers

The advantages of combinative markers are that they could be analyzed both separately and in combination, which makes the result more reasonable (Huaqiang et al., 2012). Diouf et al. (2005) used a combination of RAPD and SSR techniques to study the genetic diversity in local cowpea varieties and breeding lines from Senegal. The study found that microsatellite markers are found to be more effective than RAPD in determining the relationship among cowpea accessions and varieties. Tosti et al. (2005) studied three neighboring cowpea landraces currently cultivated in central Italy by AFLP and SAMPL markers to determine the distribution of genetic variation within and among them. The three landraces studied, although relatively similar, were highly different from one another as shown by the data obtained from the AFLP and SAMPL markers. Gillaspie et al. (2005) utilized AFLP and SSR markers to assess genetic diversity and relatedness between subspecies of *Vigna unguiculata*. Three AFLP primer combinations and 10 SSR primer sets successfully identified closely related accessions, and the presence of heterogeneity in some accessions. Results of cluster analysis between molecular markers and morphological traits are usually lacking of consistency (Nkongolo, 2003). Reasons for this could be: the limited number of traits observed, the limited variation for the traits, the number of underlying genes for the traits, which may also be limited, and possible epistatic interactions among the genes (Schut and Stam, 1997). In cowpea genetic breeding and evaluation of germplasm resources, a combination of molecular markers and classical markers is essential. Tantasawat et al., (2010) estimated genetic diversity and relatedness of 23 yard long bean (*Vigna unguiculata* spp. sesquipedalis) accessions and 7 accessions of a hybrid between cowpea (*V. unguiculata* spp. unguiculata) and dwarf yard long bean in Thailand by morphological characters, SSR and ISSR markers. Five morphological characters were diverse among most accessions. However, five groups of 2-3 accessions could not be distinguished from one another based on these morphological characters alone. The comparison of average marker index of the multilocus marker and mantel test indicated higher efficiency of ISSR for estimating the levels of genetic diversity and relationships among yard long beans and dwarf yard long beans in the study. Ghalmi et al. (2010) compared 20 landraces of cowpea grown throughout

Algeria through morphological and genetic characterization. Despite the absence of significant correlation between morphological and RAPD data, significant correlations between morphological data and both ISSR and a combined RAPD-ISSR dataset were noted. A conclusion had been made that ISSR markers were better linked to morphological variation than RAPD markers.

2.11 Current genetic linkage map of cowpea

The current map of cowpea consists of 11 LGs spanning a total of 2670 cM, with an average distance of ca. 6 cM between markers (Fig. 4). It includes 242 AFLP and 18 disease or pest-resistance related markers (Ouédraogo et al., 2002a), plus 133 RAPD, 39 RFLP, and 25 AFLP markers from the original map of Menéndez et al. (1997) for a total of 441 markers, of which 432 were assigned to a LG. Among these marker loci, genes for a number of biochemical and phenotypic traits have been located on this map. These include C, a general colour factor, and P, for purple pod color, on LG4 [according to the numbering system of Ouédraogo et al. (2002a), LGs on the bean and cowpea maps have been numbered independently; thus, LGs with the same number on the two maps probably refer to nonsyntenic groups], a 35-kDa dehydrin protein, implicated in chilling tolerance during emergence (LG2; Ismail et al., 1999), and markers for resistance to *Striga gesnerioides* races 1 and 3 (LG1 and LG6), cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV) (two distinct loci on LG2), B1CMV (LG8), Southern Bean Mosaic Virus (SBMV) (LG6), Fusarium wilt (LG3), and root-knot nematodes (gene Rk; NemR on LG1) (Ouédraogo et al., 2002a).

Above each LG is the length in centimorgans (cM) and number of markers comprising the LG. Distances (in cM) between adjacent markers are indicated to the left. Markers associated with LGs determined by Menéndez et al. (1997) are color coded in order to show their distribution on the current map. Markers linked to *Striga* resistance are given in red and marked by an asterisk. Loci for biological resistance/tolerance loci and resistance gene analogs (RGAs) are boxed in red. Markers that could not be placed with a LOD 3 score are listed under the LG they have the greatest affinity to. Unlinked markers are AAC-CTA-3, Parthcarp, AAC-CTT-10, ACA-CTA-7, ACG-CAA-10, AGG-CAT-1, R25, AAGCTT-9 (Fig 4).

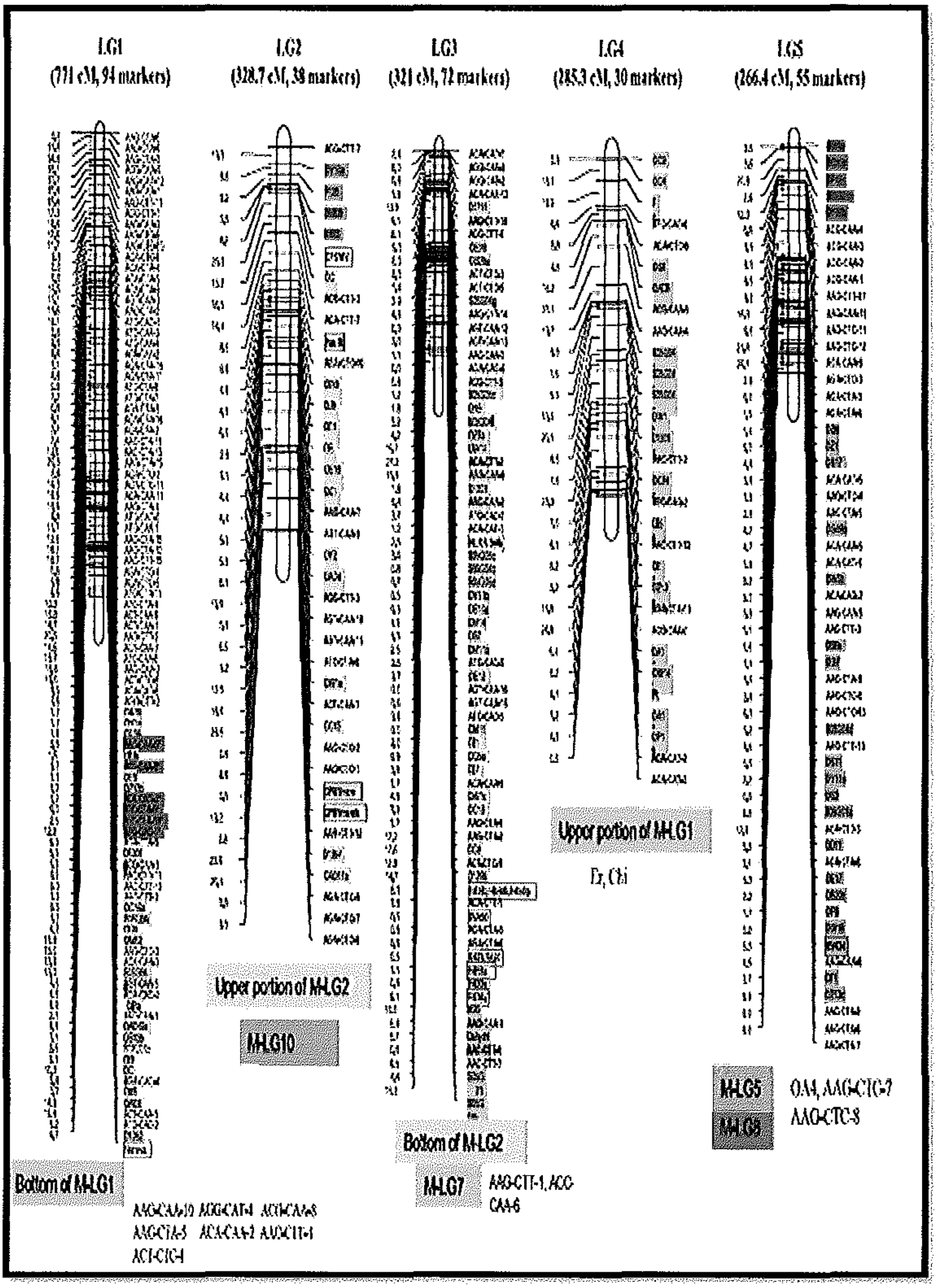


Figure 4: Current genetic linkage map of cowpea. Shown are the 11 LGs comprising the genetic linkage map of cowpea as published by Ouédraogo et al.(2002 a).

At least five distinct races of the parasitic weed *Striga gesnerioides* have been identified within the cowpea-growing regions of West Africa (Lane et al., 1996; 1997) based on the differential response of various cowpea genotypes (cultivars and breeding lines) carrying specific resistance genes (Table 10). Similarly, "resistance-breaking" strains of the root knot nematode *Meloidogyne* incognita, cowpea aphid (*Aphis craccivora*), cowpea weevil (*Callosobruchis maculatus*), and Fusarium wilt (*Fusarium oxysporum* f. sp. tracheiphilum) have been recognized in specific cowpea production areas. Markers for genes conferring resistance to the various strains of these pests would allow efficient development of varieties with resistance that is more broadly effective using MAS. Currently, useful markers in cowpea for implementation of MAS are only available for some of the *Striga* resistance genes, and these are the first candidates for broad application in cowpea breeding programs (Fig. 5 and Table 8). Ouédraogo et al. (2001, 2002 b) found three AFLP markers linked to Rsg2-1, a gene that confers resistance to *Striga* Race 1 (SG1) present in Burkina Faso, and six AFLP markers linked to gene Rsg4-3, a gene that provides resistance to *Striga* Race 3 (SG3) from Nigeria. Two of the AFLP markers were associated with both Rsg2-1 and Rsg4-3. (Ouédraogo et al., 2002 a) were able to convert one of these markers to a SCAR (sequence-characterized amplified region) that has proven to be an effective and remarkably reliable marker for resistance to Striga SG1 and SG3 conferred by Rsg2-1 and Rsg4-3. This SCAR marker, designated 61R (E-ACT/M-CAA), detects a single polymorphic band linked to SG1 and SG3 resistance in the resistant cultivars B301, IT82D-849, and Tvu 14676 and is being tested for use in breeding trials. Recently, two AFLP markers were identified that are closely linked to Rsg1-1, a gene that also confers resistance to SG3 in Nigeria (Boukar et al., 2004). One of the AFLP markers, designated EACT/ M-CAC115 and determined to be 4.8 cM from Rsg1-1, was converted to a SCAR marker for ease of use in breeding programs (Boukar et al., 2004).

Table 8: Agronomic, growth habit, and disease and pest resistance trait loci currently placed on the cowpea genetic map of Ouédraogo et al.,. (2002) and other traits mapped to probable nonanalogous linkage groups [1]

Trait	Locus designation	Linkage group/reference map
Pod pigmentation	P	LG1; (LG1-Menéndez et al. 1997)
Resistance to *Striga gesnerioides*-Race 1	*Rsg2-1*	LG1
Resistance to *Striga gesnerioides*-Race 3	*Rsg1-3, Rsg1-1*	LG1
Root-knot nematode (*Meloidogyne incognita*) resistance	*Rk*	LG1
Nodes to 1st Flower (D1301a)	NTF	LG2; (LG2-Menéndez et al. 1997)
Dehydrin protein	Dhy	LG2; (LG7-Menéndez et al. 1997)
Resistance to cowpea mosaic virus	CPMV	LG2
Resistance gene analog (pathogen unknown)	RGA-138	LG2
Resistance gene analog (pathogen unknown)	RGA-168	LG2
Resistance gene analog (pathogen unknown)	RGA-190	LG2
Resistance to *Fusarium oxysporum*	*Fus*R	LG3
Cowpea severe mosaic virus resistance	CPSMV (*ims*)	LG3
Cowpea mosaic virus resistance	CPMV	LG3
Resistance gene analog (pathogen unknown)	RLRR3-4B	LG3
General flower color factor	C	LG4; (LG1-Menéndez et al. 1997)
Seed weight (OB6a)	SW	LG5; (LG5-Menéndez et al. 1997)
Resistance gene analog (pathogen unknown)	RGA-131	LG5
Resistance to southern bean mosaic virus	SBMV (*sbc-1,2*)	LG6
Resistance to *Striga gesnerioides*-Race 1	*Rsg3-1, Rsg-994*	LG6
Resistance to blackeye cowpea mosaic virus	BICMV	LG8
Resistance gene analogs (pathogen unknown)	RLRR3-4T	LG9
Traits mapped in other populations with probably nonanalogous linkage groups to map of Ouédraogo et al. 2002		
Resistance to cowpea aphid (*Aphid craccivora*)	*Rac1*	(LG1-Myers et al. 1996)
50% Flowering	50%FL	(LG7-Fatokun et al. 1993)
Seed weight	SW	(LG7-Fatokun et al. 1993)
Plant height	HT	(LG8-Fatokun et al. 1993)
Pod number per plant	PodN	(LG9-Fatokun et al. 1993)

[1] Adapted from genetic maps and data of Ouédraogo et al. (2002) and Menéndez et al. (1997) that used the same genetic population. There is insufficient marker data to integrate LGs of the maps of Fatokun et al. (1993) and data from Myers et al. (1996) with the map of Ouédraogo et al. (2002)

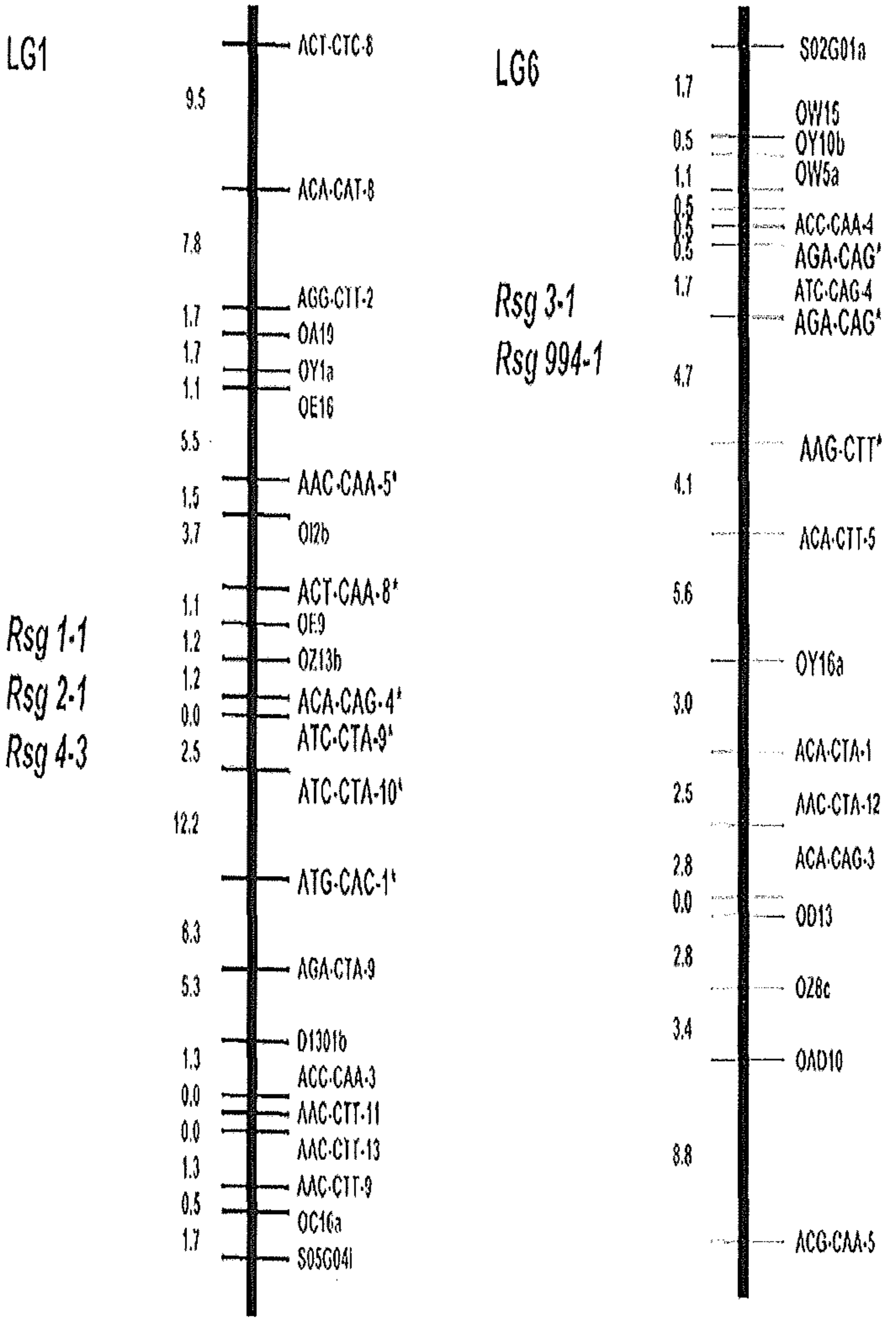


Figure 5: Partial maps showing linkage of molecular markers to S. gesnerioides race-specific resistance genes in cowpea.

In the above (Fig. 5) are shown are the linkage of AFLP, RAPD, and other markers to *S. gesnerioides* race 1 (Rsg2-1 and Rsg1-1) and race 3 (Rsg4-3) resistance genes on the partial map of LG1 of the cowpea genetic map (left) and the linkage of AFLP, RAPD, and other markers to *S. gesnerioides* race 1 (Rsg3-1) and race 3 (Rsg994-1) resistance genes on partial map of LG6 of the cowpea genetic map (right). AFLP markers linked to *Striga* resistance as reported in Ouédraogo et al. (2001) and Ouédraogo et al. (2002 b) are indicated by an asterisk. Map distances are shown in cM.

3. Materials and Methods

Two sets of materials for two sets of experiments were used in the present study. The first set of cowpea materials was used to study the genetic relationship of Ethiopian cowpea accessions, using SSR markers. The second set of materials was used to analyse the genetic relationship of East African cowpea accessions, and IITA inbred lines based on SSR and SNP markers. The details of the two sets of material and methods used are presented as follows:

3.1 Genetic diversity of Ethiopian cowpea accessions (*Vigna unguiculata* (L.) Walp.) based on SSR markers

3.1.1 Plant material

Eighty-three cowpea accessions, which were representatives of improved lines, local landraces, and a few semi-domesticated wild forms, were acquired from Ethiopian Biodiversity Institute (EIB) and as well additional collections were made from different regions of Ethiopia. Twenty one accessions were found to be uniform and the remaining 62 accessions showed considerable within accession variation with respect to seed color and size. Thus, each accession that showed within-accession variability was further sub-divided into 2 to 8 subtypes making 189 sub-type accessions. In total, 210 accessions (189 + 21) accession were used for diversity analysis. The collection location of accessions 211441 was unknown according to the information obtained from Ethiopian Biodiversity Institute (EIB). We have also used all of the cowpea accessions available at IBC, Ethiopia including two (210856 and 210857) accessions collected from northern part of Ethiopia bordering Eritrea (Table 9, Table 10 and Fig 6); beside this the Botswana local accession B301 was used for comparison purpose of *Striga gesnerioides* resistance with the Ethiopian accession.

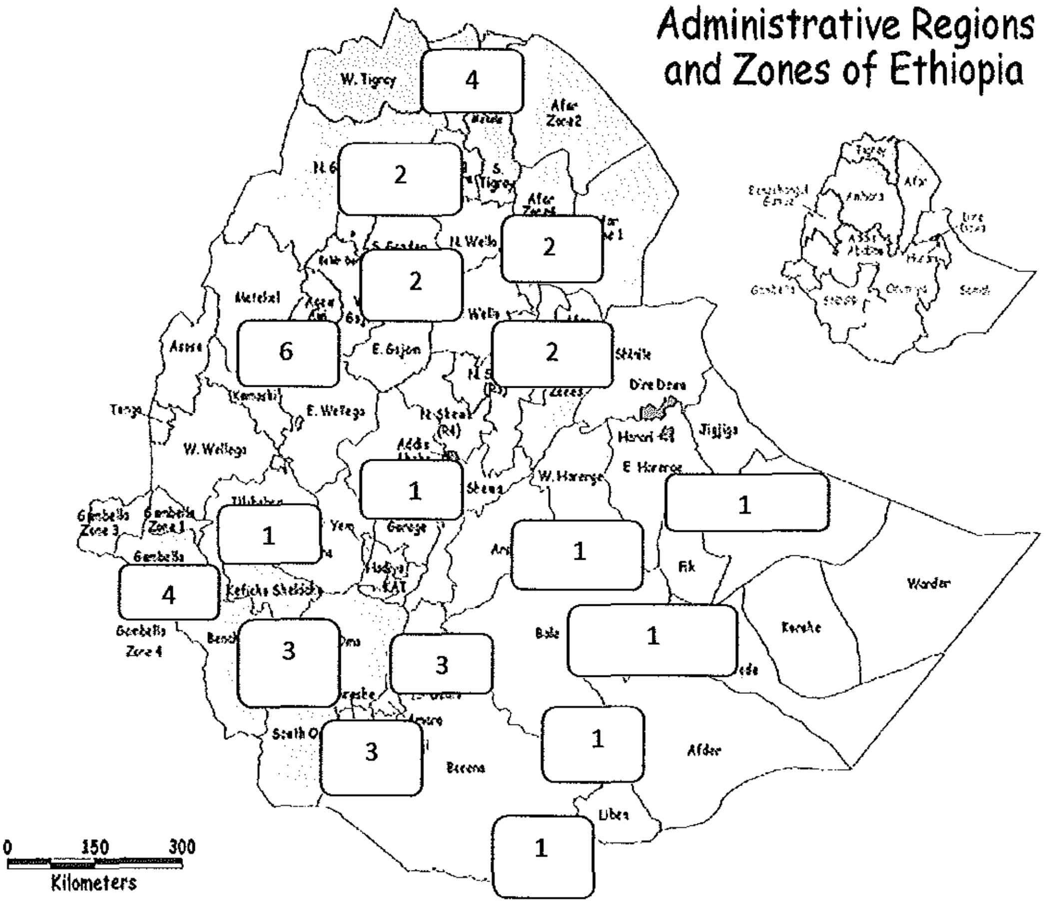


Figure 6: Map of Ethiopia showing the collection sites of cowpea accessions used in the present study (key: 1= Oromia, 2=Amhara, 3=SNNP,4=Tigray, 5=Gambella 6= Benishangul and Gumeze)

Table 9: List of Ethiopian cowpea accessions used in diversity study

No.	Accession Number	No of Sub Groups	Genus name	Species name	Former administrative region	Region/State/Province	Latitude	Longitude	Altitude
1	19576	2	Vigna	Spp	Shewa	Amhara	09-55-42-N	39-59-06-E	1353
2	19577	2	Vigna	Spp	Shewa	Amhara	09-57-50-N	40-01-47-E	1260
3	208776	2	Vigna	Unguiculata	Welega	Oromia			1900
4	210856	6	Vigna	Unguiculata	Eritrea				
5	210857	3	Vigna	Unguiculata	Eritrea				
6	211287	2	Vigna	Unguiculata	Gamo Gofa	SNNP			
7	211382	2	Vigna	Unguiculata	Shewa	Amhara	09-35-00-N	39-44-00-E	
8	211383	3	Vigna	Unguiculata	Shewa	Amhara	09-35-00-N	39-44-00-E	
9	211384	6	Vigna	Unguiculata	Shewa	Amhara	09-35-00-N	39-44-00-E	
10	211429	4	Vigna	Unguiculata	Gamo Gofa	SNNP			
11	211430	2	Vigna	Unguiculata	Gamo Gofa				
12	211433	4	Vigna	Unguiculata	Gojam	Benishangul & Gumuz			1300
13	211435	2	Vigna	Unguiculata	Gojam	Amhara			
14	211436	3	Vigna	Unguiculata	Gojam	Amhara			
15	211440	4	Vigna	Unguiculata	Gamo Gofa		05-18-00-N	37-18-00-E	1810
16	211441	5	Vigna	Unguiculata	**Not Known**				
17	211443	5	Vigna	Unguiculata	Harerge	Oromia			
18	211444	4	Vigna	Unguiculata	Sidamo	Oromia			
19	211446	4	Vigna	Unguiculata	Gamo Gofa				
20	211447	5	Vigna	unguiculata	Gojam	Benishangul & Gumuz			1300
21	211490	4	Vigna	unguiculata	Gamo Gofa	SNNP	05-20-00-N	37-25-00-E	1700
22	211491	3	Vigna	unguiculata	Gamo Gofa	SNNP			1560
23	211557	7	Vigna	unguiculata	Gamo Gofa				
24	215759	4	Vigna	unguiculata	Welo	Amhara	11-5 -00-N	39-45-00-E	
25	215760	4	Vigna	unguiculata	Welo	Amhara	11-5 -00-N	39-45-00-E	
26	215761	3	Vigna	unguiculata	Welo	Amhara	11-5 -00-N	39-47-00-E	
27	215762	4	Vigna	unguiculata	Welo	Amhara	11-11-00-N	39-58-00-E	
28	215821	4	Vigna	unguiculata	Illubabor	Gambella	08-11-00-N	34-14-00-E	530
29	216746	1	Vigna	unguiculata	Illubabor	Gambella	08-11-00-N	34-13-00-E	560
30	216747	2	Vigna	unguiculata	Illubabor	Gambella	08-09-00-N	34-12-00-E	570
31	216748	2	Vigna	unguiculata	Illubabor	Gambella	07-51-00-N	34-33-00-E	550

Table 9 (cont'd)

32	216749	3	Vigna	unguiculata	Illubabor	Gambella	07-51-00-N	34-33-00-E	550
33	221727	4	Vigna	unguiculata	Tigray	Tigray			
34	222867	1	Vigna	unguiculata	Illubabor	Gambella	07-31-00-N	34-22-00-E	630
35	222890	3	Vigna	unguiculata	Illubabor	Gambella	07-31-00-N	34-22-00-E	630
36	222891	1	Vigna	unguiculata	Illubabor	Gambella	07-31-00-N	34-22-00-E	630
37	223402	2	Vigna	unguiculata	Harerge	Oromia	08-41-00-N	40-14-00-E	1880
38	223403	4	Vigna	unguiculata	Harerge	Oromia			
39	227104	2	Vigna	unguiculata	Illubabor	SNNP			1300
40	228624	1	Vigna	unguiculata	Gojam	Amhara			
41	230575	1	Vigna	unguiculata	Welega				
42	235122	2	Vigna	unguiculata	Tigray	Tigray			
43	239096	1	Vigna	unguiculata	Gojam	Amhara			
44	241098	1	Vigna	unguiculata	Shewa	Oromia	08-29-00-N	39-18-00-E	1540
45	241099	1	Vigna	unguiculata	Shewa	Oromia	08-01-00-N	39-03-00-E	1600
46	241761	3	Vigna	unguiculata	Gamo Gofa	SNNP	05-19-69-N	37-24-99-E	1350
47	244804	3	Vigna	unguiculata		SNNP			1773
48	251	2	Vigna	unguiculata	Illubabor	Gambella-1			550
49	252	2	Vigna	unguiculata	Illubabor	Gambella-2			450
50	253	2	Vigna	unguiculata	Illubabor	Gambella-3			450
51	254	2	Vigna	unguiculata	Illubabor	Gambella-4			450
52	255	1	Vigna	unguiculata	Illubabor	Gambella-5			450
53	256	2	Vigna	unguiculata	Illubabor	Gambella-6			450
54	257	3	Vigna	unguiculata	Illubabor	Gambella-7			450
55	258	2	Vigna	unguiculata	Illubabor	Gambella-8			550
56	259	1	Vigna	unguiculata	Illubabor	Gambella-9			450
57	260	1	Vigna	unguiculata	Illubabor	Gambella-10			450
58	261	2	Vigna	unguiculata	Illubabor	Gambella-11			450
59	262	1	Vigna	unguiculata	Illubabor	Gambella-12			450
60	263	1	Vigna	unguiculata	Illubabor	Gambella-13			450
61	264	1	Vigna	unguiculata	Illubabor	Gambella-14			450
62	265	1	Vigna	unguiculata	Illubabor	Gambella-15			450
63	266	2	Vigna	unguiculata	Illubabor	Gambella-16			450
64	267	2	Vigna	unguiculata	Illubabor	Gambella-17			550
65	268	3	Vigna	unguiculata	Illubabor	Gambella-18			550
66	269	1	Vigna	unguiculata	Illubabor	Gambella-19			550
67	270	8	Vigna	unguiculata	Harerge	Oromia (harar-1)			

Table 10: List of accessions by sub-groups and seed types

No	Accession number	List of sub-groups	Seed type	No	Accession number	List of sub-groups	Seed type
1	19576	19576-1	green-big	**107**	216748	216748-1	white
2		19576-2	green-small	**108**		216748-2	dark white
3	19577	19577-1	light green	**109**	216749	216749-1	white
4		19577-2	dark green	**110**		216749-21	dark white
5	208776	208776-1	Black	**111**		216749-22	dark white
6		208776-2	White	**112**	221727	221727-11	white
7	210856	210856-11	dark Gold	**113**		221727-12	white
8		210856-12	dark Gold	**114**		221727-2	gold
9		210856-2	light gold	**115**		221727-3	dark ranger
10		210856-3	Black	**116**	222867	222867	green
11		210856-4	White	**117**	222890	222890-1	white-big
12		210856-5	Ranger	**118**		**222890-2**	**black**
13	210857	210857-11	Dark white-big	**119**		222890-3	white-small
14		210857-12	Dark white-big	**120**	222891	222891	white
15		210857-2	Dark white-small	**121**	223402	223402-1	dark white
16	211287	211287-1	White-big	**122**		223402-2	ranger
17		211287-2	White-small	**123**	223403	223403-11	dark ranger
18	211382	211382-1	dark Gold	**124**		223403-12	dark ranger
19		211382-2	dark white	**125**		223403-2	light ranger
20	211383	211383-1	dark Gold	**126**		223403-3	dark white
21		211383-2	dark white	**127**	227104	227104-1	white
22		211383-3	White	**128**		227104-2	dark white
23	211384	211384-1	black-big	**129**	228624	228624	white
24		211384-2	black-small	**130**	230575	230575	black
25		211384-3	white-big	**131**	235122	235122-1	white
26		211384-4	white-small	**132**		235122-2	dark white
27		211384-5	ranger-big	**133**	239096	239096	white
28		211384-6	ranger-small	**134**	241098	241098	light Gold

Table 10 (cont'd)

29	211429	211429-1	Black	**135**	241099	241099	silver
30		211429-2	dark white	**136**	241761	241761-11	dark white
31		211429-3	light ranger	**137**		241761-12	dark white
32		211429-4	white ranger	**138**		241761-2	light gold
33	211430	211430-1	dark black-big	**139**	244804	244804-1	white
34		211430-2	dark black-small	**140**		244804-2	dark white
35	211433	211433-1	light white	**141**		244804-3	light ranger
36		211433-2	dark white	**142**	251	251-1	black-big
37		211433-3	cherry red	**143**		251-2	black-small
38		211433-4	dark gold	**144**	252	252-1	light cherry
39	211435	211435-1	White	**145**		252-2	pink
40		211435-2	Silver	**146**	253	253-1	gold
41	211436	211436-1	White	**147**		253-2	light red cherry
42		211436-21	Silver	**148**	254	254-1	brownish
43		211436-22	Silver	**149**		254-2	burgundy
44	211440	211440-1	dark white-big	**150**	255	255	red cherry
45		211440-2	dark white-small	**151**	256	256-1	gold
46		211440-3	light gold-big	**152**		256-2	light red cherry
47		211440-4	light gold-small	**153**	257	257-1	black (wild)
48	211441	211441-1	cherry red	**154**		257-2	dark ranger (wild)
49		211441-21	dark white	**155**		257-3	light ranger (wild)
50		211441-22	dark white	**156**	258	258-1	dark ranger
51		211441-31	Gold	**157**		258-2	light ranger
52		211441-32	Gold	**158**	259	259	white
53	211443	211443-1	dark white	**159**	260	260	light pink
54		211443-2	light white	**160**	261	2611	dark white
55		211443-3	Black	**161**		2612	dark white
56		211443-41	Silver	**162**	262	262	dark scarlet
57		211443-41	Silver	**163**	263	263	black

Table 10 (cont'd)

88		215759-4	light ranger	**194**		276-4	dark white
89	215760	215760-1	Black	**195**	277	277-1	light pink
90		215760-2	White	**196**		277-2	dark pink
91		215760-3	Ranger	**197**		277-3	dark white
92		215760-4	dark white	**198**	278	278-1	White
93	215761	215761-1	light ranger	**199**		278-2	light white
94		215761-2	Gold	**200**	279	279-1	White
95		215761-3	light white	**201**		279-2	Gold
96	215762	215762-1	light ranger	**202**	280	280	White
97		215762-2	White	**203**	281	281	white- dark eyed
98		215762-3	red cherry	**204**	282	282	Gold
99		215762-4	Gold	**205**	283	283	Gold
100	215821	215821-1	Black	**206**	284	284	white-black eyed
101		215821-2	White	**207**	285	285-1	Gold
102		215821-3	red cherry	**208**		285-2	light white
103		215821-4	dark white	**209**	286	286-1	light red cherry
104	216746	216746-2	red cherry	**210**		**286-2**	**dark red cherry**
105	216747	216747-1	White				
106		216747-2	dark white				

3.1.2 DNA isolation and quantification

Leaf samples for DNA isolation were collected from two 15 days old seedlings of each accession grown in screen house at Timko lab, University of Virginia, USA. Total genomic DNA was extracted following the CTAB protocol modified at Timko Lab. Approximately 200 mg leaf samples were harvested from each accession in 2 ml Eppendroff tube, polyvinyl-polypyroid powder (small amount) was added just before adding liquid nitrogen to protect the leaf from browning and then leaf sample were grounded into fine powder with liquid nitrogen using tissuelyser. Then after, CTAB (2% CTAB, 100 Mm Tris HCL, 20Mm EDTA and 1.4M NaCl) buffer was pre warmed at 60^{0}C in water a bath. Subsequently 1ml CTAB buffer + Proteinase K (10mg/ml) (30 µl) + 2% B-mercaptoethanol (1.6 µl) was added to each tube to break open cells and soluble cellular contents in the eppendorf tubes and placed in water bath at 60^{0}C for 1hr with occasional gently mixing. Afterward 200 µl 5M potassium acetate was added and left in ice for 20 min. A total of 700-ul chloroform isoamyl (24:1) was added and mixed gently and left undisturbed for 3-5 min. Then after, the samples were centrifuged for 15 min at 10000 rpm and the middle (interface) aqueous layer was transferred to properly labelled new 2ml tube using 1000ul tips. Chloroform isoamyl the same volume left in tube were added, mixed gently, and left undisturbed for 3-5 min. After centrifuging for 10 min at 10000 rpm, the supernatant removed to clearly labelled new 1.5 ml tubes. Then, 500-ul ice-cold isopropanol was added and stored in -20 ^{0}c for 30 min until white flocculent (precipitate) was obtained. The sample were centrifuged for 5 min at 1000 rpm and the supernatant was removed carefully not to lose the pellet. After getting the pellet, the samples were washed with 75% ice-cold ethanol twice. Subsequently, they were centrifuged for 5 min at 15000 rpm and the supernatant was removed, then after the pellet was left to dry at room temperature. Finally 500 ul water was added and left at 4 ^{0}C overnight; Afterward 2 ul RNas was added and left in an incubator at 37 ^{0}c for 1 hr to remove RNA, finally stored at 4 ^{0}C. The extracted genomic DNA was purified by adding potassium acetate 20 ul and left in ice for 20 min. Subsequently, chloroform isoamyl (24:1) the same volume as added and gently mixed up left for 3 min. Afterward, the sample was centrifuged for 15 min at 10000 rpm and the supernatant was transferred in to a new tube, then, the chloroform isoamyl (24:1) the same volume was added and left for 3min, subsequently, centrifuged for 15 min at 15000 rpm and then the supernatant was transferred to new tube. Then, 100% ethanol 20 µl was added and then centrifuged for 5 min at 12000 rpm and finally the supernatant was removed. Finally, 70% ethanol 20 µl was added and centrifuged for 5 min at 12000 rpm, the supernatant was removed and allowed to dry at room temperature. Then, TE buffer was added and left overnight at 4 ^{0}C. The quality of extracted genomic DNA samples was checked by running 2 µl samples on 1% agarose gel and the concentrations of genomic DNA samples were estimated using NanoDrop1000 spectrophotometer (Thermo Scientific) (Table 11) finally stored at 4 ^{0}c.

Table 11: Nano-drop readings of partial DNA sample

DNA Sample s	Nucleic Acid Concentration (ng/ul)	260/280
1	184	2.18
2	170	2.23
3	174	2.21
12(2)	284	1.98
13	206	2.16
14	184	2.22
15	160	2
16	377	1.98
17	112	2.07
138	160	2.19
139	193	2.23
140	259	2.2
140	258	2.19
141	116	1.81
142	126	2.01

3.1.3 SSR markers selection

A total of 203 microsatellite markers developed for cowpea and related *Vigna* species at Timko Laboratory were screened to find the most polymorphic markers (http://cowpeagenomics.med.virginia.edu/CGKB/ developed under the Cowpea Genomics Initiative maintained by Professor Michael P. Timko, Department of Biology at University of Virginia. Copyright (C) 2009 Dr. Michael P. Timko). Twenty-three primer pairs were selected based on their level of polymorphism, the quality, and repeatability of the amplified bands (Table 12). Three of the 23 primers (SSR1, 61RM2, and C42-2B) are associated with the resistant gene to the root parasitic weed *Striga gesnerioides* were used to check for existence of the resistance gene among Ethiopian accessions.

3.1.4 Polymerase chain reaction (PCR) amplifications

PCR amplification was carried out in 10 µl final volume mixture containing 1 µL 10X PCR buffer, 1 µL (10 µM) of each primer, 0.4 µL (10mM) of each dNTPs, 0.4 µL (5u/ul) U Taq DNA polymerase and 2µl of 50 ng DNA and 5.2 µl double distilled water on Eppendroff Mastercycler Gradient thermocycler. The PCR cycle was programmed for initial denaturation at 94°C for 4 min followed by 35 cycles of 30 s at 94°, 30 s at (54°C - 56°C), 30 sec at 72°C, and a final extension of 10 min at 72°C. PCR products were resolved on a polyacrylamide gel (6%), using 0.5 X TBE containing 1 mg/ mL ethidium bromide with a vertical, and horizontal electrophoresis apparatus (C.B.S. Scientific Co., Delmar, CA, USA, and model C-DASG-400-50) at 300 V. The fragments in the gel were visualized using AlphaImager 2200 (Alpha Innotech, Santa Clara, CA, USA) under UV transilluminator.

3.1.5 Scoring of data and statistical analysis

The size of the most intensely amplified fragments was determined by comparing their migration distance relative to the molecular weight of bands of known size markers, 100 kb plus base pairs (bp) DNA ladder using Alpha-Ease FC 5.0 software (Alpha Innotech, USA) ran in adjacent lanes in the same gel for fragment length estimation.

The individual SSR fragments were scored for size and polymorphism. Monomorphic bands or invariable markers were not included in the statistical analysis because they are not informative. The informative/polymorphic bands were scored on the basis of the DNA fragment length generating a matrix of raw data; which was subjected to different diversity analyses.

Table 13: List of cowpea accessions used for molecular evaluations of East African and IITA inbred lines

No.	Accession name	Country of origin
1	IT-99K-1122	Nigeria (IITA)
2	IT-97K-356-1	Nigeria (IITA)
3	IT-99K-1060	Nigeria (IITA)
4	IT-96K-719	Nigeria (IITA)
5	IT-93K-556-7	Nigeria (IITA)
6	IT-98K-1111-1	Nigeria (IITA)
7	IT-93K-452-1	Nigeria (IITA)
8	IT-95K-207-22	Nigeria (IITA)
9	IT-93K-428-3	Nigeria (IITA)
10	IT-95K-268-1-4	Nigeria (IITA)
11	IT-97K-569-9	Nigeria (IITA)
12	IT-97K-569-9	Nigeria (IITA)
13	IT-99K-1245	Nigeria (IITA)
14	IT-97K449-38	Nigeria (IITA)
15	82D-889(CH)	Ethiopia
16	BOLE (CH)	Ethiopia
17	208776	Ethiopia
18	211443	Ethiopia
19	211435	Ethiopia
20	211446	Ethiopia
21	211491	Ethiopia
22	211444	Ethiopia
23	211557	Ethiopia
24	211490	Ethiopia
25	211436	Ethiopia
26	211430	Ethiopia
27	241761	Ethiopia
28	211429	Ethiopia
29	211385	Ethiopia
30	211441	Ethiopia
31	211433	Ethiopia
32	230575	Ethiopia
33	230044	Ethiopia
34	221727	Ethiopia
35	223403	Ethiopia
36	223402	Ethiopia
37	TUV-1977	Ethiopia
38	WWT	Ethiopia
39	Black eye bean	Ethiopia
40	Assebot	Ethiopia
41	TVu-433	Kenya
42	TVu-114	Kenya
43	TVu-115	Kenya
44	TVu-139	Kenya
45	TVu-552	Kenya
46	TVu-1190	Kenya
47	TVu-2651	Kenya
48	**TVu-6378**	**Kenya**
49	TVu-8450	Kenya
50	TVu-8767	Kenya
51	TVu-11414	Kenya
52	TVu-11419	Kenya
53	TVu-11422	Kenya
54	TVu-11431	Kenya
55	TVu-13448	Kenya
56	TVu-13454	Kenya
57	TVu-13457	Kenya
58	TVu-13467	Kenya
59	TVu-13469	Kenya
60	TVu-13470	Kenya
61	TVu-13473	Kenya
62	TVu-13475	Kenya
63	TVu-13485	Kenya
64	TVu-13490	Kenya
65	TVu-13501	Kenya
66	TVu-13511	Kenya
67	TVu-13516	Kenya
68	TVu-14160	Kenya
69	TVu-16410	Kenya
70	TVu-16031	Somalia
71	TVu-16038	Somalia
72	TVu-16041	Somalia
73	TVu-16043	Somalia
74	TVu-16044	Somalia
75	TVu-16050	Somalia
76	TVu-16053	Somalia
77	TVu-16054	Somalia
78	TVu-16061	Somalia
79	TVu-16073	Somalia
80	TVu-16078	Somalia
81	TVu-16083	Somalia
82	TVu-16086	Somalia
83	TVu-16174	Somalia
84	TVu-16176	Somalia
85	TVu-11955	Sudan
86	TVu-11957	Sudan
87	TVu-11978	Sudan
88	TVu-11979	Sudan
89	TVu-11982	Sudan
90	TVu-11983	Sudan
91	TVu-11984	Sudan
92	TVu-11986	Sudan
93	TVu-11987	Sudan
94	Tvu 7778	Nigeria (IITA)
95	Danilla	Nigeria (IITA)

Afterward 2 ul RNAs was added and left in the incubator at 37^{0}C for 1 hr to remove RNA, and finally stored at 4^{0}C. The quality of extracted genomic DNA samples were checked by running 2 µl samples on 1% agarose gel and the concentrations of genomic DNA samples were estimated using NanoDrop1000 spectrophotometer (Thermo Scientific) (Table 14); finally stored at 4^{0}C.

Table 14: Nano-drop readings of partial DNA sample

List of DNA Sample	Nucleic Acid Concentration (ng/ µl)	260/280
1	431.49	1.94
2	482.66	2.01
3	341.29	2.01
4	115.87	1.99
5	200.07	1.97
6	467.98	1.98
7	216.24	2.03
8	236.6	1.97
42	1501.98	2.16
43	1022.62	2.13
46	460.62	1.99
47	1502.87	2.18
51	2065.02	2.16
55	1090.31	2.18
57	3132.35	2.03

The number refers to the corresponding number of accession in Table 14

3.3.3 PCR amplification

PCR amplification was carried out in 10.5 µl final volume mixture containing 1 µL 10X PCR buffer, 1.25 µM of each primer, 0.8 mM of each dNTPs, 0.06 uL (0.3U) U Taq DNA polymerase and 2 µl of 20 ng DNA and 3.34 µl double distilled water on Eppendorf Mastercycler Gradient thermocycler. The PCR cycle was programmed for initial denaturation at 94°C for 1 min followed by 35 cycles of 30 s at 94°, 30 s at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C.

3.3.4 Fragment analysis using ABI sequencer

Fragment analyses of SSR primers that yielded polymorphism in the PCR analyses were carried out using 13 selected fluorescent primers out of 16 fluorescent primers. The primers were run in multiplexes, based on their fluorescence dye and allele size using BIONEER ACCUPOWER® Multiplex PCR Premix Kits (Table 15). PCR products were run on an ABI PRISM 3730xl fragment analyzer (Applied Biosystems, Foster City, CA, USA) at the IITA, and allele peaks were sized and alleles were called using the Genemapper v. 3.7.3.7 software.

Table 15: List of Fluorescent SSR primers

No	Fluorescent primer	Primer Sequence (5'-3')	Group	Product Size (bp)	Colour	Conc. (µl)
1	Vm39	5' GAT GGTTGTAATGGGAGAGTC-3' 5' AAAAGGATGAAATTAGGA GAG CA-3'	1	212	yellow	2
2	Vm40	5' TAT TAC GAG AGG CTA TTT ATT GCA-3' 5' CTC TAA CAC CTC AAG TTA GTG ATC-3'	1	200	Blue	3
3	Vm53	5'-GAG TTC CGT TCG TTG TGA GTA GAG-3' 5'-ACA GAG GAG GAA AAG GAA GTA TGC-3'	1	288	Red	4
4	Vm35	5'GGT CAA TAG AATAATGGAAAGTGT-3' 5' ATGGCTGAAATAGGTGTCTGA-3'	2	127	Green	2
5	Vm9	5' ACCGCA CCC GAT TTATTT CAT-3' 5' ATCAGCAGA CAG GCAAGACCA-3'	2	271	Red	1
6	Vm70	5'-AAA ATC GGG GAA GGA AAC C-3' (AG) 5'-GAA GGC AAA ATA CAT GGA GTC AC-3'	3	186	Green	1.6
7	Vm94	5'-TCG AAC TTT GGC TTG AGG-3' 5'-TGT CGT TTT GTC CCC CAT TA-3'	3	253	Blue	3
8	Bmd2	5'-AGCGACAGCAAGAGAACCTC-3' 5'-CAACAAACGGTGATTGACCA-3'	3	106	Blue	2.6
9	Bmd17	5'-GTTAGATCCCGCCCAATAGTC-3' 5'-AGATAGGAAGGGCGTGGTTT-3'	3	98	Yellow	3
10	Vm31	5'-CGC TCT TCG TTG ATG GTT ATG-3' 5'-GTG TTC TAG AGG GTG TGA TGG TA-3'	4	200	Blue	1.4
11	Vm37	5' TGTCCGCGTTCTATAAAT CAG C-3' 5' CGAGGATGAAGTAACAGATGATC-3'	4	289	Red	2.2
12	Vm51	5'-CAT TGC CAC TGG TTT CAC TTA-3' 5'-GAG GCT CAG CAT TTT GTT TCT AT-3	4	256	Yellow	4
13	Vm74	5' CTGCTACACCTTCCATCATTC-3' 5' CCTTTGCTGTGTGGTGGTTT-3'	4	135	Green	1.2

3.3.5 Statistical analysis

Allele frequency, genetic diversity, and polymorphism information content (PIC) were determined for each of 13 SSR markers using PowerMarker Version: V3.25 software (Liu and Muse 2005). Neighbor joining dendrogram was generated with the software program, DARwin 5, Version: 5.0.158 (Perrier et al., 2003). In addition, the fixation indices, gene flow, the Analysis of Molecular Variance (AMOVA), and Principal Coordinate Analysis (PCoA) was performed among the cowpea accessions using GenAlex version 6.5b3 software

(Peakall and Smouse, 2006).In addition, the genetic structure of the accessions was investigated by Analysis of Molecular Variance (AMOVA), fixation indices were used to elucidate the resulting genetic structure and Principal Coordinate Analysis (PCoA) was performed to identify genetic variation patterns among the cowpea accessions using GenAlex version 6.5b3 software (Peakall and Smouse, 2006). Population structure analysis for the 95-cowpea accessions was performed using STRUCTURE v2.3.3 software (Pritchard et al., 2000) based on the 13 SSR markers. We adopted the "admixture model", burn-in period equal to 10,000 iterations and a run of 10,000 replications of Markov Chain Monte Carlo (MCMC) after burn in. For each run, three replications were performed with the number of clusters (K) varying from 2 to 20, leading to 57 Structure outputs. Longer burn-in or MCMC did not change significantly the results. The structure output results were zipped into one archive; then after the zipped file was uploaded into the Structure harvester; we then estimated the number of subpopulation and the best output based on the Structure harvester Web v0.6.94 , Plot vA.1, Core vA.2 software (Earl et al., 2012).

3.4 Genetic diversity of East African Cowpea Genotypes (*Vigna unguiculata* (L.) Walp.) Collections and IITA Inbred Lines based on SNP Markers

The same number of cowpea accessions (95) and extracted DNA used for SSR analysis were used for SNP analysis (3.3.1 and 3.3.2).

3.4.1 Single Nucleotide Polymorphism (SNP) Selection

One hundred and sixty five SNP markers were selected out of 1,122 SNP markers used to construct a linkage map for cowpea by University of California Riverside (Wellington et al., 2009; Mitchell et al., 2011) and of these 151 polymorphic SNP markers were selected for final genetic diversity analysis (Table 16). The SNP markers were selected in such a way that they would cover evenly all 11 chromosomes of cowpea as represented by the linkage groups, (Tables 16, Table 34, and Figure 26). The 95 DNA samples were prepared on ABI plate for shipping according to the requirement of SNP analysis service provider, LGC Genomics (formerly KBioscience), UK.

Table 16: List of SNPs selected from genetic linkage map of cowpea

No.	SNP IDs Marker	Chromosome No	No.	SNP IDs Marker	Chromosome No
1	1107_518	1	**77**	12568_234	6
2	12526_795	1	**78**	14654_1071	6
3	12882_709	1	**79**	14784_1653	6
4	12929_463	1	**80**	15305_818	6
5	13294_282	1	**81**	279_179	6
6	14619_471	1	**82**	3900_562	6
7	18_107	1	**83**	437_590	6
8	25_592	1	**84**	4692_429	6
9	2820_248	1	**85**	4749_1972	6
10	3787_812	1	**86**	5270_452	6
11	5735_110	1	**87**	5356_124	6
12	9432_1340	1	**88**	7233_543	6
13	9815_2051	1	**89**	7383_1042	6
14	10480_616	2	**90**	8438_669	6
15	1297_783	2	**91**	9134_1559	6
16	14497_540	2	**92**	11558_901	7
17	16946_421	2	**93**	11585_1881	7
18	2046_754	2	**94**	12349_535	7
19	3427_925	2	**95**	13586_1058	7
20	3838_830	2	**96**	13872_1420	7
21	4200_155	2	**97**	15113_1068	7
22	4273_342	2	**98**	17196_517	7
23	6580_67	2	**99**	17450_1553	7
24	708_159	2	**100**	17513_514	7
25	8044_1006	2	**101**	234_249	7
26	8253_397	2	**102**	4131_472	7
27	8395_1157	2	**103**	4778_497	7
28	8947_802	2	**104**	5692_1408	7
29	9739_495	2	**105**	1936_545	8
30	10378_737	3	**106**	1281_790	8
31	10650_1563	3	**107**	14702_888	8

Table 16 (cont'd)

32	1165_701	3	**108**	15637_1357	8
33	12505_1312	3	**109**	15875_801	8
34	12905_686	3	**110**	311_1536	8
35	13022_1425	3	**111**	3803_763	8
36	14056_564	3	**112**	5135_477	8
37	15129_553	3	**113**	6378_514	8
38	15183_436	3	**114**	7248_578	8
39	16139_2530	3	**115**	9607_1753	8
40	16566_353	3	**116**	1060_220	9
41	16655_1561	3	**117**	12126_561	9
42	2_341	3	**118**	122_468	9
43	2453_65	3	**119**	14034_820	9
44	2591_569	3	**120**	15764_405	9
45	2974_1109	3	**121**	15773_423	9
46	7068_60	3	**122**	1989_448	9
47	7087_1100	3	**123**	5137_1051	9
48	1202_1215	4	**124**	5656_680	9
49	12854_535	4	**125**	658_460	9
50	13269_270	4	**126**	7548_1327	9
51	13386_815	4	**127**	7565_739	9
52	13873_544	4	**128**	9779_613	9
53	4702_954	4	**129**	12029_2782	10
54	5268_412	4	**130**	1283_371	10
55	5503_54	4	**131**	1653_181	10
56	5652_704	4	**132**	2245_530	10
57	6867_337	4	**133**	2870_790	10
58	8166_564	4	**134**	4237_650	10
59	897_240	4	**135**	4306_482	10
60	9114_900	4	**136**	4800_500	10
61	1004_587	5	**137**	5993_278	10
62	11613_1075	5	**138**	6205_632	10
63	11920_1704	5	**139**	8877_1528	10
64	1441_128	5	**140**	10277_636	11
65	14814_511	5	**141**	11599_1036	11
66	1980_886	5	**142**	14825_288	11
67	5058_372	5	**143**	16413_395	11
68	534_355	5	**144**	3494_143	11
69	6046_661	5	**145**	4712_832	11
70	6663_368	5	**146**	5449_242	11
71	7344_500	5	**147**	5756_456	11
72	7967_1210	5	**148**	7184_257	11
73	8121_1880	5	**149**	734_340	11
74	8905_1569	5	**150**	8150_1237	11
75	10738_1400	6	**151**	8842_943	11
76	10974_245	6			

3.4.2 Statistical analysis

Allele frequency, genetic diversity, and polymorphism information content (PIC) were determined for each of 151 SNP markers using PowerMarker Version: V3.25 software (Liu and Muse 2005). Neighbor joining dendrogram was generated with the software program, DARwin 5, Version: 5.0.158 (Perrier et al., 2003). In addition, the fixation indices, gene flow, the Analysis of Molecular Variance (AMOVA), and Principal Coordinate Analysis

(PCoA) was performed among the cowpea accessions using GenAlex version 6.5b3 software (Peakall and Smouse, 2006).In addition, the genetic structure of the accessions was investigated by Analysis of Molecular Variance (AMOVA), fixation indices were used to elucidate the resulting genetic structure and Principal Coordinate Analysis (PCoA) was performed to identify genetic variation patterns among the cowpea accessions using GenAlex version 6.5b3 software (Peakall and Smouse, 2006). Population structure analysis for the 95-cowpea accessions was performed using STRUCTURE v2.3.3 software (Pritchard et al., 2000) based on the 151 SNP markers. We adopted the "admixture model", burn-in period equal to 10,000 iterations and a run of 10,000 replications of Markov Chain Monte Carlo (MCMC) after burn in. For each run, three replications were performed with the number of clusters (K) varying from 2 to 20, leading to 57 Structure outputs. Longer burn-in or MCMC did not change significantly the results. The structure output results were zipped into one archive; then after the zipped file was uploaded into the Structure harvester; we then estimated the number of subpopulation and the best output based on the Structure harvester Web v0.6.94, Plot vA.1, Core vA.2 software (Earl et al., 2012).

4. Results and Discussion

4.1 Molecular genetic diversity study of Ethiopian cowpea landrace accessions

4.1.1 SSR polymorphism

The genetic profiles of Ethiopian cowpea with 23 SSR markers detected a total of 75 alleles, including 2 alleles with allele frequency lower than 0.01, considered as rare alleles, which are effective indicators to detect the genetic differentiation within populations. The two rare alleles were obtained from Gambella accession (222890-2) and Southern Nations, Nationalities and Peoples (SNNP) accession (286-2) when tested against the SSR1 marker. The number of alleles for the overall population varied from 2 for CP93/94, CP397/398, CP403/404, Y21 and 61RM2 to 5 for CP333/334, CP335/336 and CP269/270 markers while the average is 3. The genetic diversity (D) varied from 0.06 for marker SSR1 to 0.73 for CP269/270 with the mean value of 0.47. The PIC for overall populations varied from 0.06 for marker SSR1 to 0.68 for primer CP269/270 with a mean of 0.4 (Table 17). Almost half of the markers showed average polymorphism. Highest mean PIC value (0.46) and genetic diversity (0.39) were obtained in SNNP. Slightly lower PIC and genetic diversity of (0.42,0.35), (0.42,0.37) and (0.41,0.34) were obtained for Gambella, Amhara and Oromia regions respectively, whereas lowest PIC and D values were observed for Tigray, Eritrea and Benishangul Gumuz suggesting that SNNP, Gambella, Amhara and Oromia possess abundant microsatellite diversity in their respective order (Table 18).

In the present study, polymorphic information content (PIC) analysis results ranging from 0.06 to 0.68 was found for the SSR markers, with a mean PIC of 0.4, which is comparable with the previous findings. Fatokun et al. (2008) observed PIC ranging from 0.29 to 0.87 with a mean of 0.68 among 48 wild cowpea lines using SSR markers. A study of Li et al. (2001) reported a PIC ranging from 0.02 to 0.73 with a mean of 0.47 among cultivated cowpea with a different set of SSR markers. Badiane et al. (2012) reported PIC value, which varied from 0.08 to 0.33 with a mean of 0.23 using cowpea, collected from Senegalese National Germplasm. Aaron et al. (2010) reported PIC, which varied from 0.07 to 0.66 with an average of 0.38 using cowpea collected from Ghanaian germplasm. Ogunkanmi et al. (2014), assessed 48 accessions of cultivated cowpea, the PIC from West African accessions was found to be 0.369, South African is 0.329 while North East and Central Africa with 0.332, which is evidence confirming that West Africa contains greater diversity. The low level of polymorphism detected in our study is in agreement with previous studies and may be the result of a bottleneck induced by a single domestication event in this crop (Li et al., 2001;

Tosti and Negri, 2002; Badiane et al., 2004; Diouf and Hilu, 2005) in addition to its inherent self-pollinated reproduction mechanism.

Across the entire genotype set, 75 alleles with an average of 3 alleles per locus were detected. The number of alleles amplified using SSR markers ranged from 2 to 5, which is lower than reported in previous studies for cowpea. In contrast, Asare et al. (2010) reported 4 to 13 alleles in cowpea collected from Ghana, Badiane et al. (2012) reported 1 to16 alleles in cowpea collected from Senegalese National Germplasm which was higher than that previously reported on the same germplasm which ranged from 1 to 9 (Diouf and Hilu, 2005). Sawadogo et al. (2010) reported 5 to 12 alleles in cowpea collected from Burkina Faso using cross species SSRs from *Medicago*. Diouf and Hilu (2005) reported 1 to 9 alleles for different germplasm and Li et al. (2001) reported that 27 cowpea SSR primers detected between 2 and 7 alleles among 91 cowpea breeding lines. According to Aaron et al. (2010) the number of alleles detected per primer pair varied from a minimum of 1 to a maximum of 6 with an average of 3.8, using Ghanaian germplasm. Ogunkanmi et al. (2014) reported a total of 37 alleles with mean alleles of 3.1 using 48 cultivated cowpea accession with 12 SSR markers. The low level of allele detected in Ethiopian accessions could be due to the amount of germplasm used in our study and low genetic base found in Ethiopian cowpea accessions. The number of alleles amplified by various SSR markers used in our study, varied greatly and ranged from 2 alleles per marker to a maximum of 5 alleles per marker. As the number of alleles is dependent on the number of accessions, it is difficult to compare results of different studies. Nevertheless, the number of alleles detected was within the same range as that found in other studies for other crops. For example, SSR alleles ranged from 3 to 11 in rice (Yang et al.. 1994); 11 to 26 in soybean (Rongwen et al. 1995), 3 to 16 in wheat (Plaschke et al., 1995), and 2 to 23 in maize (Pejic et al., 1998).

Genetic diversity among different populations was analyzed based on the data from 23 SSR loci, the genetic diversity (D) of overall population varied from 0.06 for marker SSR1 to 0.73 for CP269/270 with the mean value of 0.47. The genetic diversity values were slightly higher in samples from the southern Ethiopian region (D=0.46) than in Amhara (D=0.42), Gambella (D=0.42) and Oromia (D=0.41) while slightly lower genetic diversity values of 0.3, 0.28, 0.28 were obtained for Tigray, Benishangul Gumuze and Eritrea regions respectively. The comparison of the total number of alleles among different region genotypes showed no significant differences. Of 75 alleles observed, a slightly larger number of alleles were contributed by Amhara (69 alleles, 69%), SNNP (63 alleles, 63%), Oromia (59 alleles, 59%)

4.1.2 Clustering and Structure analysis based on SSR markers

Clustering analysis of the 23 SSR markers separated all the *V. unguiculata* accessions into three main clusters. Most of the accessions found in the second main cluster contained accessions from different regions, while the third main cluster contains accessions only from one region (Oromia, i.e, Harerge).

A total of 165 out of 210 (78.6%), 41 out of 210 (19.5%) and 4 out of 210 (1.9%) cowpea accessions were grouped in the main cluster group II, I, III, respectively, showing that most of the cowpea accessions were grouped together in the main cluster II.

The first main cluster was further divided into two subclusters. The second subcluster contained only one accession (2692) from Gambella, whereas the first subcluster further divided into two sub-sub-clusters each containing only one accession 241098 and 244099 from Oromia region, while the first sub-sub-cluster comprises one accession (2681) from Gambella and 22171711 from Tigray regions. More than half of the first main cluster group predominantly composed of accessions from Amhara and SNNP regions.

The second main cluster is divided into two sub groups, and both subclusters further divided into two sub-sub groups. Most of the cowpea accessions were predominantly in the main cluster group II, which is composed of 165 out of 210 (79%) accessions; of which 40 out of 46 (87%) were from Oromia region, 26 out of 38 (68%) were from Amhara region, 37 out of 48 (77%) were from SNNP region, 43 out of 49 (91%) from Gambella region and 7 out of 9 (78%) from Eritrea.

A total of 24 accessions from Oromia, 16 accessions from Amhara, 25 accessions from SNNP, 27 accessions from Gambella and 2 accessions each from Benshngul Gumuze, Tigray and Eritria were found in sub-groups II of sub-sub-cluster I, indicating that they might have an underlying genetic relationship.

Based on our results, all of the five entries (2114411, 2144121, 2144122, 2114131 and 2114132) which are subtypes of the main accession 211441 for which the collection sites are unknown, were all grouped in the main cluster II. 2144121, 2114131 and 2114132 were tightly clustered and shared a common allele with accession 2521 from Gambella region; while, 2114411 was tightly clusted with accession 211433 from Benshngul Gumuze; similarly, 2144122 closely clusterd with accession 2113842 from Amhara region.

Of the accessions analyzed, accessions: 27012, 2114432, 2705 and 27052 are the most genetically divergent. All the accessions grouped in cluster III formed an isolated sub group containing only four accessions. All of the four accessions are tightly clustered and shared a common allele that belongs to the Oromia region. This group was further divided into two groups, each containing two accessions. The first group contained accessions 27012 and 2114432 and the second sub-group contained accessions 2705 and 27052, indicating that they might have distant relationship with the rest of Ethiopian cowpea accessions (Fig 8 and Table 19).

Based on the dendrogram, only 28 (i.e. 14 pair of accession) out of the 189 (15%) sub-type accessions, as described in the material and methods section were, grouped together but the rest of 161 out of 189 (85%) accessions are grouped in different cluster groups. Therefore, the subtype accessions created based on the morphological traits were significantly supported by the SSR molecular data. For instance, from accession 270; we grouped eight subtypes namely, 2701, 2702, 2703, 2704, 2705, 27061 and 27062. Out of these sub-type accessions only subaccessions 2702 and 27062 were clustered together while the rest were grouped separately. Similarly, the five sub-accessions that were formed from accession 211447 (2114471, 21144721, 21144722, 2114473, and 2114474) were all grouped in different clustered group. Similarly, each of the original accessions 215821, 221727, 211383, 211436, 216747 and 223402 were sub-divided into 2 to 4 sub-type accessions, and each sub-type accession was grouped in different cluster group.

The results of Structure analysis were used to infer the possible number of clusters (subpopulations) using a Bayesian clustering approach. The most probable clusters is K=3, which received the strongest support as suggested by ΔK values (K ranging from 2 to 20), Thus, the structure analysis identified three sub-population groups, which was in agreement with clustering obtained by the dendrogram tree construction (Fig 9, Table 19, Fig 10).

Defining the exact genetic relationship among accessions is inherently difficult. The ability to resolve subtle genetic differences is enhanced by increasing the number of markers and improving the distribution of markers throughout the genome. Nonetheless, we were able to place the various cowpea accessions from Ethiopia into three main groups, that reflect their geographical origins and relatedness poorly. For example, our result showed that Gambella, though it has the second smallest area coverage (2.9%) as compared to other regions; however, has got genetic diversity value higher than the biggest region Oromia (27.8%), the same as the third largest region Amhara (15.2%) and a little bit lower than the fourth biggest region, SNNP (10.9%).

Hence future cowpea collection strategy should give due consideration for Gambella region. Since they share common alleles and tightly clustered together, accessions: 2144121, 2114131, and 2114132 might have been collected from Gambella region; similarly, accessions 2114411 and 2144122 might have been collected from Benshngul Gumuze and Oromia region, respectively. Ethiopian cowpea has formed three isolated major cluster groups, each of which further divided to form their own subcluster groups. The majority of the accessions (79%) were grouped in cluster group II, while cluster III contained only 1.9% of the total accessions and classified separately, indicating that this cluster group was genetically separated from the others. Except cluster group III, which tightly clustered and shared a common allele and contained accessions from only one region (Oromia), cluster group II and I were composed of accessions from almost all geographical locations. The structure analysis identified three sub-population groups, which were in agreement with sub-clustering obtained by the genetic tree construction. However, the number of accessions identified in each sub-group of the two different analysis outputs was different. At the beginning of our experiment, we identified subtypes in 62 out of 83 Ethiopian cowpea accession based on their within accessions morphological variability i.e. color and size; the grouping identified by seed color and size were further supported by the genetic relationship of the dendrogram tree construction. Therefore, the subtypes can be considered as different accessions; hence, they can stand by themselves to form isolated accessions.

Table 19: Genetic analysis identified three subpopulations among reference set accessions of cowpea.

No	**Sub-groups**	**I**			**II**				**III**
	Individuals (No.) (main	**41**			**165**				**4**
	Individuals (No.) (sub-	**I (40)**		**II (1)**	**I (126)**		**II (39)**		
	Individuals (No.) (sub-	**I (25)**	**II**		**I**	**II**	**I**	**II**	
1	Oromia (O)(46)	1	1	-	24	7	7	2	4
2	Amhara (A) (38)	7	5	-	16	3	5	2	-
3	South (SNNP) (48)	11	-	-	25	8	4	-	-
4	Gambella (G) (49)	1	4	1	27	5	9	2	-
5	Benishangul and	4	-	-	2	-	3	-	-
6	Tigray (T)(6)	1	3	-	2	-	-	-	-
7	X-location (X) (5)	-	-	-	5	-	-	-	-
8	Eritrea (E) (9)	-	2	-	2	-	5	-	-

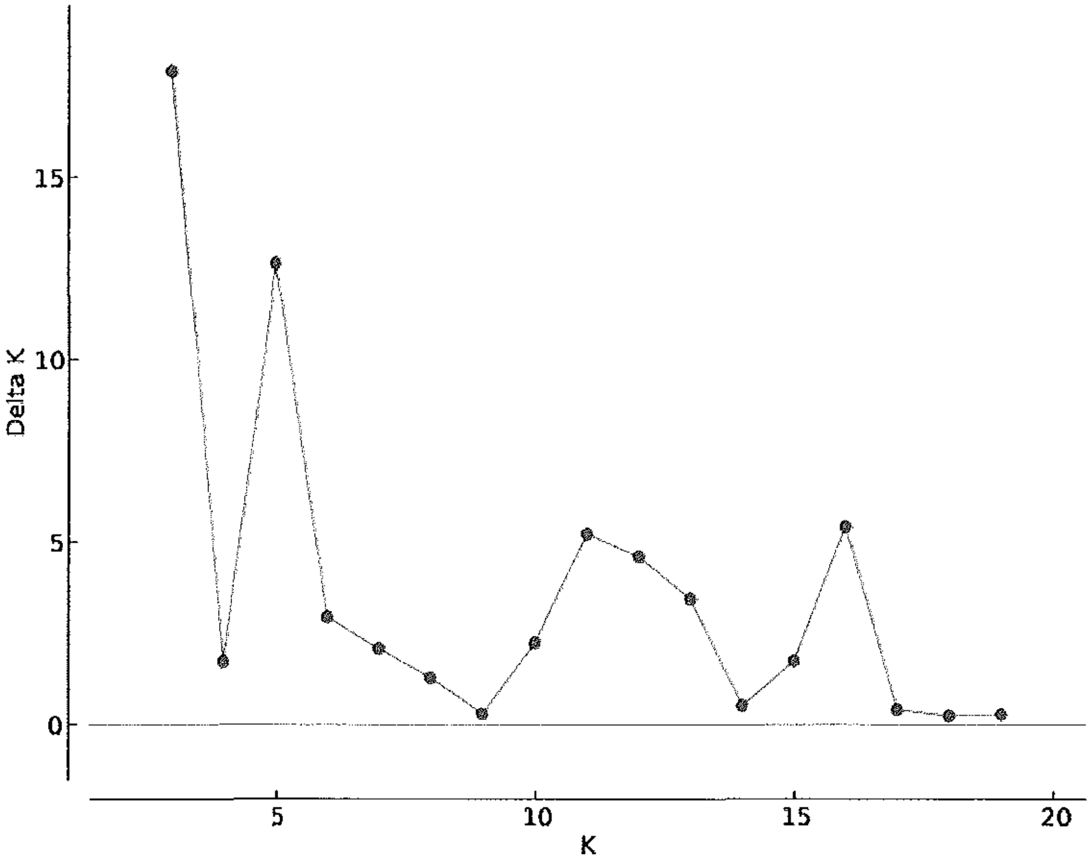


Figure 9: Estimation of the number of populations for K ranging from 2 to 20 by calculating ΔK

4.1.3 Analysis of Molecular Variance (AMOVA)

The analysis of molecular variance (AMOVA) showed that all sources of variation were highly significant ($P < 0.001$) and approximately 7% of the overall variation was attributed to genetic differentiation among populations; 60% was explained by differences among individuals within populations and 32% was attributed to genetic differentiation within individuals (Table 21). This indicates that the Ethiopian accessions in this study possess wide diversity within both individuals and populations. The fixation index (F_{st}) was 0.075, which showed moderate differentiation accompanied by a higher gene flow (N_m=3.176) between regions, representing the presence of germplasm exchange among regions. In other studies different AMOVA results were obtained when 81 Kenyan cowpea were studied; the total accessions among the geographical regions revealed 3.99% genetic variation while the variation among the accessions within geographical region suggested 52.33% variation within individual accession explained 43.67%, the fixation index (F_{st}) was low 0.04, indicating low differentiation among cultivated cowpea accessions in Kenya (Kuruma et al., 2008) and different AMOVA results were obtained for 312 Tanzanian cowpea accessions; for the geographic data, only the regions within the zones were significant, and they explained 3% of the genetic variance and had F_{st} value of 0.033, the groups found by structure explained a much higher percentage of the genetic variance (97%) (Sariah et al., 2010). In our result is in agreement with the genetic result, which showed groupings of accessions from different regions in to one cluster. According to Wright (1978), IPGRI and Cornell University (2003) and Kiambi et al. (2005) suggested that F_{st} in a range of 0–0.05, indicates little differentiation, 0.05–0.15 moderate differentiation, 0.15-0.25 large differentiation and above 0.25 indicates a very large differentiation, demonstrating the majority of variations are found within population groups rather than between population groups.

Table 21. Analysis of molecular variance (AMOVA) among cowpea populations

Source of variation	DF	Sum of squares	Var component	Variation (%)	**F statistics (Fst)**
Among populations	8	281.2	0.54	7^{***}	0.075
Among individuals within populations	201	2292.6	4.49	60^{***}	
Within individuals	210	508	2.42	32^{***}	
Total	419	3081.7	7.46	-	

N_m=3.176

4.2 Cloning the Race Specific *Striga generiods* 3 (RSG3) gene and Diversity analysis based on (SSR) Striga resistance markers

4.2.1 Diversity analysis of among Ethiopian accessions based on SSR Striga resistance markers

Three well-characterized markers (SSR1, 61RM2 and C42-2B) associated with resistance to the root parasitic weed *S. gesnerioides* were used to characterize Ethiopian cowpea populations. The number of amplification products generated ranged from 2 (61RM2) to 4 (C42-2B) with an average of 3 bands per primer. The size of the amplified fragments ranged from 150 to 550 bp for SSR1, 250 to 500bp for C42-2B and 300 to 1250bp for 61RM2; similarly, the overall size ranged from 150 to 1250. 9 (100%) polymorphic bands were observed among the Ethiopian cowpea accessions tested (Table 23).

Table 22:Allele frequency, allele number, genetic diversity, and polymorphism information content (PIC) of the three SSR striga markers studied

No	Markers	Major Allele Frequency	Allele No	Gene Diversity	Heterozygosity	PIC
1	SSR1	0.97	3	0.06	0.04	0.06
2	61RM2	0.90	2	0.19	0.18	0.17
3	C42-2B	0.73	4	0.43	0.29	0.39
	Mean	0.87	3	0.23	0.17	0.21

4.2.2 Similarity and genetic among Ethiopian cowpea accessions with B301 based on three SSR *striga* markers

We used three well-characterized markers (SSR1, 61RM2 and C42-2B) associated with resistance to the root parasitic weed, *Striga gesnerioides* to characterize the Ethiopian accessions. Dendrogram of 3 markers separated all the *V. unguiculata* accessions from Ethiopia and multi-race *striga* resistant accession B301 from Botswana into three main clusters. Most of the accessions, along with B301 fall in the first main cluster group containing accessions from all of the geographical regions, while the second main cluster contains accessions only from one region; Amhara. Accordingly 178 out of 211 (84.36%), 32 out of 211 (15.16%) and only one cowpea accession from Amhara region was clustered in the main cluster group of I, III and I respectively, showing most of the cowpea accessions were grouped in the main cluster I (Table 23 and Fig 11).

The first main cluster was further divided into two sub-clusters. The first sub-cluster contains 84 accessions from all of the regions of which 83 accessions were grouped in the first sub-sub cluster, while only one accession (2167462) from Gambella formed isolated second sub-sub cluster. The second sub cluster contained 94 accessions from all of the regions, of which 88 and 6 accessions were grouped in the first and second first sub-sub cluster group. The third main cluster group consisted of 32 accessions from all regions except from Tigray and Benishangul Gumuz regions, of which 19 accessions are from Oromia region; whereas, the second main cluster contains one accession (2157624) from Amhara region (Table 23 and Fig 11).

Most of the Ethiopian accessions, 178 out of 211 (84.36%) were grouped in the first main cluster group showing greater similarity with the multi-race *Striga* resistant accession B301 from Botswana as compared with main cluster group II and III. 27 out of 46 (58.69%) accessions from Oromia region, 33 out of 46 (71.74%) from Amhara region, 45 out of 48 (93.75%) from SNNP region, 45 out of 49 (91.83%) from Gambella region, 7 out of 9 (77.78%) from Eritiria and all accessions from Benishangul Gumuze and Tigray regions were grouped in the first main cluster group together with B301. This is indicating that accessions shared common allele and have an underlying genetic relationship; whereas all of the accessions found in main cluster group II and III are distantly clustered with B301 showing that they shared less common allele and showed less similarity with multi-race resistant accession, B301 (Table 22 and Fig 11).

Of all the accessions, 3 accessions (195761, 195762 and 195771) from Amhara region, one accession each from Gambella (2228902) and SNNP (2862) regions were grouped together with B301; the aforementioned accessions were grouped in the second sub-sub cluster group. Most interestingly, we identified a small number of local landraces 3 (195761, 195762 and 195771) accessions from Amhara region, 1 accession each from Gambella (2228902) and SNNP (2862) regions among the various accessions that tightly clustered and shared a common allele with the multi-race striga resistant accession B301from Botswana (Table 23 and Fig 11).

B301 accession is considered as a multi-race *striga* resistant accession (Sawadogo et al., 2010) because of their resistance to all races of *Striga gesnerioides* in cowpea, except Race-4 from Benin (Lane et al., 1996, 1997). Most of the Ethiopian accession showed greater similarity and shared common allele with the multi-race *striga* resistance accession, B301, from Botswana; whereas, the highest similarity was obtained between five Ethiopian accessions from Amhara, Gambella and SNNP regions; intriguingly these Ethiopian cowpea accessions are tightly clustered and shared a common allele with the multi-race *striga* resistant accession B301from Botswana. This is surprising since these Ethiopian landraces are genetically distant from B301 suggesting that either this is a novel allele of the resistance allele or that the allele introgressed a long time ago.

4.2.3 Genetic variation among Ethiopian accessions and B301 of Botswana based on 23 SSR markers

A genetic dendrogram showed the relationship between B301 which is resistant for *Striga gesneriods* of SG3 race with the Ethiopian accessions (2228902 and 2862), which showed resistance to SG3 race. The dendrogram of the 23 SSR markers delineated all the 211 *V. unguiculata* accessions into three main clusters (Fig.12). 175 out of 211 (82%), 23 out of 211 (11%) and 15 out of 211 (7%) of the cowpea accessions were detected in the main cluster groups of I, II, and III respectively, showing most of the cowpea accessions are in the main cluster I.

B301 and the Ethiopian accessions of 2228902 and 2862 were tightly clustered and shared a common allele, grouped in the first main cluster group indicating that they might have an underlying genetic relationship and share a high level of similarity.

Table 23. Genetic analysis of three striga markers identified three subpopulations among reference set accessions of cowpea.

No	Sub-groups	I				II	III
	Individuals (No.) (main group)	178				1	32
	Individuals (No.) (sub group)	I (84)		II (94)			
	Individuals (No.) (sub sub group)	I (83)	II (1)	I (88)	II (6)		
1	Oromia (O)(46)	16		11			19
2	Amhara (A) (38)	11		19	3	1	4
3	South (SNNP) (48)	22		22	1		3
4	Gambella (G) (49)	26	1	17	1		4
5	Benishangul and Gumeze (BG) (9)	1		8			
6	Tigray (T)(6)	2		4			
7	X-location (X) (5)	3		2			
8	Eritrea (E) (9)	2		5			2
9	B301				1*		

*= the Botswana accession B301

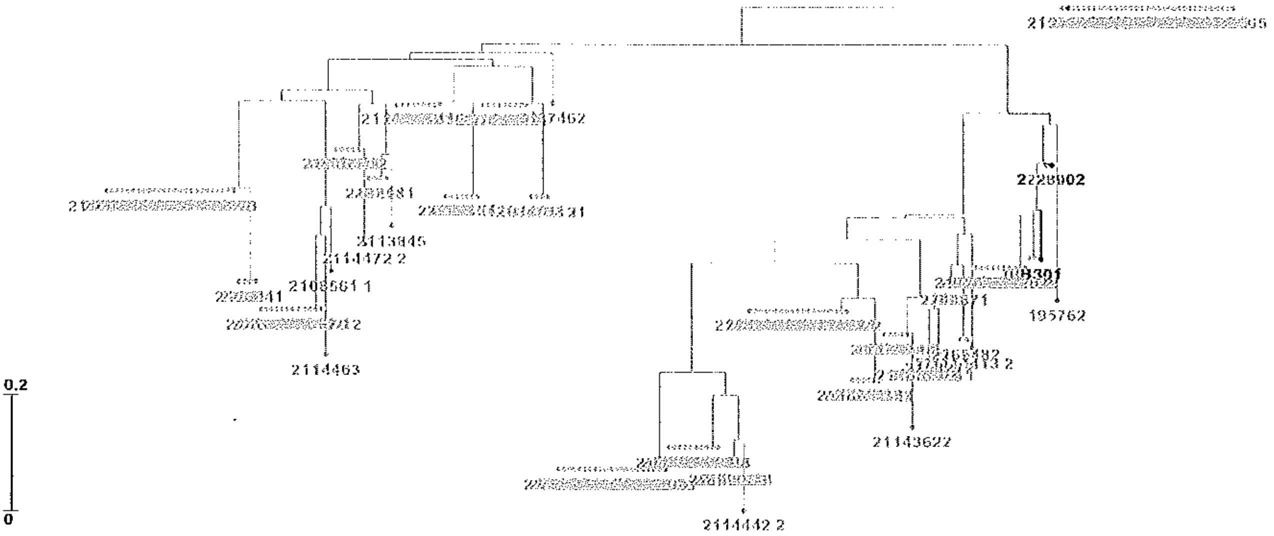


Figure 11: *Genetic relationship among cowpea accessions of Ethiopia based on striga markers (Red: B301, Blue= 2228902 (Gambella), Green=2862 (SNNP), Pink=195761, 195762 and 195771 (Amhara) and the rest of accessions (Gray)*

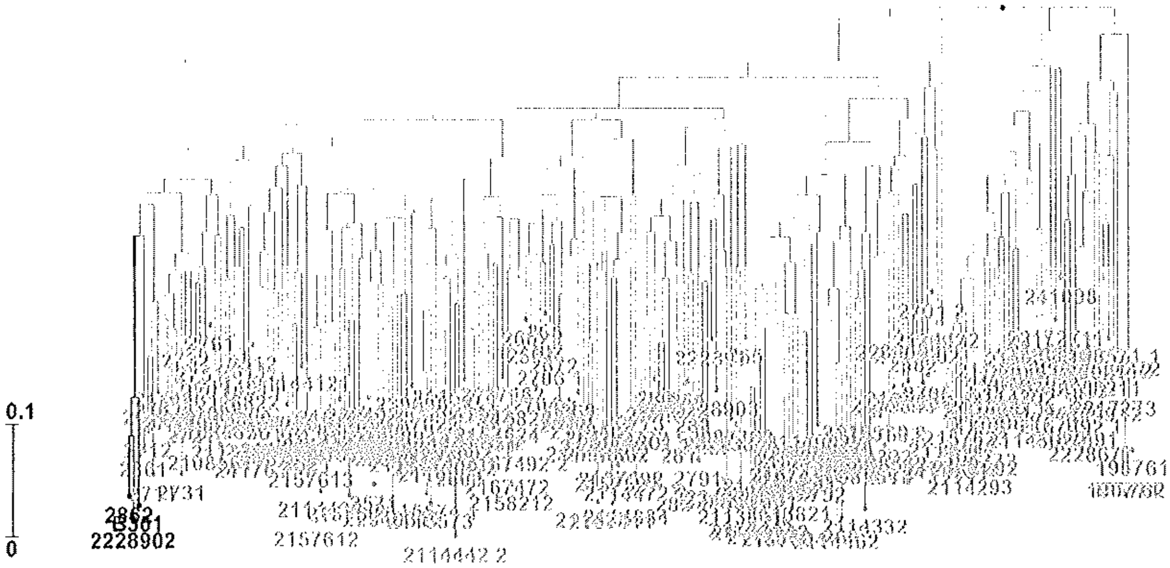


Figure 12: Dendrogram showing similarities between 210 Ethiopian and Botswana (B301) cowpea accessions (Red) based on 23 SSR markers

4.2.4 Polyacrylamide gel electrophoresis (PAG) results of SSR1

SSR1 is associated with SG3 race specific resistance of cowpea to *Striga*. This marker was used to screen all 210 Ethiopian cowpea accessions (Figs 13 and 14). Accessions 2228902 and 2862 showed a 150 bp fragment band consistent with resistance against SG3 race as previously described (Li and Timko, 2011).

Cloning of the 150 bp fragments and subsequent sequencing data confirmed that the RSG3 sequence isolated from Ethiopian materials in our study matched the earlier isolated gene from Botswana (Figs 13 and 14). Intriguingly, this resistance is only associated with the B301, a cowpea accession from Botswana and has not been observed in any other populations.

The SSR1 marker detected two alleles in two of the accessions analyzed. SSR1 marker is one of the three well-characterized markers in cowpea that are known to be associated with resistance to the root parasitic weed *Striga gesnerioides* race SG3 (Li and Timko, 2009). The rare alleles are assumed to be present within the 150 bp region of the resistance gene in the leucine rich repeat domain (Li and Timko, 2009). Therefore, the two accessions 2228902 (Gambella) and 2862 (SNNP) carrying the rare alleles might have the resistance (RSG3-301) gene against *Striga gesnerioides* race SG3; however, this result requires confirmation using sequencing of the region containing the gene and phenotypic characterization of the accessions (Figs 27, 28, 29 and 30).

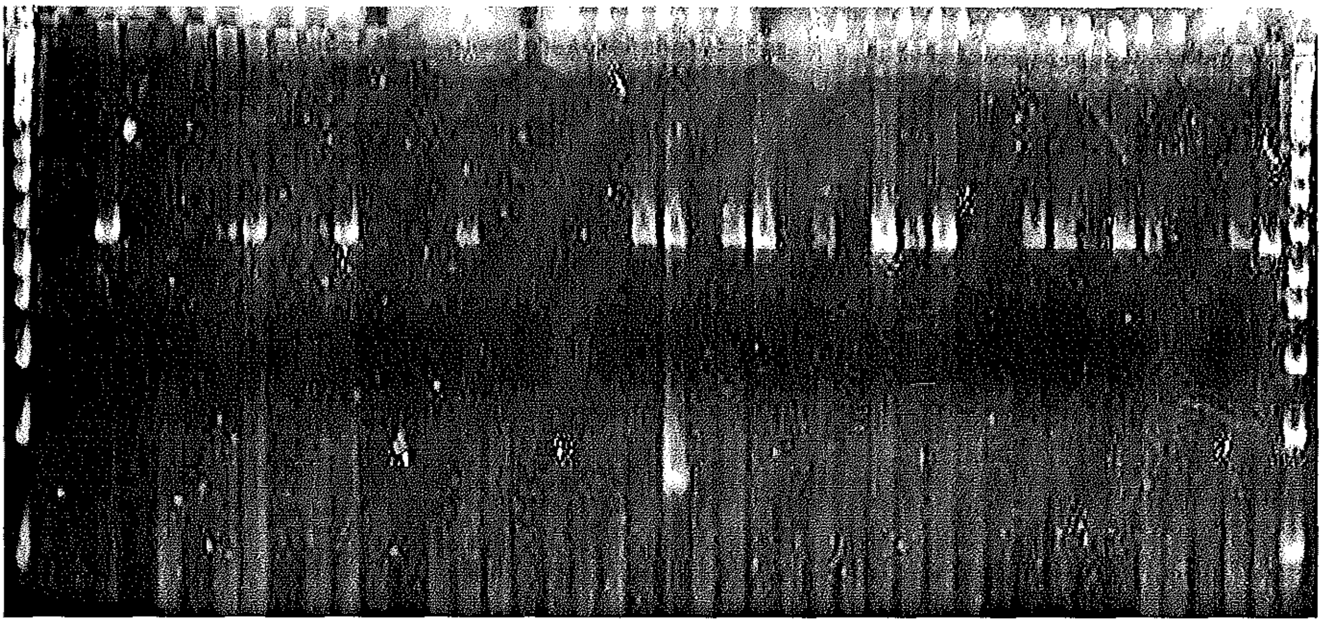


Figure 13: Polyacrylamide gel electrophoresis (PAG) analysis SSR1 (87(1) to 130) (**2228902** accession with 550bp (above) and 150bp (below) band tested against 100kb pulse ladder

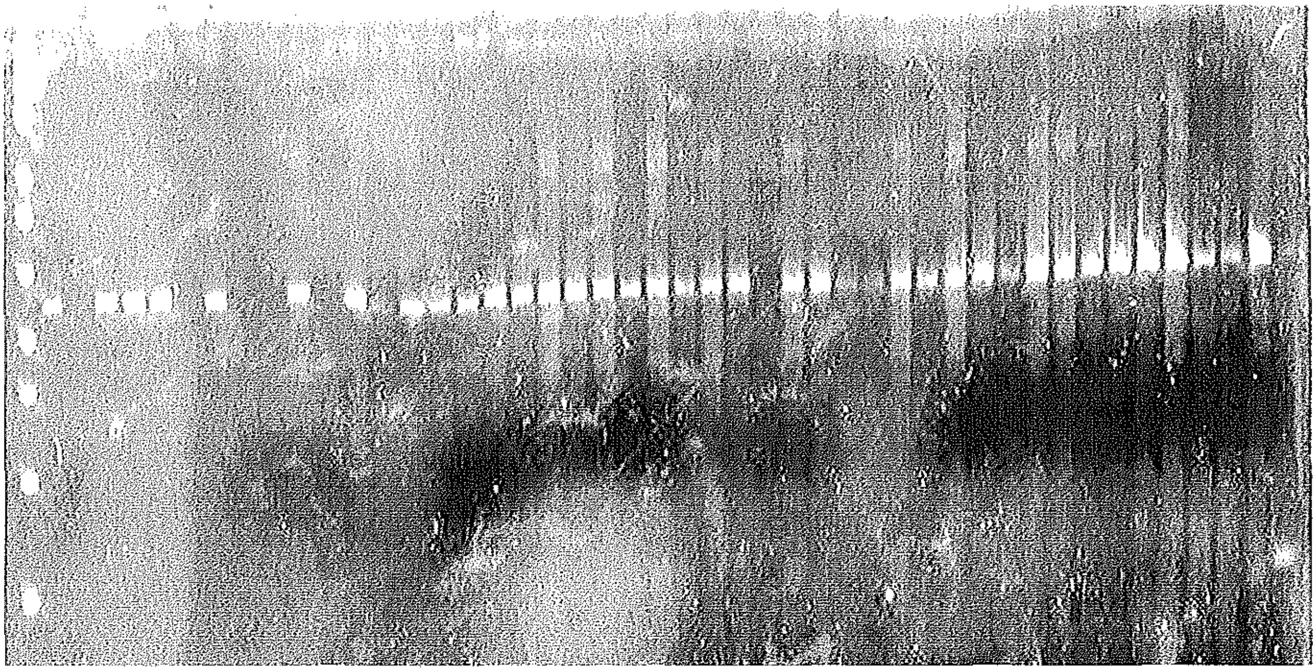


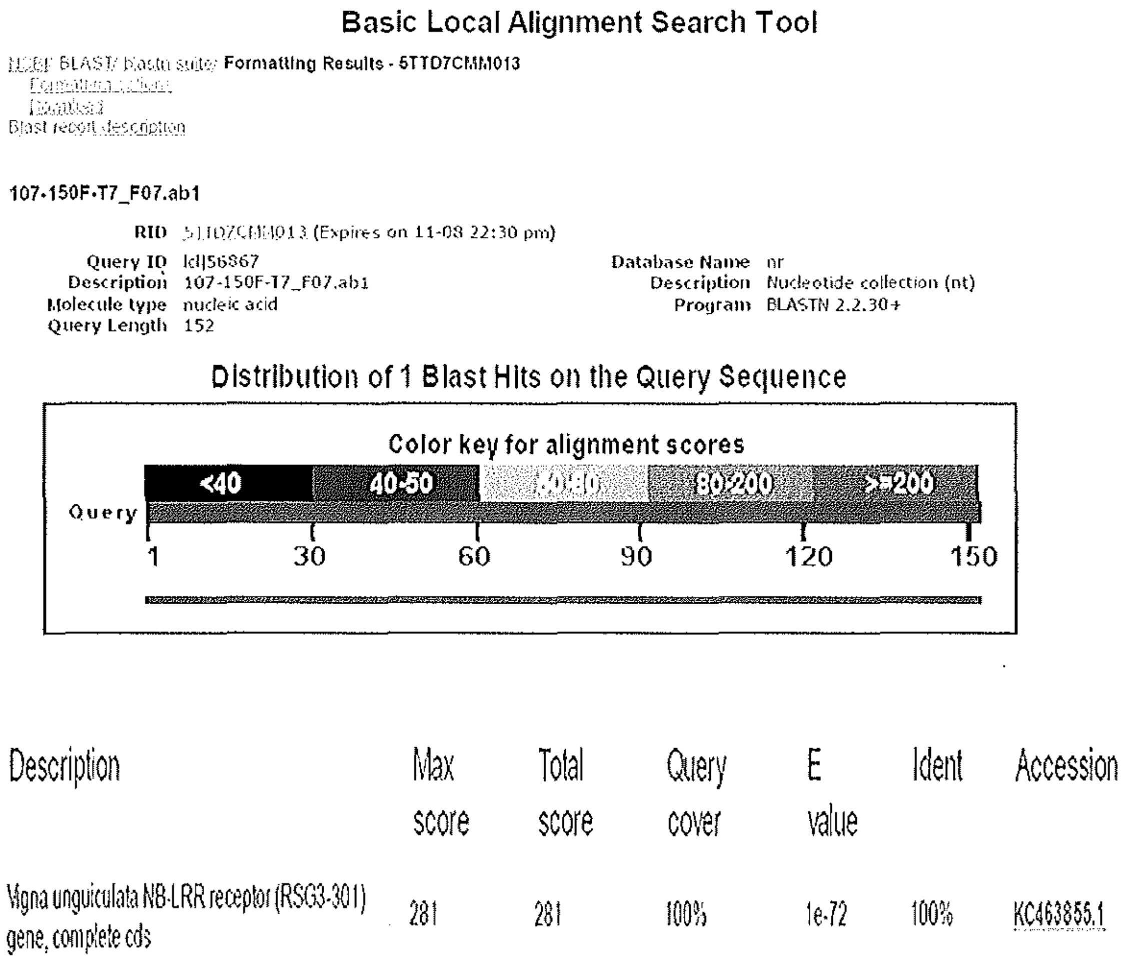
Figure 14: Polyacrylamide gel electrophoresis (PAG) analysis SSR1 (149 to 194) (2862 accession with 550bp (above) and 150bp (below) band tested against 100kb pulse ladder

4.2.5 The nucleotide sequence of RSG3 gene identified from the Ethiopian cowpea accessions

The nucleotide sequence of both of the Ethiopian accessions of the 150 bp fragment after editing the nucleotide sequence obtained from Genewiz Company is as follows:

CAAGAAGGAGGCGAAGACTGGGAAAAAGTTGCTCACATTCGAAACCTATATATATGTT
CTAGAAAGAATCTATATAAGATAGAGTTATTGAAATAGAGTTATTGAAATGTAAAGAA
ACTTCTAAAGTATTGGAGTTGGAGAAAAGCTTAGGA

Figure 15. Results Basic Local Alignment Search Tool



Basic Local Alignment Search Tool

NCBI/ BLAST/ blastn suite/ **Formatting Results - 5TTD7CMM013**
[illegible]
[illegible]
Blast report description

107-150F-T7_F07.ab1

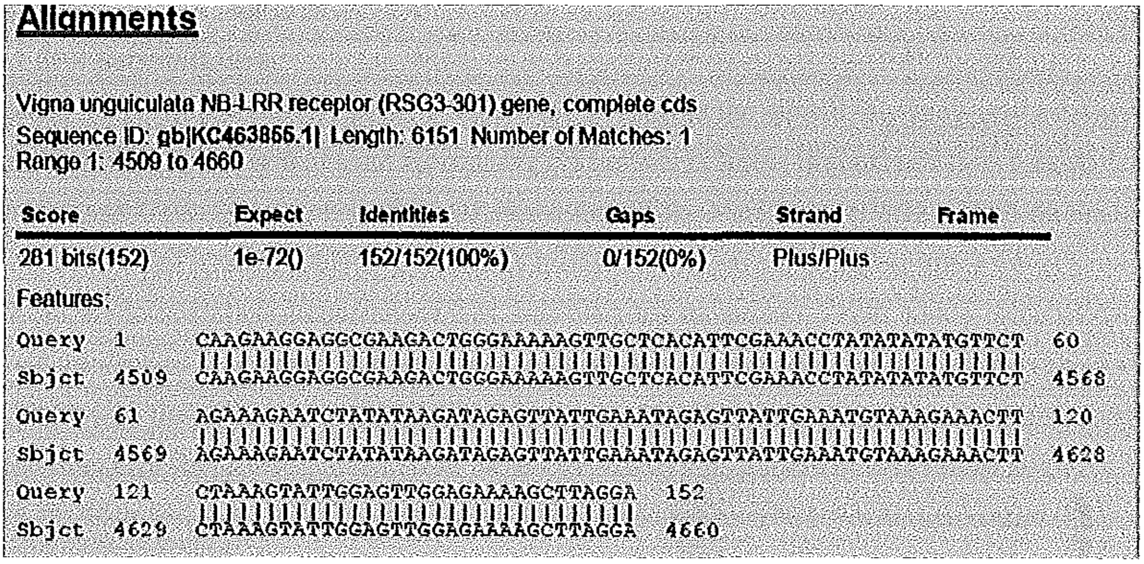
RID 5TTD7CMM013 (Expires on 11-08 22:30 pm)

Query ID	lcl|56867	**Database Name**	nr
Description	107-150F-T7_F07.ab1	**Description**	Nucleotide collection (nt)
Molecule type	nucleic acid	**Program**	BLASTN 2.2.30+
Query Length	152		

Distribution of 1 Blast Hits on the Query Sequence

Description	Max score	Total score	Query cover	E value	Ident	Accession
Vigna unguiculata NB-LRR receptor (RSG3-301) gene, complete cds	281	281	100%	1e-72	100%	KC463855.1

Figure 16: Result of conserved domains of protein sequence



Alignments

Vigna unguiculata NB-LRR receptor (RSG3-301) gene, complete cds
Sequence ID: gb|KC463855.1| Length: 6151 Number of Matches: 1
Range 1: 4509 to 4660

Score	Expect	Identities	Gaps	Strand	Frame
281 bits(152)	1e-72()	152/152(100%)	0/152(0%)	Plus/Plus	

Features:

```
Query  1     CAAGAAGGAGGCGAAGACTGGGAAAAAGTTGCTCACATTCGAAACCTATATATATGTTCT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4509  CAAGAAGGAGGCGAAGACTGGGAAAAAGTTGCTCACATTCGAAACCTATATATATGTTCT  4568

Query  61    AGAAAGAATCTATATAAGATAGAGTTATTGAAATAGAGTTATTGAAATGTAAAGAAACTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4569  AGAAAGAATCTATATAAGATAGAGTTATTGAAATAGAGTTATTGAAATGTAAAGAAACTT  4628

Query  121   CTAAAGTATTGGAGTTGGAGAAAAGCTTAGGA  152
             ||||||||||||||||||||||||||||||||
Sbjct  4629  CTAAAGTATTGGAGTTGGAGAAAAGCTTAGGA  4660
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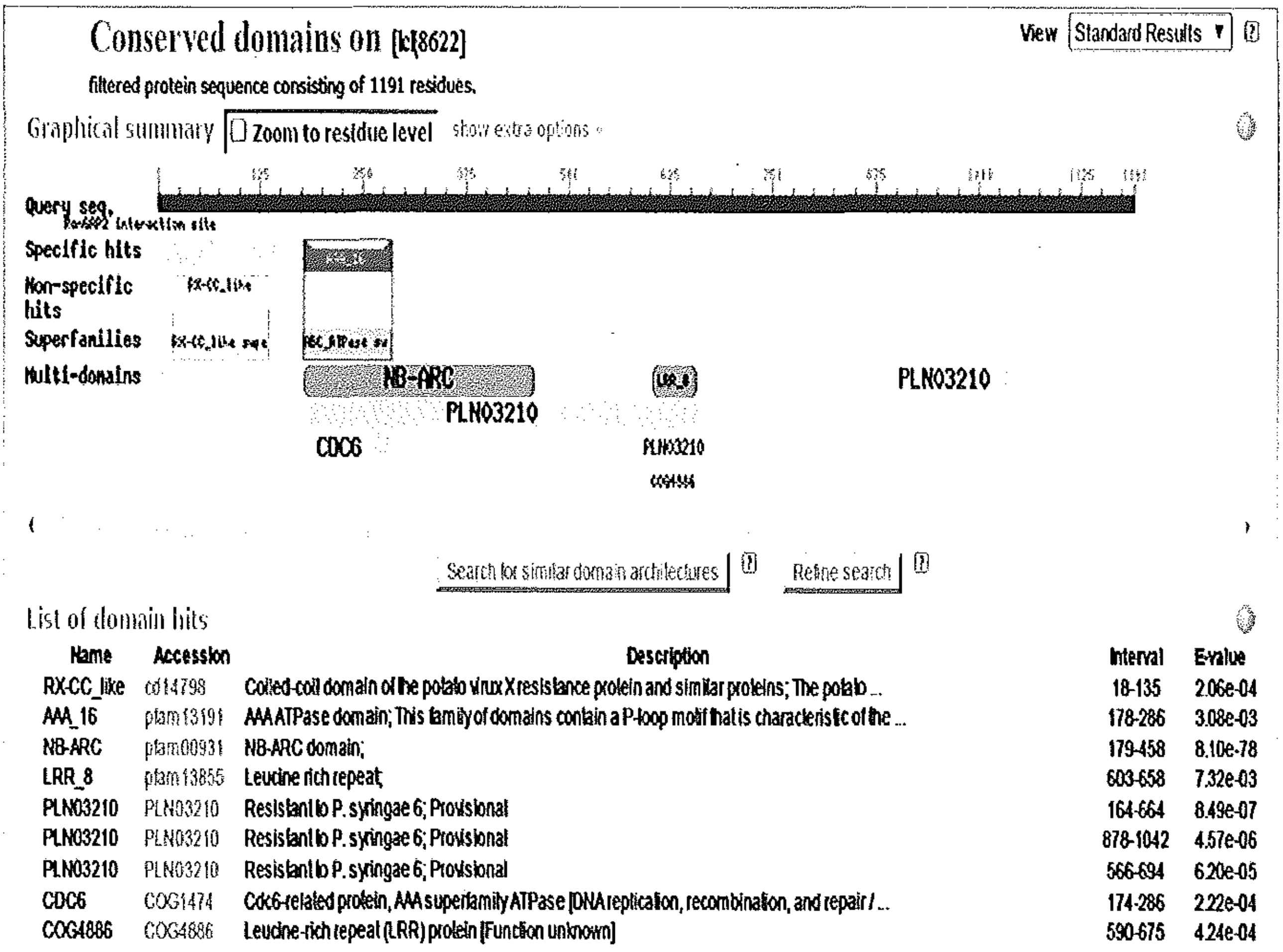

Conserved domains on [lcl|8622]

filtered protein sequence consisting of 1191 residues.

List of domain hits

Name	Accession	Description	Interval	E-value
RX-CC_like	cd14798	Coiled-coil domain of the potato virus X resistance protein and similar proteins; The potato ...	18-135	2.06e-04
AAA_16	pfam13191	AAA ATPase domain; This family of domains contain a P-loop motif that is characteristic of the ...	178-286	3.08e-03
NB-ARC	pfam00931	NB-ARC domain;	179-458	8.10e-78
LRR_8	pfam13855	Leucine rich repeat;	603-658	7.32e-03
PLN03210	PLN03210	Resistant to P. syringae 6; Provisional	164-664	8.49e-07
PLN03210	PLN03210	Resistant to P. syringae 6; Provisional	878-1042	4.57e-06
PLN03210	PLN03210	Resistant to P. syringae 6; Provisional	566-694	6.20e-05
CDC6	COG1474	Cdc6-related protein, AAA superfamily ATPase [DNA replication, recombination, and repair / ...	174-286	2.22e-04
COG4886	COG4886	Leucine-rich repeat (LRR) protein [Function unknown]	590-675	4.24e-04

diversity of 81 Kenyan cowpea accessions and reported PIC value of varying from 0.09 to 0.82 with a mean of 0.34. The low level of polymorphism detected in our study is in agreement with previous studies and may be the result of a bottleneck induced by a single domestication event in this crop (Li et al. 2001; Tosti and Negri, 2002; Badiane et al., 2004; Diouf and Hilu, 2005) in addition to its inherent self-pollinated reproduction mechanism.

A total of 83 alleles were detected for SSR markers with an average of 6 alleles per locus. The number of alleles amplified using SSR markers ranged from 3 in SSR marker Vm51 and BMD2 to 15 in SSR marker Vm70. Similarly Asare et al. (2010) reported 4 to 13 alleles in cowpea collected from Ghana, while Sawadogo et al. (2010) reported 5 to 12 alleles in cowpea collected from Burkina Faso using cross species SSRs from Medicago. Badiane et al. (2012) reported 1 to16 alleles in cowpea collected from Senegalese national germplasms. Diouf and Hilu (2005) reported 1 to 9 alleles for different germplasm which ranged from 1 to 9 and Li et al. (2001) 5 reported that 27 cowpea SSR primers detected between 2 and 7 alleles among 91 cowpea breeding lines. According to Aaron et al. (2010) the number of alleles detected per primer pair varied from a minimum of 1 to a maximum of 6 with an average of 3.8 using Ghanaian germplasm. Ogunkanmi et al. (2014) reported 37 alleles with mean alleles of 3.1 when they used 48 cultivated cowpea accessions with 12 SSR markers. Kuruma et al. (2008) reported allele number ranging from 2 to 14 and with mean allele of 4.5 using 81 cowpea accessions collected from Kenya. In our study, gene diversity (D) ranged from 0.32 to 0.87 using 13 SSR markers with an average 0.56; Aaron et al. (2010) reported the genetic diversity (D) which ranged from 0.12 to 0.68 with an average of 0.44 using Ghanaian germplasm. On the other hand, the genetic diversity value reported by Badiane et al. (2012) varied from 0.08 to 0.42 with an average of 0.28 using cowpea collected from Senegalese national germplasm. This result showed that the genetic diversity (D) of East African accession is more diverse than the West African cowpea collections.

4.3.2 Genetic relationship analysis based on SSR markers

Dendrogram of the 13 SSR markers separated all the *V. unguiculata* accessions into three main clusters. We observed a clear genetic structure in the analysed 95-cowpea accessions from East African countries and IITA inbred lines. The total number of the main cluster group is different from the total number of the population; nonetheless, the geographic distance and the genetic background of the accessions were clearly reflected in the genetic grouping (Table 25 and Fig 17).

A total of 36 out of 95 (38%), 40 out of 95 (42%) and 19 out of 95 (20%) of the cowpea accessions were detected in the main cluster group of I, II, and III respectively, showing most of the cowpea accessions were grouped in the main clusters I and II. Almost half of the main cluster group I

Both the drought resistant (Danilla) and drought susceptible (Tvu-7778) are closely clustered in main cluster III. Local accessions Tvu-13490 and Tvu-6378 from Kenya, local accession 2305675 from Ethiopia and Tvu-160073 from Somalia and the inbred line Danilla from IITA were all tightly clustered and shared a common allele, indicating that they might have an underlying genetic relationship; similarly local accession Tvu-11957 from Somalia and the inbred line Tvu-7778 from IITA were tightly clustered and shared a common allele, indicating that they might have an underlying genetic relationship.

All of the local accessions from each country and the IITA inbred lines were distributed in all of the three main cluster groups, i.e we have clearly observed the grouping of accessions from different countries and IITA inbred lines in the same cluster group. The grouping with in the cluster identified a substantial degree of association between provenance and genotype. Almost all of the accessions from each country and inbred line from IITA were tightly clustered together with each other within the three cluster groups identified. Therefore, the genetic relation tree construction tend to show the geographical distance of the counties and the genetic background of our cowpea material; despite the fact that the grouping of accessions from different countries was observed, there was clear groupings of accession from different countries, this clearly showed the presence of germplasm exchange between East African countries and the IITA center. The released varieties Black eye bean, WWT, TUV-1977 tightly clustered and showed close genetic relationship with some of the local accession of Ethiopia from Borena, Gamogofa, Benchmaji, Gojjam, Borena, Shoa, Hararge, Debub omo, Metekel, Wollega and Bali, hence the result showed the existence of high genetic relation between the aforementioned released and the local cowpea accession of Ethiopia. However, the released varieties Asebot and 82D-889(CH) were tightly clustered with two local Ethiopian accessions (211444 and 211490) while, the released variety Bole (CH) isolated and grouped along with a single cowpea accession 230567; this also showed the existence of high genetic relation between the released variety and the local cowpea accession of Ethiopia. Danilla and Tvu-7778 were inbred lines released by IITA for their high resistance and susceptible to drought stress resistance respectively; based on our genetic result, Danilla was tightly clustered and shared a common allele with the local cowpea accessions Tvu-13490 and Tvu-6378 (Kenya), 2305675 (Ethiopia) and Tvu-160073 (Somalia); similarly Tvu-7778 was tightly clustered and shared a common allele with the local cowpea accessions, Tvu-11957 from Somalia, hence the local accessions should be further checked for their drought tolerance characteristics using well characterized marker which is associated with drought tolerance.

Table 25: Genetic analysis identified three subpopulations among reference set accessions of cowpea.

No	**Sub-groups**	**I**				**II**				**III**			
	Main group	**36**				**40**				**19**			
	Sub group	**I (22)**		**II (14)**		**I (25)**		**II (15)**		**I (9)**		**II (10)**	
	Sub- sub- group	**I (14)**	**II (8)**	**I (7)**	**II (7)**	**I (15)**	**II (10)**	**I (8)**	**II (7)**	**I (8)**	**II (1)**	**I (5)**	**II (5)**
1	Ethiopia (26)	4	-	-	-	15	1	1	3	2	-	-	-
2	Kenya (29)	8	3	-	3	-	3	6	2	2	-	-	2
3	IITA inbred lines (16)	-	-	1	-	-	5	1	-	2	1	5	1
4	Somalia (15)	2	5	-	4	-	1	-	-	1	-	-	2
5	Sudan (9)	-	-	6	-	-	-	-	2	1	-	-	-

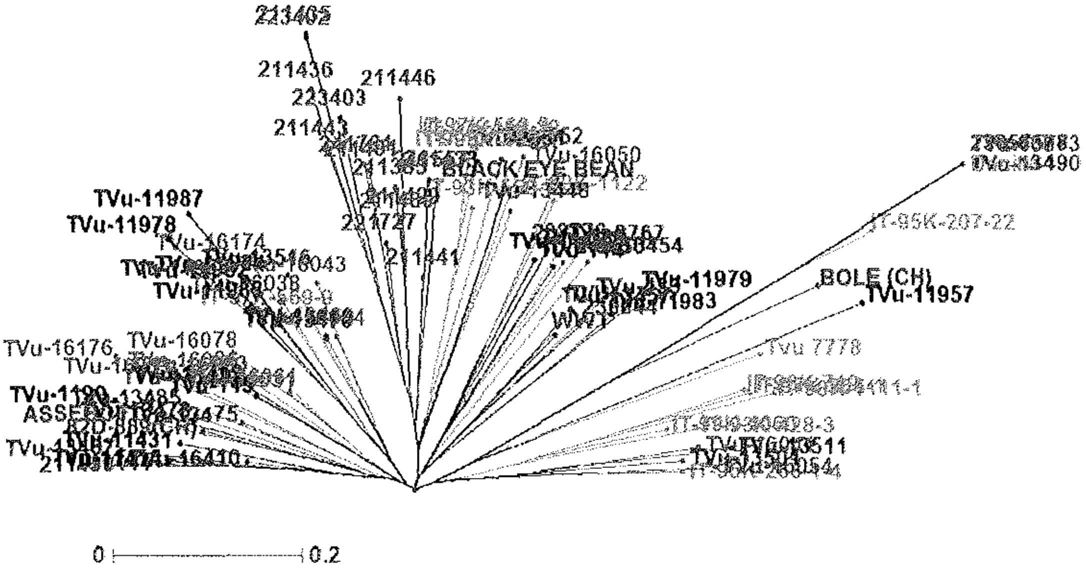

Figure 17: Dendrogram showing similarities between 95 cowpea accessions based on 13 SSR markers (Red= Ethiopia, Blue=Kenya, IITA inbred line= light green, pink= Somalia, green= Sudan)

4.3.3 Genetic differentiation between populations

The three principal coordinates together described 65.87% of the total variation for the traits evaluated (Fig. 18), in which the three principal coordinates (PC1, PC2 and PC3) explained 32.9%, 17.18% and 16.54% of the total molecular variance, respectively. The local cowpea accessions from Ethiopia, Kenya, Somalia and Sudan as well as the inbred lines from IITA, each tend to form their own isolated separate cluster group; however, there was a clear grouping of accessions from different countries together. We have observed also 5 outliers 2 inbred lines from IITA, and one accession each from Ethiopia and Somalia. Similar trend was observed as in genetic tree grouping, i.e., clustering of accessions from different countries grouped together; this showed the presence of germplasm material exchange between countries and with IITA center. The second principal component (32.9%) is effective as compared with the first principal component (17.18%) in separating each population group in to left and right side of the plot.

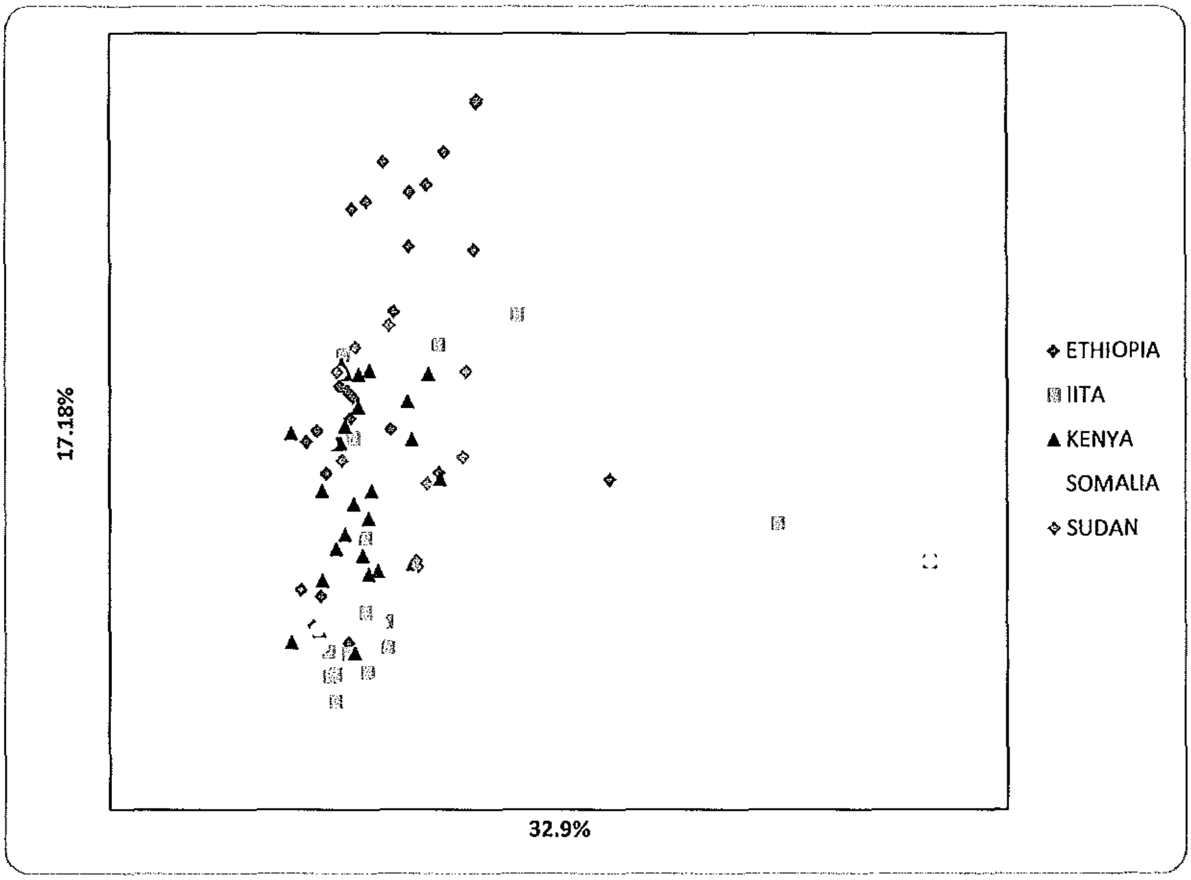

Figure 18: Principal component analysis (PCoA) based on 13 SSR markers.

4.3.4 Results of Analysis of Molecular Variance (AMOVA) for 13 SSR markers

The analysis of molecular variance (AMOVA) showed that all sources of variation were highly significant ($P < 0.001$) and approximately 10% of the overall variation was attributed to genetic differentiation among populations; 84% was explained by differences among individuals within populations and 6% was attributed to genetic differentiation within individuals (Table 26). This indicates that the accessions in this study possess wide diversity within both individuals and populations. The fixation index (F_{st}) was 0.095 which showed moderate differentiation between countries and there was higher gene flow between countries (N_m) 2.38; illustrating the existence of germplasm exchange among East African countries and with IITA. In other studies different AMOVA results were obtained when 81 Kenyan cowpea were studied; the total accessions among the geographical regions revealed 3.99% genetic variation while the variation among the accessions within geographical region suggested 52.33% variation within individual accession explained 43.67%, the fixation index (F_{st}) was low 0.04, indicating low differentiation among cultivated cowpea accessions in Kenya (Kuruma et al. 2008) and different AMOVA results were obtained for 312 Tanzanian cowpea accessions; for the geographic data, only the regions within the zones were significant, and they explained 3% of the genetic variance and had F_{st} value of 0.033, the groups found by structure explained a much higher percentage of the genetic variance (97%) (Sariah et al. 2010). Hence, the moderate differentiation results obtained in our study demonstrating the majority of variations are found within population groups rather than between population groups.

Table 26: Analysis of molecular variance (AMOVA) among populations

Source of variation	DF	Sum of squares	Var. components	Variation (%)	F statistics (Fst)
Among populations	4	89.540	0.408	10**	0.095
Among individuals within populations	90	672.002	3.589	84**	
Among individuals in all the populations	95	27.500	0.289	6**	
Total	189	789.042	4.286	-	

N_m=2.38

4.3.5 Structure analysis based on 13 SSR markers

The results of Structure analysis based on 13 SSR markers were used to infer the possible number of cluster (sub-populations) using a Bayesian clustering approach. The most probable clusters is K=3, which received the strongest support as suggested by ΔK values (K ranging from 2 to 20). (Fig 19; Table 27; Fig 20). Structure based analysis allowed the identification of three sub-groups. Eighteen of 95 (19%), 19 out of 95 (20%) and 58 out of 95 (61%) cowpea accessions were detected in the main cluster group of I,II, III respectively, showing that most of the cowpea accessions were grouped in the main cluster II. Twelve out of 29 (41.4%) Kenya, 3 out of 16 (18.7%) IITA, 2 germplasm from Somalia and one germplasm from Ethiopia and no accession from Sudan were detected in sub-groups I. Similarly, 13 out of 26 (50%) Ethiopia, 3 out of 29 (10.3%) Kenya, and one accession each from Somalia, Sudan and IITA were detected in sub-groups II. Whereas, most of the accession were identified in sub cluster group III; 14 out of 29 (48.3%), 12 accession each from Ethiopia, Somalia and IITA accessions were detected in sub-groups III; almost all of the accessions 8 out 9 (89%), were grouped in sub-cluster group III. The structure analysis based on 13 SSR markers identified three sub-population groups, which was in agreement with sub-clustering obtained by the genetic tree construction, however; the number of accession identified in each sub-group of the two different analysis were different.

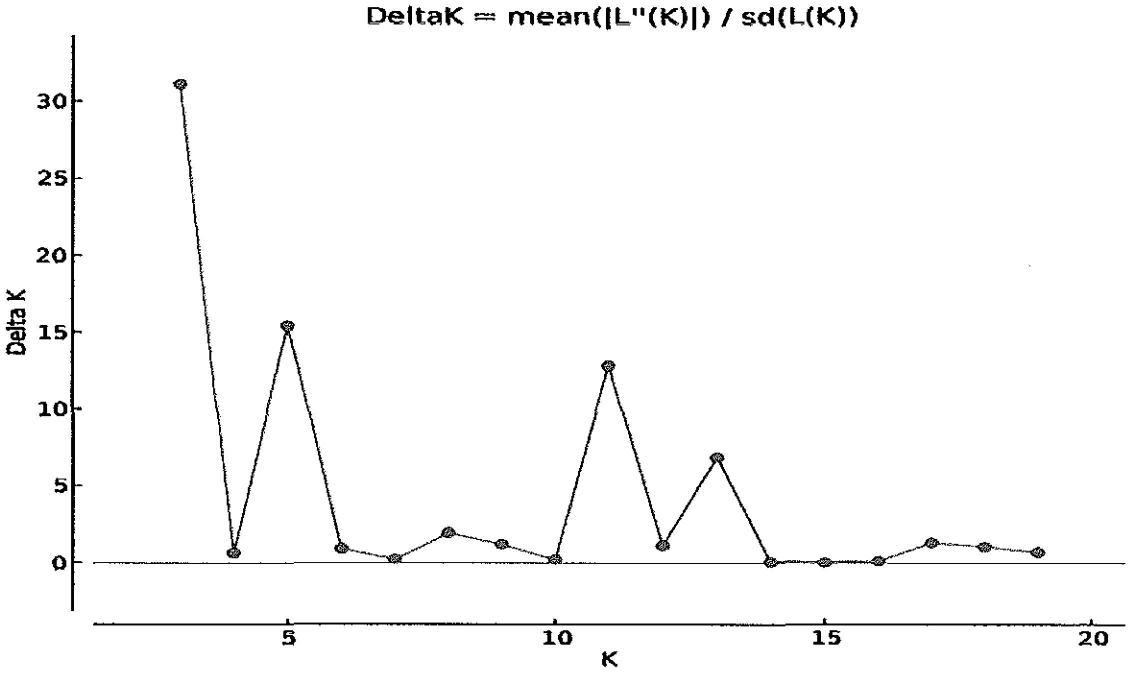

Figure 19: Estimation of the number of populations based on 13 SSR markers for K ranging from 2 to 20 by calculating ΔK

Table 27: STRUCTURE analysis based on 13 SSR markers identified three subpopulations among reference set accessions of cowpea

Sub-groups	**I (Red)**	**II (Green)**	**III (Blue)**
Individual (95)	**18**	**19**	**58**
ETHIOPIA (26)	1	13	12
KENYA (29)	12	3	14
SOMALIA (15)	2	1	12
SUDAN (9)	0	1	8
IITA (16)	3	1	12

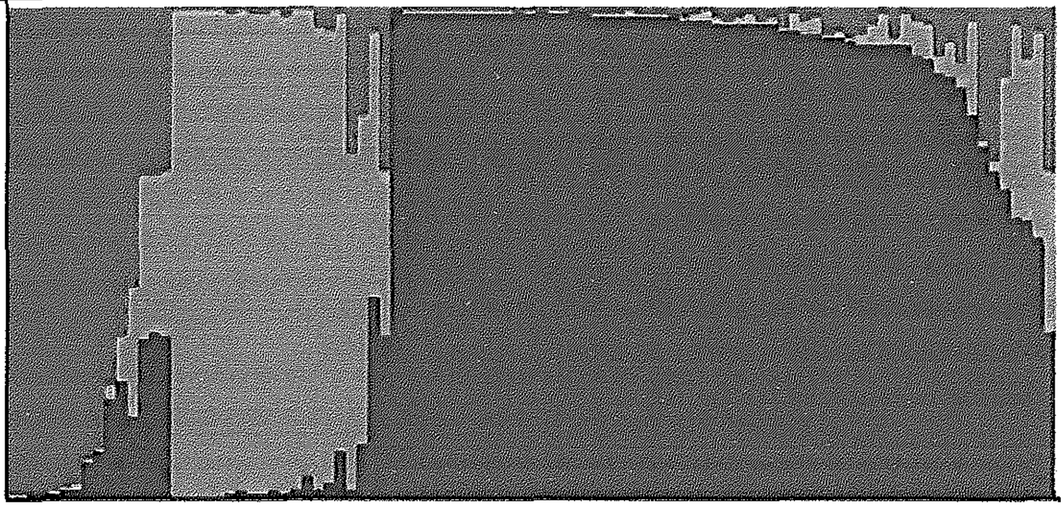

Figure 20: STRUCTURE analysis based on 13 SSR markers identified three subpopulations among reference set accessions of cowpea

4.4. Genetic diversity of East African Cowpea Genotypes Collections and IITA Inbred Lines based on SNP Markers

4.4.1 SNP polymorphism

Among the 164 SNPs markers, 151 SNPs were polymorphic. Seven SNPs primers were monomorphic, and therefore, were excluded from the analysis. In addition, 6 SNPs failed to produce a genotype call because of poor signals (Table 28), hence these were also excluded from the analysis. All possible SNP types were found in the landraces and inbred lines from cowpea collections; the majority included A/G (or T/C) followed by small but equal SNP types of G/C and T/A, and a very small numbers for C/A followed by G/T (Table 29). The polymorphic information content (PIC) representing the allele diversity for a specific locus varied from 0.02 to 0.38 with a mean of 0.27. SNP markers 10974_245, 1202_1215, 12505_1312, 14619_471, 14702_888, 16413_395, 2870_790, 4237_650, 4712_832, 5356_124, 6580_67, 7383_1042, 8121_1880, 8877_1528, and 9134_1559 have showed high PIC value 0.37. Gene diversity (D) was 0.34 on average and ranged from 0.02 to 0.5; SNP marker 6580_67 exhibited highest gene diversity (D) with 0.5 while the least was 18_107 with a value of 0.02 (Table 29). Almost all of the SNP markers have allele frequency above 0.5; SNP 122_468 detected the highest level of allele frequency 1, whereas SNP 2870_790 detected the lowest allele frequency 0.5 (Table 29).

Table 28: Polymorphism of 164 SNPs based on 95 cowpea accessions

Class	No. of Marker	Percentage (%)
Polymorphic	151	92.1%
Monomorphic	7	4.3%
No amplicons (banding)	6	3.6%
Total	164	100%

One hundred and fifty one polymorphic SNP and 13 SSR markers were used to genotype 95 cowpea accessions of local cultivars from Ethiopia, Kenya, Somalia, Sudan, and inbred lines from IITA. In the present study, the SNP loci had lower PIC values than the SSRs; we found polymorphic information content (PIC) ranging from 0.01 to 0.02 for the SNP and 0.28 to 0.86 for the SSR marker systems, with mean PIC of 0.27 and 0.51, respectively. Fatokun et al. (2008) observed PIC ranging from 0.29 to 0.87 with a mean of 0.68 among 48 wild cowpea lines using SSR markers. Study of Li et al. (2001) reported a PIC ranging from 0.02 to 0.73 with a mean of 0.47 among cultivated cowpea with a different set of SSR markers. Badiane et al. (2012) reported PIC value, which varied from 0.08 to 0.33 with a mean of 0.23 using cowpea, collected from

Senegalese national germplasm collection. Aaron et al. (2010) reported PIC, which varied from 0.07 to 0.66 with an average of 0.38 using cowpea collected from Ghanaian germplasm collection. Ogunkanmi et al. (2014) assessed 48 accessions of cultivated cowpea, and found that the PIC from West African accessions was 0.369, South African had 0.329 while North East and Central Africa with 0.332, which is another evidence confirming that West Africa contains greater diversity of cowpea. Kuruma et al. (2008) assessed the genetic diversity of 81 Kenyan cowpea accessions and reported PIC value varying from 0.09 to 0.82 with a mean of 0.34. The low level of polymorphism detected in our study is in agreement with previous studies and may be the result of a bottleneck induced by a single domestication event in this crop (Li et al. 2001; Tosti and Negri, 2002; Badiane et al., 2004; Diouf and Hilu, 2005) in addition to its inherent self-pollinated reproduction mechanism.

A total of 302 alleles were detected for SNP markers highlighting the already established number of alleles per locus, i.e., 2 alleles. Almost all of the SNP markers had allele frequencies above 0.5. SNP marker 122_468 showed the highest allele frequency of 0.98 while SNP marker 2870_790 showed the lowest allele frequency of 0.5. Similarly Asare et al. (2010) reported 4 to 13 alleles in cowpea collected from Ghana, while Sawadogo et al. (2010) reported 5 to 12 alleles in cowpea collected from Burkina Faso using cross species SSRs from Medicago. Badiane et al. (2012) reported 1 to16 alleles in cowpea collected from Senegalese national germplasm. Diouf and Hilu, (2005) reported 1 to 9 of alleles for different germplasm which ranged from 1 to 9 and Li et al. (2001) 5 reported that 27 cowpea SSR primers detected between 2 and 7 alleles among 91 cowpea breeding lines. According to Aaron et al. (2010) the number of alleles detected per primer pair varied from a minimum of 1 to a maximum of 6 with an average of 3.8 using Ghanaian germplasm. Ogunkanmi et al. (2014) reported 37 alleles with mean alleles of 3.1 when they used 48 cultivated cowpea accessions with 12 SSR markers. Kuruma et al. (2008) reported allele number ranging from 2 to 14 and with mean allele of 4.5 using 81 cowpea accessions collected from Kenya.

In this study, gene diversity (D) ranged from 0.02 to 0.49 for the 151 SNP markers, averaging 0.3. According to Delphine et al. (2010), the theoretical considerations show that the maximum gene diversity D observable with biallelic markers is 0.5, Aaron et al. (2010) reported the genetic diversity (D) which ranged from 0.12 to 0.68 with an average of 0.44 using Ghanaian germplasm. On the other hand, the genetic diversity value reported by Badiane et al. (2012) varied from 0.08 to 0.42 with an average of 0.28 using cowpea collected from Senegalese national germplasm. Although we have used SNP markers, the genetic diversity of East African cowpea accession is lower than the genetic diversity of Ghanaian cowpea and higher than the Senegalese cowpea accessions both tested using SSR markers.

Table 29: Allele frequency, Allele number, Allele type, genetic diversity and polymorphism information content (PIC) of the SNPs used in this study

Marker	Allele Frequency	Allele Number	Genetic Diversity		SNP type	PIC	Marker	Allele Frequency	Allele Number	Genetic Diversity	Allele type	PIC
1936_545	0.76	2	0.37		G/A	0.30	**2820_248**	0.90	2	0.17	T/A	0.16
1004_587	0.85	2	0.26		G/A	0.22	**2870_790**	0.50	2	0.50	C/A	0.38
10277_636	0.78	2	0.35		G/C	0.29	**2974_1109**	0.79	2	0.33	G/A	0.28
10378_737	0.61	2	0.48		G/A	0.36	**311_1536**	0.82	2	0.30	G/T	0.26
10480_616	0.65	2	0.45		G/A	0.35	**3427_925**	0.91	2	0.17	G/A	0.15
1060_220	0.83	2	0.28		C/T	0.24	**3494_143**	0.80	2	0.32	C/T	0.27
10650_1563	0.57	2	0.49		C/A	0.37	**3787_812**	0.71	2	0.41	C/T	0.33
10738_1400	0.59	2	0.48		G/A	0.37	**3803_763**	0.71	2	0.41	G/A	0.32
10974_245	0.54	2	0.50		G/A	0.37	**3838_830**	0.86	2	0.25	G/A	0.22
1107_518	0.97	2	0.05		G/A	0.05	**3900_562**	0.70	2	0.42	C/T	0.33
11558_901	0.66	2	0.45		C/T	0.35	**4131_472**	0.90	2	0.19	G/A	0.17
11585_1881	0.87	2	0.23		C/T	0.20	**4200_155**	0.60	2	0.48	C/T	0.37
11599_1036	0.95	2	0.10		T/A	0.10	**4237_650**	0.54	2	0.50	T/A	0.37
11613_1075	0.67	2	0.44		G/A	0.35	**4273_342**	0.77	2	0.36	G/A	0.29
1165_701	1.00	2	0.46		G/C	0.35	**4306_482**	0.71	2	0.41	G/A	0.33
11920_1704	0.65	2	0.27		G/A	0.23	**437_590**	0.71	2	0.41	T/A	0.33
1202_1215	0.84	2	0.50		G/A	0.37	**4692_429**	0.82	2	0.30	C/T	0.25
12029_2782	0.54	2	0.40		G/T	0.32	**4702_954**	0.85	2	0.26	C/T	0.22
12126_561	0.73	2	0.06		T/A	0.06	**4712_832**	0.54	2	0.50	C/T	0.37
122_468	0.97	2	0.03		C/A	0.03	**4749_1972**	0.60	2	0.48	C/T	0.37
12349_535	0.98	2	0.17		T/A	0.16	**4778_497**	0.70	2	0.42	C/T	0.33
12505_1312	0.90	2	0.50		G/A	0.37	**4800_500**	0.93	2	0.13	C/T	0.12
12526_795	0.52	2	0.41		C/T	0.32	**5058_372**	0.74	2	0.38	G/C	0.31
12568_234	0.72	2	0.18		G/T	0.16	**5135_477**	0.78	2	0.35	G/A	0.29
1281_790	0.90	2	0.37		C/T	0.30	**5137_1051**	0.76	2	0.36	C/T	0.30
1283_371	0.75	2	0.47		C/T	0.36	**5268_412**	0.77	2	0.36	G/A	0.29
12854_535	0.62	2	0.43		T/A	0.34	**5270_452**	0.80	2	0.32	G/T	0.27
12882_709	0.69	2	0.43		G/A	0.34	**534_355**	0.71	2	0.41	G/A	0.33
12905_686	0.69	2	0.27		C/T	0.23	**5356_124**	0.53	2	0.50	C/A	0.37
12929_463	0.84	2	0.30		G/A	0.26	**5449_242**	0.73	2	0.39	C/T	0.32
1297_783	0.82	2	0.48		T/A	0.37	**5503_54**	0.61	2	0.47	G/C	0.36
13022_1425	0.59	2	0.39		G/A	0.31	**5652_704**	0.76	2	0.36	C/T	0.30
13269_270	0.74	2	0.43		C/T	0.34	**5656_680**	0.97	2	0.06	G/T	0.06
13294_282	0.69	2	0.45		C/T	0.35	**5692_1408**	0.65	2	0.46	G/A	0.35
13386_815	0.65	2	0.19		G/A	0.18	**5735_110**	0.79	2	0.33	G/A	0.28
13586_1058	0.89	2	0.43		C/T	0.34	**5756_456**	0.73	2	0.40	T/A	0.32
13872_1420	0.69	2	0.29		G/A	0.25	**5993_278**	0.90	2	0.18	C/T	0.17
13873_544	0.82	2	0.44		G/A	0.34	**6046_661**	0.73	2	0.40	T/A	0.32
14034_820	0.68	2	0.49		C/T	0.37	**6205_632**	0.83	2	0.28	C/A	0.24

4.4.2 Clustering analysis based on SNP markers

The dendrogram derived from the combined data of 151 SNP primers delineated all the cowpea accessions into ten main clusters (Fig.21 and Table 30). We observed a clear genetic structure in the analyzed 95-cowpea accessions from East African countries and IITA inbred lines. The total number of main cluster group is different from the total number of the population; nonetheless, the geographic distance and the genetic background of the accessions were clearly reflected in the genetic group relation of dendrogram tree construction.

Out of the 95 accessions 45 (47.4%), 37 out of 95 (39%) and 13 (13.6%) cowpea accessions were detected in the main cluster group of II, I, III respectively, showing most of the cowpea accessions grouped in the main cluster I. More than half of the main cluster group I contains accessions from Kenya (25 out of 45), followed by 7 accession from Ethiopia and 3 accession each from the Sudan and Somalia but only one accession from IITA inbred lines (IT-99K-1122) . The main cluster group II contains half of the accession (19 out of 37) from Ethiopia, almost all of the accession (15 out of 16) from IITA inbred lines, only 3 accessions (Tv-265, Tv-13448 and Tv-552) were from Kenya; however, Somalia and the Sudan were not represented in this group. The main cluster group III contains almost all of its accession from Somalia 12 out of 13 (92%), and only one accession (Tvu-13511) from Kenya.

All of the Ethiopian local accessions formed two sub-groups, which tightly clustered together. Sub-groups II is the largest, containing 21 out of 26 (77%) accession, which tightly grouped in the main cluster group I. Sub-groups I consists of 6 out of 26 (23%) accessions, which formed 3 tightly clustered sub groupings in the main cluster group I.

All of the Kenyan local accessions formed one bigger sub-groups which closely clustered together consisting most of the accessions, 25 out of 29 (86%), grouped in main cluster I, while four accessions (Tvu-552, Tvu-13448, Tvu-265 and Tvu-13511) were loosely clustered in main cluster II and I.

Similarly, all of the inbred lines from IITA formed one sub-groups which tightly clustered together containing of all inbred lines in main group II; except one outliers inbred lines IT-99K-1122 which is isolated and grouped in the main cluster group I.

Somali local accession formed two sub-groups, I and II. sub-groups II the bigger which tightly clustered together and grouped in main cluster group III containing accessions 11 out of 15 (74%), while sub-groups II consists of Tvu-16053, Tvu-16054, Tvu-16176 and Tvu-16410 which loosely clustered together in main cluster group I.

All of the Sudan local accessions formed one sub-groups, which tightly clustered together in main cluster group I consisting of 8 out of 9 (89%) accessions, except for one accession (Tvu-11957) which is isolated in the main cluster group II.

The six released cowpea varieties from Ethiopia were distributed and clustered in the main clusters II and I. The released varieties, Bole (CH), Black eye bean, Asebot and 82D-889(CH) closely clustered with the local Ethiopian accession of the biggest sub-groups II. Similarly, the released varieties WWT and Tvu-1977 tightly clustered together to each other but loosely clustered with four local Ethiopian accessions (211557, 211490, 211444, and 211441) of sub-groups I.

Both the drought resistant (Danilla) and drought susceptible (Tvu-7778) are closely clustered in the main cluster II. The local accession, Tvu-13448 from Kenya, was tightly clustered and shared a common allele with Danilla and Tvu-7778, indicating that they might have an underlying genetic relationship.

All of the local accessions from each country and the IITA inbred lines were distributed in all of the three main cluster groups i.e accessions from different region grouped together. Although the number of cluster wasn't equal with the number of population groups; the clustering exercise identified a substantial degree of association between provenance and genotypes. All of the accessions within the three cluster groups tend to tightly clustered together with each other, except few accessions; similarly the inbred lines from IITA tend to form their own isolated cluster group when analyzed using 151 SNP markers. Therefore, within the cluster group the neighbor-joining tree construction clearly showed the geographical distance of the countries and the genetic background of our cowpea material, which was clearly showed in the grouping of IITA inbred lines. The present result clearly showed the presence of germplasm exchange between East African countries and the IITA center. The released varieties Bole (CH), Black eye bean, Asebot and 82D-889(CH) are closely clustered and shared a common allele with local accession collected from all of the collection areas; Wolega, East and West Hararage, Borena, Gamogofa, Benchmaji, Debub Omo, Semen Shoa, Debubawi Enderta and Bali, hence the result showed the existence of high genetic relation between the aforementioned released and the local cowpea accession of Ethiopia. However, the released varieties WWT and TUV-1977 are tightly clustered to each other but distantly clustered with the local Ethiopian accessions, therefore, these varieties might have less phylogenetic relation. Tvu-7778 is one of the inbred lines released by IITA for its high susceptibility to drought stress; based on our SNP genetic result Tvu-7778 was tightly clustered and shared a common allele with the local cowpea accessions Tvu-13448 from Kenya; hence, this local accession should be further checked for its drought susceptible characteristics using well characterized marker which is associated with drought tolerance.

Table 30: Phylogenetic analysis based on SNPs data identified three subpopulations among reference set accessions of cowpea

No	**Sub-groups**	**I**			**II**				**III**	
	Main group	45			37				13	
	Sub group	**I (37)**		**II**	**I (33)**		**II (4)**		**I (8)**	**II**
	Sub-sub-group	**I (35)**	**II**	-	**I**	**II**	**I (4)**		-	-
1	Ethiopia (26)	7	-	-	15	2	2		-	-
2	Kenya (29)	23	-	2	2	-	1		1	-
3	IITA inbred lines (16)	1	-	-	2	12	1		-	-
4	Somalia (15)	1	2	-	-	-	-		7	5
5	Sudan (9)	3	-	6	-	-	-		-	-

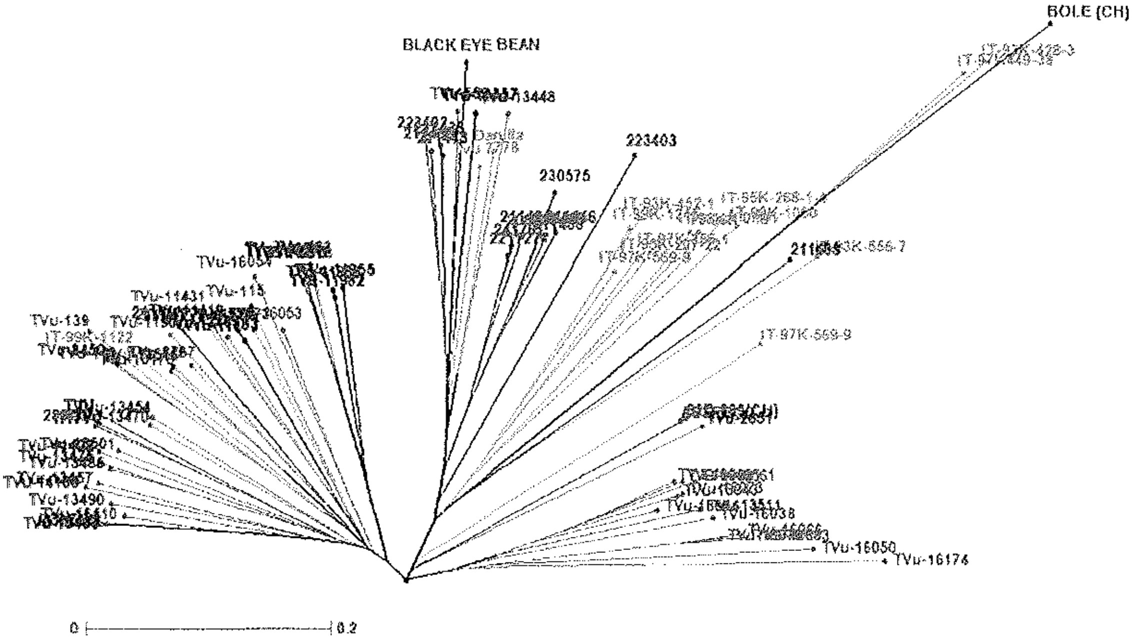

Figure 21: Dendrogram showing similarities between 95 cowpea accessions based on 151 SNP markers (Red= Ethiopia, Blue=Kenya, IITA inbred line= light green, pink= Somalia, green= Sudan)

4.4.3 Genetic Differentiation between populations groups based on SNP marker

The three principal coordinates together described 68.42% of the total variation for the traits evaluated (Fig. 22), in which the three principal coordinates (PC1, PC2 and PC3) explained 32.9%, 20.38% and 15.14% of the total molecular variance, respectively. The local cowpea accessions from Ethiopia, Kenya, Somalia and Sudan as well as inbred lines from IITA each tend to form its own isolated separate cluster group; however, there was a clear grouping of accessions from different country together. We have also observed 3 outliers, 2 inbred lines from IITA and one accession from Ethiopia. Similar trend was observed as in genetic grouping, i.e., clustering together of accessions from different countries grouped together; this showed the presence of germplasm material exchange between East African countries and with IITA center. The second principal component (32.9%) is effective as compared to the second PCoA (20.38%) in separating each population group in to left and right side of the plot.

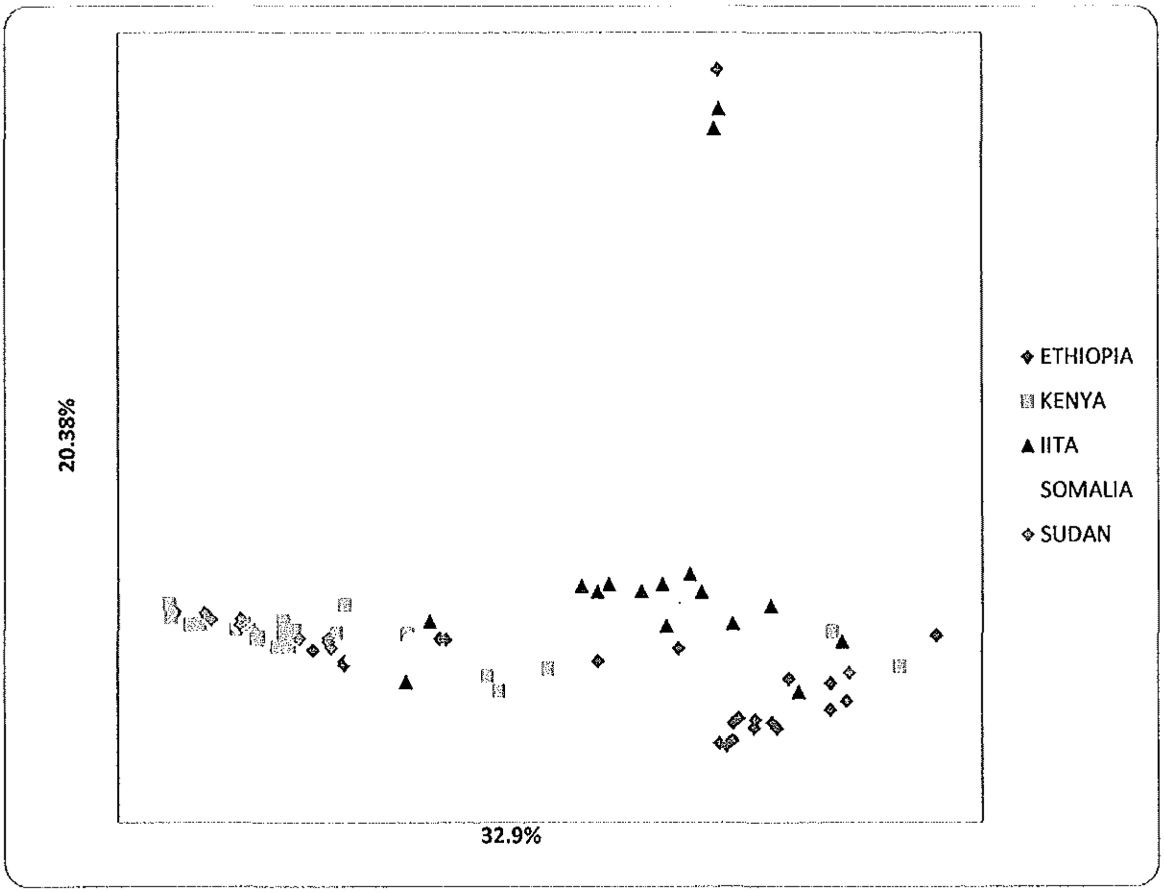

Figure 22: Principal component analysis (PCoA) based on 151 SNP markers.

4.4.4 Analysis of Molecular Variance (AMOVA) SNP markers

The Analysis of molecular variance (AMOVA) showed that all sources of variation were highly significant ($P < 0.001$) and approximately 17% of the overall variation was attributed to genetic differentiation among populations; 77% was explained by differences among individuals within populations and 7% was attributed to genetic differentiation within individuals (Table 31). This indicates that the accessions in this study possess wide diversity both within and among populations. The fixation index (F_{st}) was 0.15, which showed moderate differentiation and moderate gene flow (N_m) =1.2 between East African countries and with IITA. In other studies different AMOVA results were obtained when 81 Kenyan cowpea were studied; the total accessions among the geographical regions revealed 3.99% genetic variation while the variation among the accessions within geographical region suggested 52.33% variation within individual accession explained 43.67%, the fixation index (F_{st}) was low 0.04, indicating low differentiation among cultivated cowpea accessions in Kenya (Kuruma et al. 2008) and different AMOVA results were obtained for 312 Tanzanian cowpea accessions; for the geographic data, only the regions within the zones were significant, and they explained 3% of the genetic variance and had F_{st} value of 0.033, the groups found by structure explained a much higher percentage of the genetic variance (97%) (Sariah et al., 2010). According to Wright (1978), IPGRI and Cornell University (2003) and Kiambi et al. (2005), F_{st} in a range of 0–0.05 indicates little differentiation, 0.05–0.15 moderate differentiation, 0.15-0.25 large differentiation and above 0.25 indicates a very large differentiation. The result we have obtained was in agreement with the genetic tree construction, where accessions from different regions clustered together rather than isolating and forming their own cluster groups demonstrating that there was germplasm exchange between East African countries and with IITA CGIR center, demonstrating the majority of variations are found within population groups rather than between population groups.

Table 31: Analysis of Molecular Variance for East African cowpea accessions and IITA Inbred lines

Source of variation	DF	Sum of squares	Var component	Variation (%)	**F statistics (F_{st})**
Among populations	4	949.6	5.17	17***	0.15
Among individuals within populations	90	4350.1	23.31	77***	
Within individuals	95	163.0	1.72	6***	
Total	189	5462.7	30.2	-	

N_m=1.2

4.4.5 Structure analysis based on SNP markers

The results of Structure analysis based on 151 SNP markers were used to infer the possible number of cluster (subpopulations) using a Bayesian clustering approach. The most probable clusters is K=4, which received the strongest support as suggested by ΔK values (K ranging from 2 to 20) (Fig 23, Table 32 and Fig 24). Structure based analysis allowed the identification of four sub-groups. 9 out of 95 (9.5%), 39 out of 95 (41%), 12 out 95 (12.6%) and 34 out of 95 (35.8%) accessions were detected in the main cluster group of I, II , III and IV, respectively, showing most of the cowpea accessions are clustered in the main cluster II. 6 out of 9 (66.7%) accessions from Sudan, 2 germplasm from Kenya and one germplasm from IITA were detected in sub-groups I, while no accession from Ethiopia and Somalia is found in this cluster. 23 out of 29 (79.3%) germplasm from Kenya, 6 from IITA, 5 from Ethiopia, 3 from Somalia and 2 from Sudan were detected in sub-groups II. The main cluster group III contains, 12 out of 15 (80%) accessions from Somalia and 21 out 26 (80.76%) from Ethiopia, 9 out of 11 (81.8%), 4 from Kenya, and only one germplasm from Sudan and no germplasm from Somalia were detected in sub-groups IV. The structure analysis based on 151 SNP markers identified four sub-population groups, unlike three sub-clustering obtained by the genetic tree construction. Besides, the number of accessions identified in each sub-groups of the two different analyses was different.

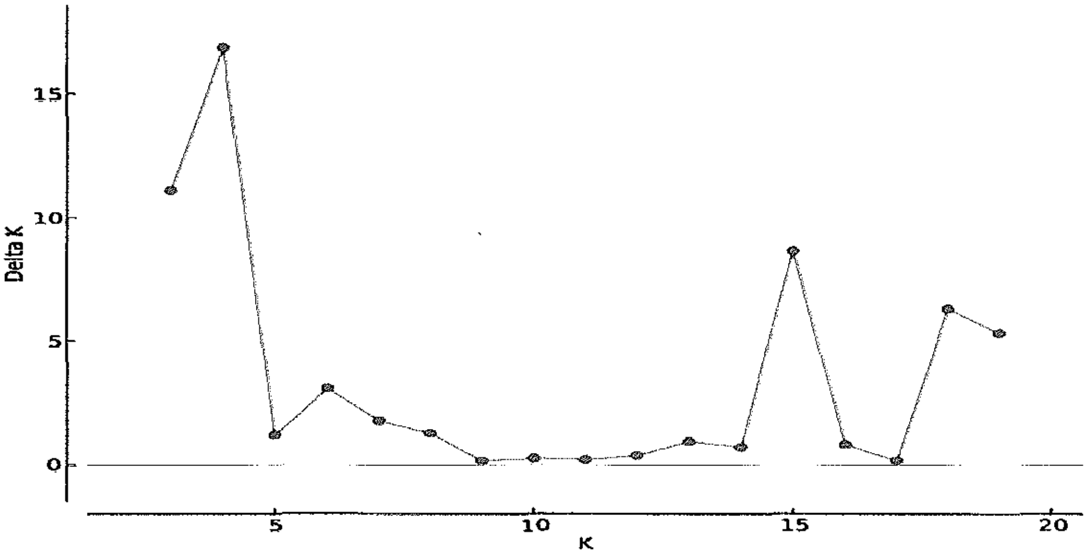

Figure 23: Estimation of the number of populations based on 151 SNP markers for K ranging from 2 to 20 by calculating ΔK

Table 32: STRUCTURE analysis based on 151 SNP markers identified four subpopulations among reference set accessions of cowpea

Sub-groups	**I (Red)**	**II (Green)**	**III (Blue)**	**IV (Yellow)**
Individual (95)	**9**	**39**	**12**	**34**
ETHIOPIA (26)	0	5	0	21
KENYA (29)	2	23	0	4
SOMALIA (15)	0	3	12	0
SUDAN (9)	6	2	0	1
IITA (16)	1	6	0	9

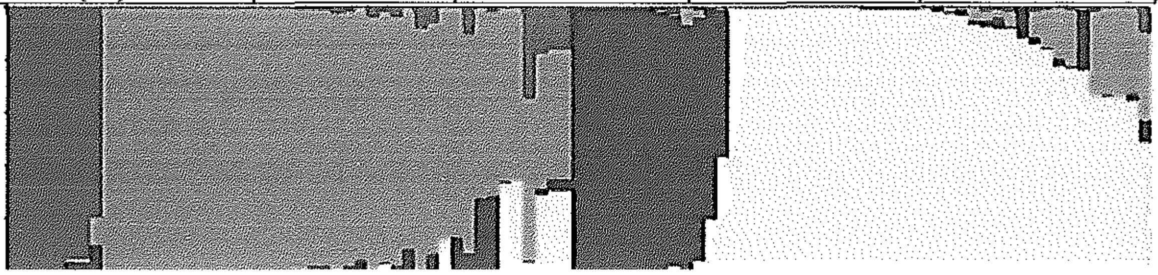

Figure 24: STRUCTURE analysis based on 151 SNP markers identified four subpopulations among reference set accessions of cowpea

4. 5 Comparisons of SSR and SNP markers

The number of alleles per locus ranged from 3 to 15 for SSR markers and the number of allele registered for SNP was 2 across all cowpea samples; the average number of alleles per locus was 6.3 for the SSRs and 2 for the SNPs. Considering each accessions group separately, the average number of SSRs alleles per locus was 3.8 for the IITA inbreds; 3.5 for Sudan, 3 for Somalia; 3.7 for Kenya; 4 for Ethiopia and similarly; the average number of SNP allele per locus was 1.86, 1.65, 1.69, 1.9 and 1.88 for the inbred lines, Sudanese, Somali, Kenyan and Ethiopian accessions respectively (Table 33 and Fig 25). The average number of alleles per SSR locus was twice higher than that for the SNPs; this is due to the fact that the SNPs are usually biallelic (Vignal et al., 2002), whereas SSRs are multi-allelic markers. This multi-allelism has established SSRs as the effective marker platform in the current crop diversity studies (Gupta and Varshney, 2000).

The total gene diversity (D) was 0.56 for the SSRs and 0.34 for the SNPs. D estimates of each population ranged from 0.44 (Kenya) to 0.54 (Sudan) for the SSRs and from 0.22 (Sudan) to 0.29 (Ethiopia) for the SNPs. The genetic diversity values were higher in samples from Sudan (D=0.54) than in samples from IITA (D=0.51), Somalia (D=0.48) and Ethiopia (D=0.47) a little bit lower D values were obtained for Kenya (D=0.44) when screened with SSR markers. Different and lower D values were observed when screened with SNP markers; higher D values for Ethiopia (D=0.29), IITA inbred lines (D=0.28), Kenya (D=0.28) but little bit lower D values were observed for Somalia (0.23) and the Sudan (0.22). The PIC value for SSR was 0.51 and 0.27 for SNP. The PIC values for each population group ranged from 0.39 (Kenya) to 0.48 (Sudan) for the SSRs and from 0.18 (Sudan and Somalia) to 0.23 (inbred lines) for the SNPs; in each of the population the PIC value registered for SNP markers were half of SSR markers. The genetic diversity values were slightly higher in samples from IITA inbred lines (D=0.51) than in samples from Ethiopia (D=0.47), Kenya (D=0.44) and the Sudan (D=0.54); a little bit lower D values were obtained for Somalia (0.48) when screened with SSR markers. Similar trend but lower D value was observed when screened with SNP markers; a higher D value Ethiopia (D=0.29), IITA inbred lines (D=0.28), Kenya (D=0.28) but little bit lower D value was observed for Somalia (0.23) and Sudan (0.22). The high gene diversity D values of SSR markers were supported by the results of large number of alleles per locus observed in this study. Despite the difference in D estimates calculated for SSRs and SNPs, we observed for both markers the same trend when gene diversity D was studied across the population groups. According to Delphine et al. (2010) the theoretical considerations show that the maximum gene diversity D observable with biallelic markers is 0.5, whereas for multi-allelic markers such as SSRs the maximum can approach 1. Another factor, which contributes to the observed difference in the D estimates of SSRs and SNPs, is the selection history of the two marker types. The SSRs were selected over years with respect to their PIC value in various sets of cowpea accessions, whereas the SNPs have not undergone such a selection procedure. Thus, this property of SNPs explains together with the definition of gene diversity D that, D values found for SNPs are lower than those for SSRs (Jones et al., 2007). Hence, the two theoretical considerations would be more applicable for cowpea diversity studies as compared to other crops. Cowpea being ''an orphan crop'' has not been a subject of study using the new sequencing technologies (Delmer, 2005). Therefore, it is expected that in the future the D estimates of the SNPs increase towards the above mentioned theoretical maximum of 0.5 (Delphine et al., 2010). For the time being due to the above mentioned reasons, SSR markers reported to be the most frequently used marker than SNP markers in cowpea diversity study (Huaqiang et al., 2012).

The overall fixation index (F_{st}) was 0.09 and 0.15 for the SSR and SNP markers, respectively (Table 26 and Table 33). In both cases, there were moderate differentiation and high gene flow between population groups. In both cases there was high among individuals within population variation. In PCoA based on Nei genetic distance estimates of all 95 cowpea genotypes, the first and second principal coordinates (PC) explained 32.9 and 20.38 % of the molecular variance for SNPs and 32.9 and 17.18%, respectively, of the molecular variance for SSRs (Fig. 18 and Fig. 22). In both cases the local cowpea accessions from Ethiopia, Kenya, Somalia and Sudan cowpea accessions as well as inbred lines from IITA each tend to form their own isolated separate cluster group; however, there was a clear grouping of accessions from different coutry together. The result showed the presence of germplasm material exchange between East African countries and with the IITA center.

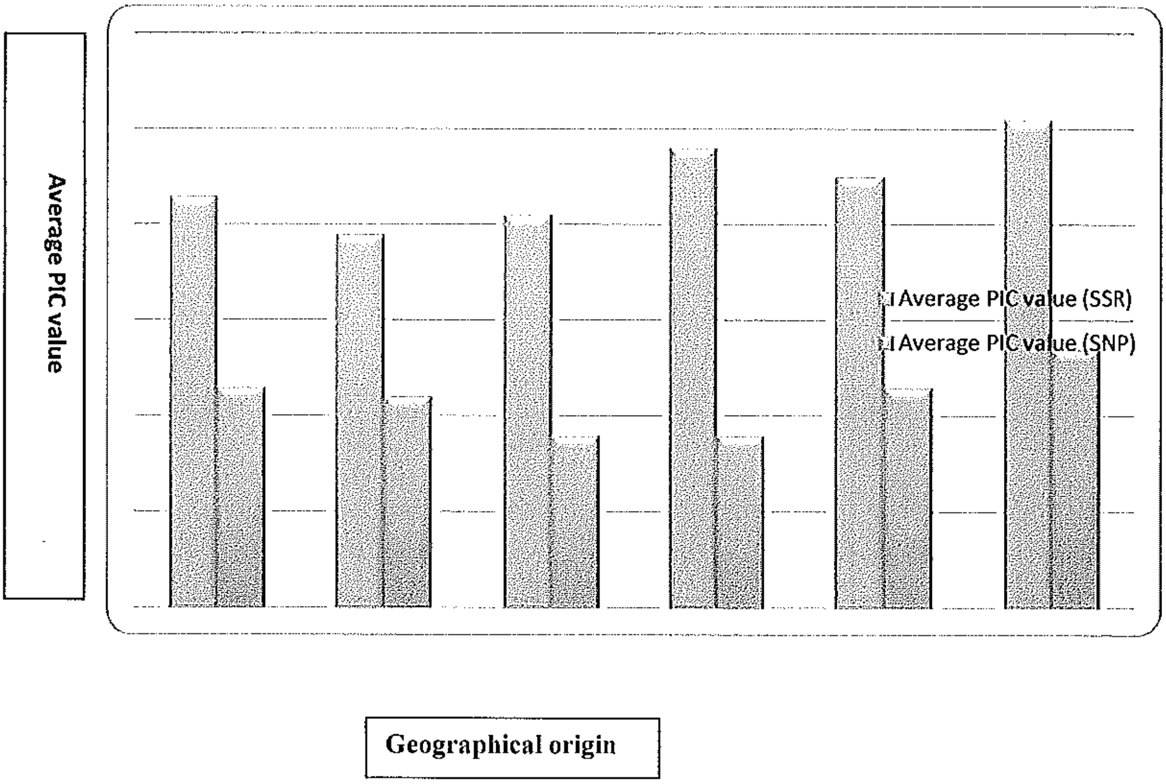

Figure 25: SSR and SNP-based PIC values for accessions originating from Ethiopia, Kenya, Somalia, Sudan, inbred lines and all population

Table 33: Average and range of the number (N_b) of alleles per locus, gene diversity (D), and Fst for 95 cowpea accessions belonging to five population groups

	Population group					
	Ethiopia (n=26)	Kenya (n=29)	Somalia (n=15)	Sudan (n=9)	Inbred line (n=16)	All (n=95)
	SSR (n=12)	**SSR (n=13)**	**SSR (n=10)**	**SSR (n=11)**	**SSR (n=13)**	**SSR (n=13)**
Mean Nb	4	3.7	3	3.5	3.8	6.3
Range	2 to 10	2 to 9	2 to 6	2 to 6	2 to 9	3 to 15
D	0.48	0.47	0.48	0.54	0.51	0.56
PIC	0.44	0.43	0.41	0.48	0.45	0.51
Fst	0.055	0.012	0.036	0.01	0.28	0.095
SNP (n=151)	**SNP (n=151)**	**SNP (n=151)**	**SNP (n=151)**	**SNP (n=151)**	**SNP (n=151)**	**SNP (n=151)**
Mean Nb	1.88	1.905	1.69	1.65	1.86	2
Range	2	1 to 2	1 to 2	0 to 2	1 to 2	2
D	0.29	0.28	0.23	0.22	0.28	0.34
PIC	0.23	0.22	0.18	0.18	0.23	0.27
Fst	0.037	0.067	0.059	0.2	0.039	0.15

5. Conclusions and recommendation

Cowpea is an important food grain legume in tropical Africa. Although the crop is not widely grown in Ethiopia, it is an important food legume for many people in some parts of Southern Ethiopia and pocket areas across the country. Besides, the crop can be promoted for cultivation and consumption as a cheap source of protein particularly, in drought prone areas. There is insufficient study on the diversity and characterization of cowpea germplasm in Ethiopia. One of the objectives of the present study was, therefore, to assess the extent of genetic diversity and relationships among 210 cowpea landraces conserved and cultivated by the local communities in various regions of Ethiopia, possessing different soil and climatic patterns, using SSR markers. This study revealed for the first time, how Ethiopian cowpea accessions can be classified based on 23 informative SSR markers. The cowpea accessions exhibited a clear genetic structure that reflected their narrow genetic base. Although the collection area covered was vast, (>1 million km^2), the genetic structural grouping did not reflect the geographic distribution of the accessions. Hence, future sampling for conservation purposes will not benefit from a strategy of widespread and evenly distributed collection site. There was little evidence to suggest that clustering of any sort is related to the reported phenotypic characteristics, such as seed color or seed size. The sub-groups identified by genetic relationship tree were in good accordance with the clusters revealed by the structure analysis, and it was in agreement with moderate fixation index value showing moderate differentiation between regions and high gene flow among regions. This demonstrates the presencs of germplasm exchange among regions. Therefore, clustering methods are equally appropriate for the assignment of genotypes to subgroups. Based on the morphological observation and molecular analysis, the total number of cowpea collection reported at IBC, Ethiopia should be modified to 210 instead of 83 to avoid redundancy of accessions. The PIC value of Ethiopian cowpea accessions was higher than that of the West African countries such as Ghana and Senegal and the East African country, Kenya, but was considerably lower than the PIC of the wild cowpea types. Thus, the results obtained in the present study reinforce the need to protect and conserve valuable genetic resources. The genetic diversity of Ethiopian cowpea should also be increased by introducing accessions with desirable alleles. The present study also identified promising five Ethiopian cowpea accessions from Amhara, Gambella, and SNNP, regions, which tightly clustered and shared a common allele with the multi-race striga resistant accession B301 of Botswana. This finding is intriguing since these Ethiopian landraces are genetically distant from B301, suggesting that the allele the present study has identified in the Ethiopian materials is either a novel allele of the resistance gene or that the allele was introgressed a long time ago and the determination of the direction of introgession to be investigated in the future. This could help in identifying additional parental lines for *Striga gesnerioides* resistance in Ethiopian cowpea germplasm.

In the other component of the PhD study, the genetic relationship of East African Cowpea collections and inbred lines of IITA were analyzed based on SSR and SNP Markers. This study demonstrated that SSR and SNP markers are very useful markers to classify accessions according to their geographic origin and genetic background, and provided meaningful genetic diversity pattern. It may be recommended that one should consider both SSR and SNP markers in future cowpea genetic diversity studies. Accessions collected from Sudan showed higher PIC value, while those collected from Somalia showed lower PIC value when screened with SSR markers. On the other hand when screened with SNP markers, the accessions from Sudan displayed lower PIC value, and the accessions from Ethiopia showed higher PIC value. In most of the population groups, the PIC values observed based on the SNP markers were lower than the PIC values calculated based on the SSR markers by about half. A larger number of markers should be employed in order to increase the genetic information obtained when using SNP markers than when using SSR marker. High throughput next generation sequencing technologies based genotyping approach such as genotyping by sequencing (GBS) (Elshire et al., 2011) can be used to generate large number of SNP markers across the cowpea genome.

In both SSR and SNP analysis, the clustering identified a substantial degree of association between region of collection and genotype. However, the geographic grouping was not reflected in the genetic structural grouping; i.e. accessions originating from different countries clustered together, showing the existence of germplasm exchange among East African countries and with IITA. The geographic distance and the genetic background of the accessions were clearly reflected in the grouping of within cluster of phylogeny tree construction and principal component analysis, when analyzed using both SSR and SNP markers. The phylogeny analysis of both SSR and SNP markers consistently showed that most of the accessions from each country tend to cluster together and shared common alleles with each other. This result was further supported by the moderate fixation index (F_{st}) demonstrating moderate differentiation and high gene flow between regions. The sub-groups identified by the phylogeny tree were in good accordance with the clusters revealed by the PCoA indicating that the assignment of genotypes to subgroups using both clustering methods are equally appropriate.

Based on the SSR and SNP analysis the released Ethiopian varieties Bole (CH), Black eye bean, Asebot and 82D-889(CH) closely clustered with the Ethiopian local cowpea accessions, indicating that they share common alleles.

The five East African cowpea accessions (Tvu-13490 and Tvu-6378 (Kenya), 2305675 (Ethiopia) and Tvu-160073 (Somalia)), tightly clustered and shared common alleles with the drought tolerant check variety, Danilla. Another accession fom Somalia (Tvu-11957), tightly clustered and shared common alleles with the drought susceptible check variety, Tvu-7778. These local accessions should be further assessed for their drought tolerance characteristics using well-characterized markers that are known to be associated with drought tolerance.

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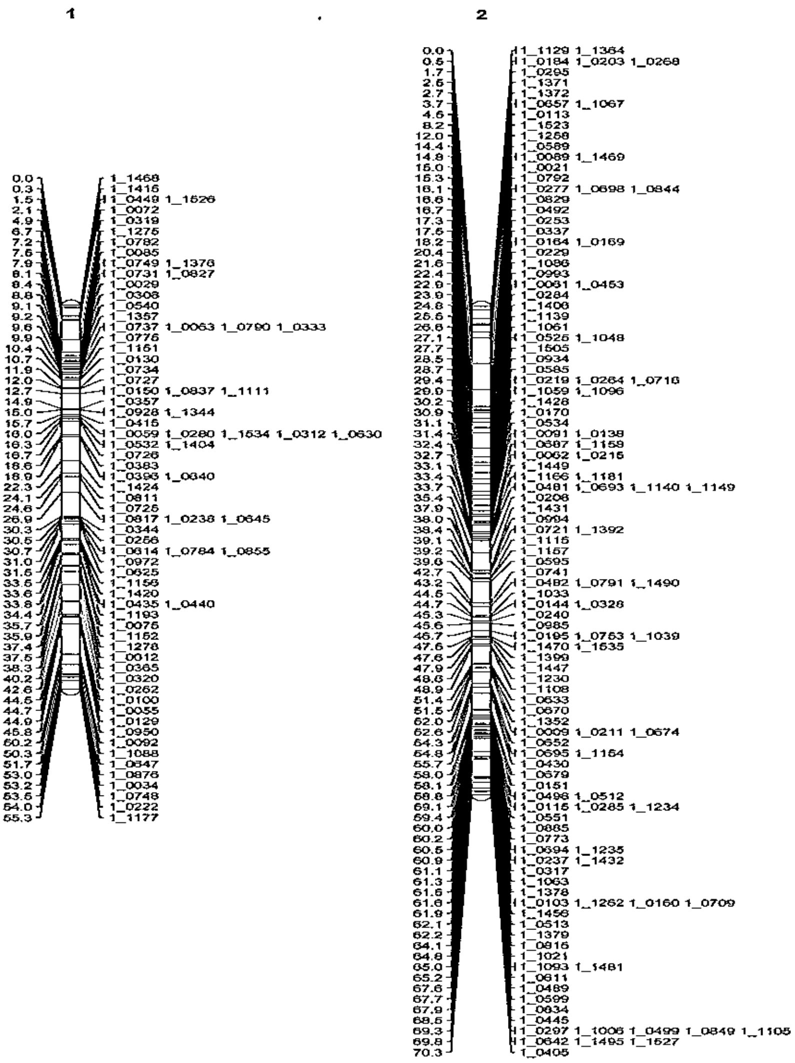
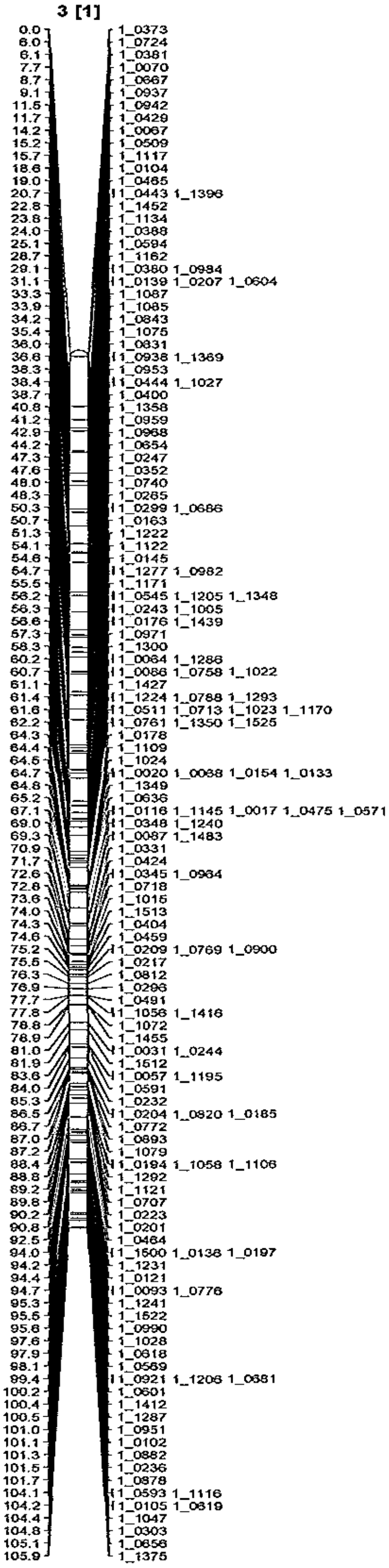
7. Appendices

Appendix 1: A linkage map of 1122 KASPar SNPs on cowpea genome

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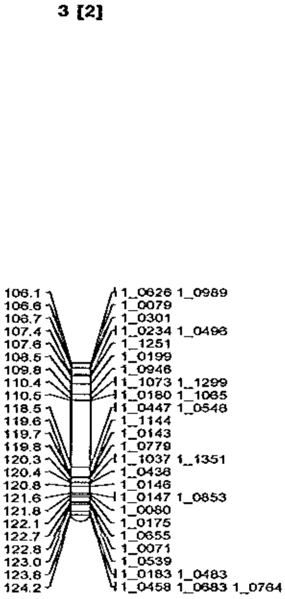
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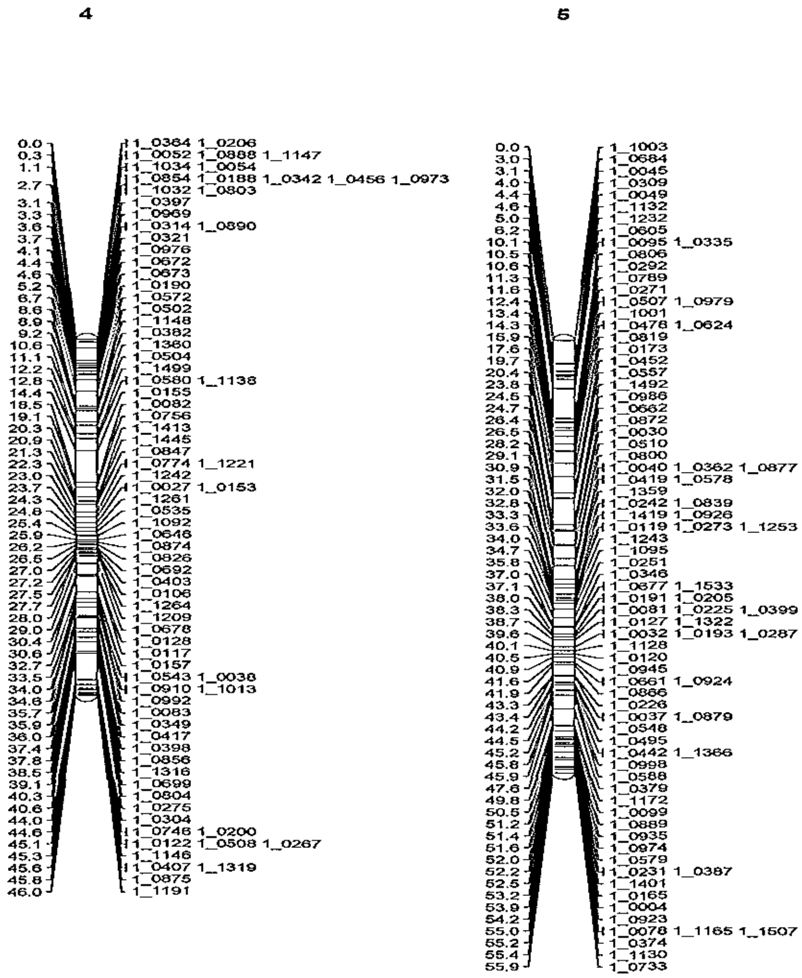
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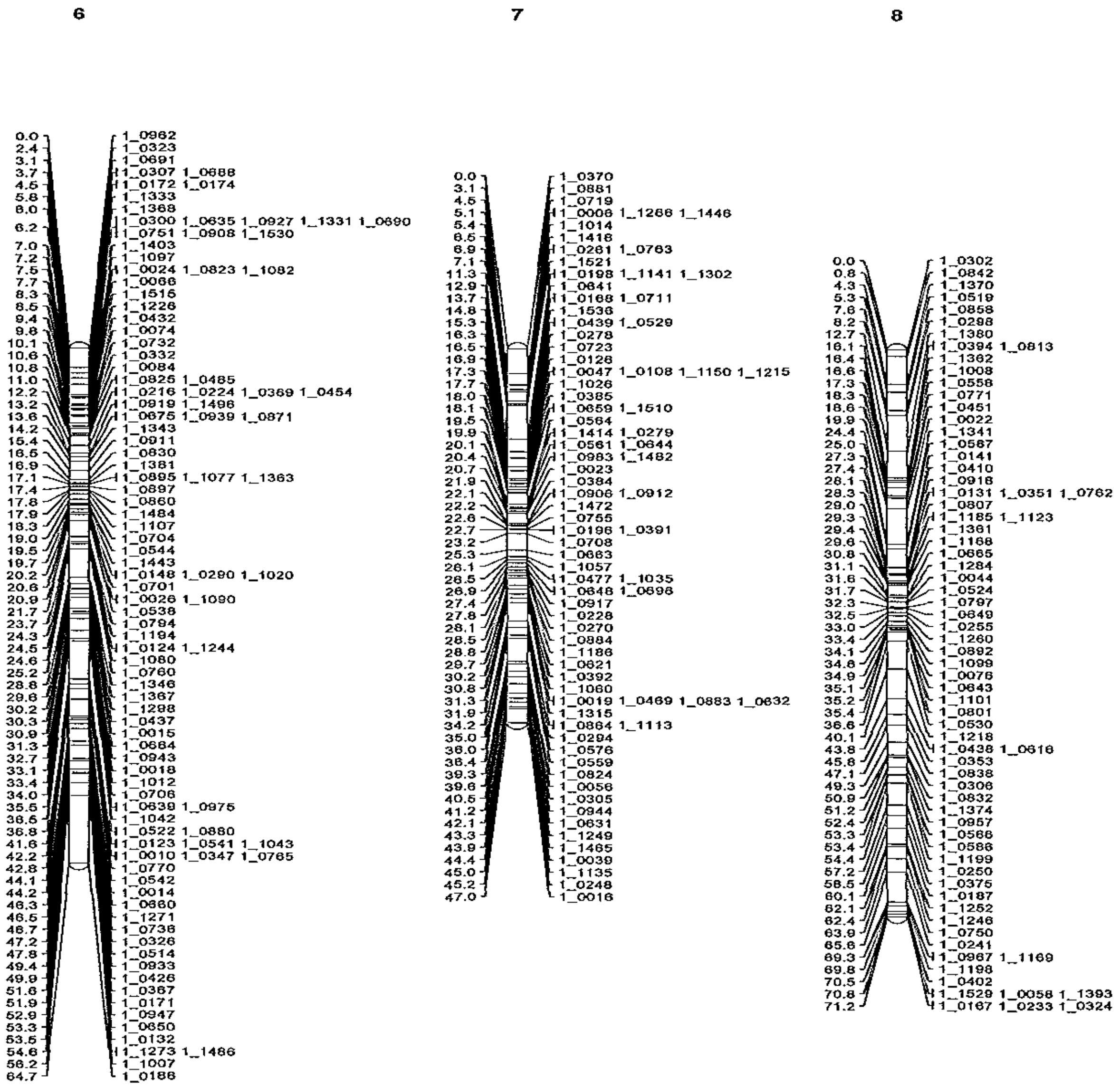
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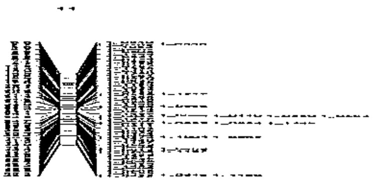
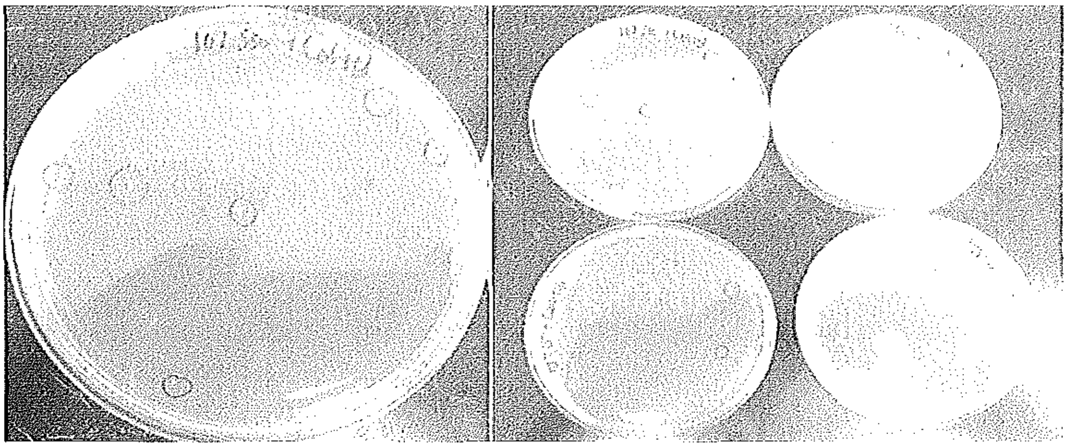


Appendix 2: Ecoli +insert DNA+Kanamicin+LBmedia (accession 2228902 and 2862))



Appendix 3: The nucleotide sequence of (accession 2228902 and 2862) (before editing)

A)The nucleotide sequence of (accession 2228902) (before editing)

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B) The nucleotide sequence of (accession 2862) (before editing)

Sequence File : 194-150H-T7.seq (accession 2862)

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CCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC
AGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCT
GTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCG
TGGNGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCG
CTCCNAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCNCCT
TATCCGGTAACTATCGTNNNAGTCCNACCCNGNANANNCGACTTANNNCCNCTG
GNAGCAGCNACTGGNAACAGNANTANCAGANNNNANGNNNNNNNNNNGNGCT
ANNNNNTNCTGNNNNNNNNCNNACNNCNGCTACNCTANAANANNGNATNNNN
NCTGNNNNNNNNNNNNANNCNGNTNNNNNNNNNAANNANNNNNNANNNTNNN
NNNNNANNNNNCNCNNNNNNNGNNGNNNTNNNGNNNNNGNN

C. The nucleotide sequence (after editing)

The nucleotide sequence of both of the Ethiopian accessions of the 150bp fragment after editing is as followes:

CAAGAAGGAGGCGAAGACTGGGAAAAAGTTGCTCACATTCGAAACCTATATATAT
GTTCTAGAAAGAATCTATATAAGATAGAGTTATTGAAATAGAGTTATTGAAATGTAA
AGAAACTTCTAAAGTATTGGAGTTGGAGAAAAGCTTAGGA

The protein sequence (before editing)

1. The protein sequence (before editing)

>filtered protein sequence consisting of 1186 residues.

maaemvtgvlvstflgrtidtlasrlfdifhqkkhkkqlrnlkmkllaidvvafdaeqkq

ftdscvrdwllrakdvvidaedlldeldyelsksqveaesqsaakkvwsslnssfliene

iesrmaqviedlddladesnilglkkgggvevgsgssskltysslpnesdicgrdddkes

ifkwltsdthnnlsilsivgmgglgktslaqhvfndprlegkfdinvwvsvpqefdvlkv

sraildtiasstdhsipkeviqkrlkenlmgkkfllvlddvwnensskwedvqkplvfgg

qgsrilvtargekvadsmrsekyrlevlkedycwelfakhafqganpqqdpdfveiakki

vkkcdglplalktmgsllhnksflwewenimrsdiwdlsenesgilpalklsylhlpshl

kkcfafcallpkgyqfdkdiliqwwmaqnfleshvqikspievgeqyfndllswsffqqs

snqdeerfimhdllndlakyvckdvcirigvdepegiskttrhcsflsselyfdgfgssi

dtqklhtftrtdpnsgwiwyckmsiddlfsrfklirilslnhylnltevpesignlkhlr

sldlsctnieelpdsmsllyklqilklnkcrrlkqfpsclhklqnlrclelvgigvenva

ahlgklknvqvsmssfhvekskemniqrlgqfnlygsltiddlqnienpsdaleadlksk phlmglhlewnfigsssvdstkaediienlrpskylkklsirnyigkqfpnwllhnslpn

lvslvlddcrscerlpplgllpflkelriyrldgivsidadfhgnnsssfkslqtlwfsd

mrqwekwdcqdvtgafprledfwikncpklkaylpkfvalkylyvsnceqlealivsaie

lllqdcgklqldcstmekltmdghdmaassvamvghmlfntsledlsicstletisddcv

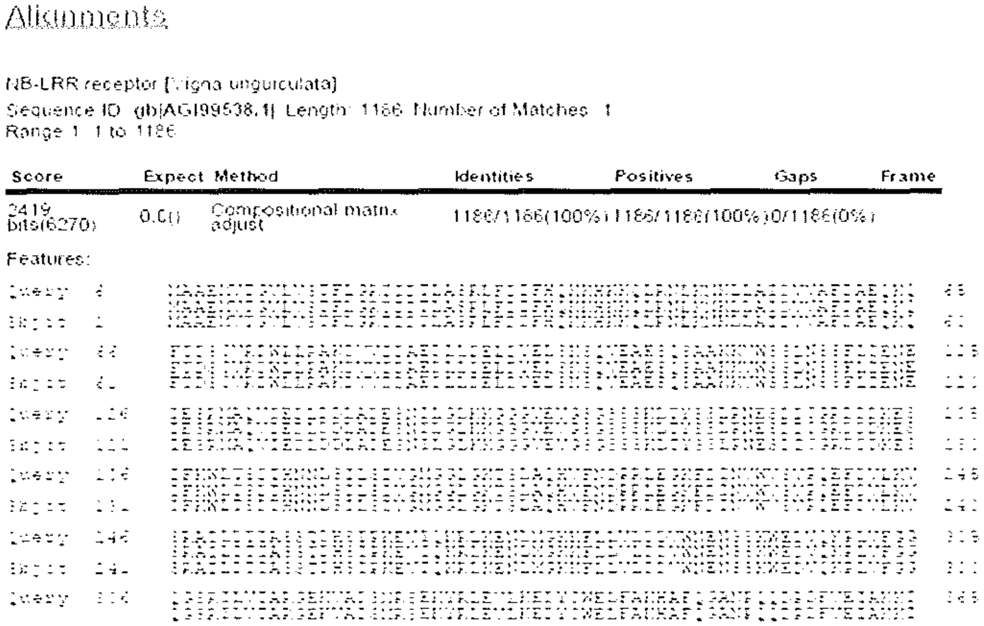
slrifpldffptlkrlelsgfpnlqmisqdhvhnhlqdltiekcpkfeslpanmhmllps

lvglhiedcptlesfpdgglpsnlnyirlgncfrlvgllkgalgdssslesleslgistpdae

cfldggllptsltelqflecqnlekldykgllqlsslrrlylldcpnlqrlpeeglpksi

sslhiidcpllkqrcqcggedwekvahirnlyicsrknlykiellk

2. The protein sequence Alignment



Alignments

NB-LRR receptor [Vigna unguiculata]
Sequence ID: gb|AGI99538.1| Length: 1186 Number of Matches: 1
Range 1: 1 to 1186

Score	Expect	Method	Identities	Positives	Gaps	Frame
2419 bits(6270)	0.0()	Compositional matrix adjust	1186/1186(100%)	1186/1186(100%)	0/1186(0%)	

Features:

```
Sbjct  301   [illegible]  360
Query  366   [illegible]  425
Sbjct  361   [illegible]  420
Query  426   [illegible]  485
Sbjct  421   [illegible]  480
Query  486   [illegible]  545
Sbjct  481   [illegible]  540
Query  546   [illegible]  605
Sbjct  541   [illegible]  600
Query  606   [illegible]  665
Sbjct  601   [illegible]  660
Query  666   [illegible]  725
Sbjct  661   [illegible]  720
Query  726   [illegible]  785
Sbjct  721   [illegible]  780
Query  786   [illegible]  845
Sbjct  781   [illegible]  840
Query  846   [illegible]  905
Sbjct  841   [illegible]  900
Query  906   [illegible]  965
Sbjct  901   [illegible]  960
Query  966   [illegible]  1025
Sbjct  961   [illegible]  1020
Query  1026  [illegible]  1085
Sbjct  1021  [illegible]  1080
Query  1086  [illegible]  1145
Sbjct  1081  [illegible]  1140
Query  1146  [illegible]  1191
Sbjct  1141  [illegible]  1186
```

Appendix 4: Recovery of DNA using Zymoclean gel DNA recovery kit

All centrifugation steps were performed between 10,000 - 16,000 x g.

a. The DNA fragment from the agarose gel was excised using a razor blade device and transferred it into a 1.5 ml microcentrifuge tube.

b. Three volumes of ADB was added to each volume of agarose excised from the gel (e.g. for 100 µl (mg) of agarose gel slice add 300 µl of ADB).

c. The samples were incubated at 37-55 °C for 5-10 minutes until the gel slice is completely dissolved2. For DNA fragments > 8 kb, following the incubation step, one additional volume was added (equal to that of the gel slice) of water to the mixture for better DNA recovery (e.g., 100-µl agarose, 300 µl ADB, and 100 µl water).

d. The melted agarose solution was transferred to a Zymo-Spin™ Column in a Collection Tube.

e. Then after it was centrifuge for 30-60 seconds and followed by the discard of the flow-through3.

f. 200 µl of DNA Wash Buffer was added to the column and centrifuged for 30 seconds and followed by the discard of the flow-through afterward the wash step was repeated.

g. 6 µl water were added directly to the column matrix and the column was placed into a 1.5 ml tube followed by centrifuging the sample for 30-60 seconds to elute DNA.

The concentration of the fragment DNA was checked on Nanodrop measurements (if the concentration is > 50ng/ul you can directly sequence the DNA but if not you have to clone it to increase the concentration of the fragment DNA). In our case, we have got DNA conc. of less than 20ng/ul hence we proceed to the gene cloning to increase the concentration of fragment DNA.

1. Lysogeny broth (LB) media preparation

10g of Bacto-trypton, 5g of Yeast extract, 10g of Nacl, 15g Bacto-agaros and 1 liter ddH_2O was mixed then after, it was autoclaved for 45min then allowed to cool down for 10-15min, then the Antibiotics (Ampicilin) was added into the LB media. Afterward the mix was distributed in to the culture plate and waited for while till it solidifies.

2. The ligation mix preparation

5ul of PCR product (from the extracted DNA), 1ul of 10xligation buffer, PCR vector 2ul, ddH_2O 1ul and 1ul of T4 DNA ligase was mixed and the mix was kept at room temperature for 20min; then 50ul of Xgal and ATPG were added and was spread on the plate using sterile spreader and then kept for 30min.

3. Heat shock

The tube samples was heat shocked for 30 min then after 250 SOC and LB media were added then covered with par film; afterward, the sample was then put for 1hrs in 37^0c shaker.

4. Platting

The liquid mix was spread on the surface of the LB-media plate until the liquid is gone. Then after it was put in 37^0c over night afterward, the sample was checked for the presence of bacteria colony. White colony contains bacteria, plasmid, and insert DNA then blue colony contains only bacteria and plasmid. The bacteria colony was transferred in to LB media (without Agarose) and Ampicilin mix, then left over night in a shaker and then checked for the presence of change in colure; finally the media with white cloudy colour was selected to extract plasmid DNA (Fig 26).

5. Plasmid extraction and DNA sequencing

GeneJet plasmid miniprep kit was used to extract the plasmid; then after nano drop measurement was taken to know the concentration of the plasmid DNA. Afterward the plasmid DNA along with the insert was sent to GENEWIZ Company for sequencing.

6. Analysis of sequenced DNA data

The sequence result of the insert DNA from GENEWIZ Company was used in the blast searches against the available public sequence databases (nucleotide and protein database). The DNA sequence data were first blasted against the nucleotide database afterward, the nucleotide blast result was blasted on protein database to get the protein sequence. The web link http://www.bioinformatics.org/sms2/filter_protein.html were used to filter protein sequence data (to remove unwanted numbers and letters from the sequence data). After editing and filtering the nucleotide sequence data the web link http://blast.ncbi.nlm.nih.gov/Blast.cgi were used to blast the nucleotide sequence and to get the matching sequence form the nucleotide database.