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Genetic diversity analysis of Ethiopian *Orobanche Crenata* population using microsatellite markers (SSR)

Gashaw Belay

A thesis submitted to Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

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Genetic diversity analysis of Ethiopian *Orobanche crenata* population using microsatellite markers (SSRs)

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ABSTRACT: *Orobanche crenata* (Broomrape) is a parasitic weed which imposes considerable yield losses on food legume crops particularly on faba bean. In Ethiopia more than 0.5 million ha of faba bean production have been at risk of *Orobanche* infestation. Attempts have been made on the development of *O. crenata* control strategies; however there is no single approach to control such parasitic weed. Information on genetic diversity and virulence genetics of *O. crenata* are limited in our country. Moreover, understanding such information has valuable input on the control and diagnosis of parasitic weeds. However, no attempts were made to study population genetics of parasitic weed on faba bean in Ethiopia. Hence, this study was designed with the aim to assess the genetic diversity of *O. crenata* populations from Ethiopia; using transferable SSR markers. A total of 96 samples were collected from South Tigray (30), South Gondar (30), South Wollo (31), and North Wollo (5). Genomic DNA was isolated from Silca gel dried *O. crenata* samples using modified CTAB method. Out of 79 *O. cumana* SSR markers 30 SSR markers were selected for cross amplification on *O. crenata*. Reaction amplification was performed by using touchdown PCR program and amplicon were processed to QIAxcel gel electrophoresis. From 30 tested SSR loci; 11 SSR markers were identified and determined as functional and transferable markers. Different biological software was used for molecular data analysis. An average number of allele, gene diversity, heterozygosity, and PIC values for the SSR loci were 9.6, 0.82, 0.38, and 0.80, respectively. An average of 59 percent of polymorphic loci per population was recorded; the mean observed and expected heterozygosity, allelic range, gene number and diversity, and Garza-Williamson index values for the all population was 0.37, 0.78, 21.68, 48, 0.77 and 0.45, respectively. South Wollo population was exhibited high gene diversity as compared to others. The Nie's genetic distance revealed that population of South Wollo and South Gondar has 88% similarity whereas individual from North Wollo showed divergent to others. The Analysis of Molecular Variation (AMOVA) has also shown that 97% and 3 % of the total variation was due to the within and among populations variation, respectively. Cluster analysis demonstrated that the 96 samples clearly divided into two major group and six sub-groups. Principal coordinate analysis (PCO) also separated the entire sample into three major groups and fully supported by STRUCTURE, that assigned all samples in to three sub-populations. However, there is no clear distinction were made about the distribution of genotypes in different groups based on their geographical origins. Overall, high level of genetic diversity achieved from this study could be used as pioneer information for *O. creanta* control strategies and diagnosis mechanism in Ethiopia. Furthermore the findings could be applied for the improvement of faba bean breeding.

Key words: *Orobanche crenata*; Broomrape; SSR markers; Genetic diversity

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LIST OF ABBREVIATIONS

AB	Applied Biosystems
AMOVA	Analysis of molecular variance
ARARI	Amhara Regional Research Institute
bp	Base pair
CTAB	Cetyltrimethyl ammonium bromide
Cm	Centimeter
^{o}C	Degree centigrade
CSA	Central Statistics Agency
DNA	Deoxyribonucleic acid
dNTP	Dexynuclieotid triphosphate
EIAR	Ethiopian Institute of Agricultural Research
g	Gram
ISSR	Inter Simple Sequence Repeat
Kb	killo base
m a.s.l	Meter above sea level
mm	milli meter

mM	milli mole
MSc.	Master of Science
ng	Nano gram
nm	Nano meter
µl	Micro litter
µM	Micro Mole
OD	Optical density
PCR	Polymerase chain reaction
PIC	polymorphic information content
pM	Pico mole
PCO	principal coordinate axis
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
S	Second
SNP	Single nucleotide polymorphism
SSR	*Simple Sequence Repeat / Microsatellite*
TA	Annealing temperature
Taq	Thermophilus Aquaticus
U	Unit
UPGMA	Unweighted Pair Group Method with arithmetic Mean
UV	Ultra violet
V	Volte
w/v	Weight by volume ratio

1. Introduction

The parasitic weed (*Orobanche spp*.) imposes considerable yield losses on faba bean *(Vicia faba),* lentil (*Lens culinaris*), carrot (*Daucus carota*), pea (*Pisum sativum*), chickpea (*Cicer arietinum*), vetches (*Vicia spp.)* and other crops in Mediterranean countries (Parker and Riches, 1993). Broomrapes (*Orobanche spp.*) are native to the Mediterranean region (North Africa, the Middle East, and southern Europe) and western Asia. Their range extends to similar climates in Asia, Africa, Australia, and North and South America where they also cause significant crop damage (Kemal *et al.,* 2008). Crenate broomrape (*Orobanche crenata*) is obligate chlorophyll lacking root parasite, which is widely distributed in the Mediterranean region, the Middle East, and Eastern Europe (Parker, 1994).

The first collection and identification of *O. crenata* in Ethiopia was in 1993 at Amhara district in one site on 10ha of land (Barakat and Abu 2008). Previous report indicates that the suspected occurrence in Ethiopia with alarming expansion where food legumes are the major production area (Teklay *et al.,* 2013b). Currently the distribution of the weed in Ethiopia is expected almost throughout the whole country except that its infestation` is extremely high in some localized areas. Moreover, highland food legumes growing areas of the country, two regions (Tigray and Amhara) where sever infestation of *O. crenata* has been reported (Rezene and Gerba, 2003).

In Ethiopia, more than 400,000ha of faba bean produced by small holder farmers is at risk of *Orobanche* infestation (Assefa, 2008). In some cases this parasitic weed (*O. crenata*) causes 75 - 100 % yield losses in food legume production particularly in faba bean (Teklay *et al.,* 2013a).

O. crenata has diploid chromosome numbers (n=19, 2n=38) (Gerald *et al.,* 2005, Mathieu *et al.,* 2012). Crenata broomrape seeds remain dormant in the soil, often for many years (Kebreab and

Murdoch, 1999), *O. crenate* seed production is variable ranging from 31, 000 (Teryokhin, 1997) to 500,000 seeds per plant (Cubero *et al.,* 1979). The small size of seeds (0.25–0.35 mm) enables wind to act as the main dispersion agent of the weed (Castejon, 1989, Teklay *et al.,* 2013). Depending on the climate conditions during the growing season, an infestation level of 2.1– 4.0 emerged *O. crenata* shoots per faba bean at harvest causing approximately 50-100% reduction in crop yield (Mesa-Garcia and Garcia-Torres, 1984; Habimana, 2014).

Currently revolution of molecular marker development is becoming very fast; thereby several makers such as Isozymes, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence repeats (ISSR), Simple Sequence Repeats (SSR), and Single Nucleotide Polymorphism (SNP) were developed for various molecular purposes by different researchers. In the past decades molecular studies conducted so far in *Orobanche spp* have been based on isoenzymes (Castej Munoz *et al.,* 1991) or RAPD markers (Gagne *et al.,* 1998). Whereas isozyme and RFLP has become outdated due to marker selection criteria such as polymorphism, mode of inheritance, capacity to measure genetic variability, and transferability of marker nature. Instead SSRs are currently considered the markers of choice in many areas of molecular genetics, mainly due to their co-dominant and polymorphic nature (Pineda *et al.,* 2013). SSR markers have proven to be useful tools for genetic diversity studies, which are reproducible, neutrally evolving, and multi-allelic. Accordingly, SSR markers were chosen as a priority marker for this study

1.1. Rationale

Attempts have been made on the development of some broomrape (*Orobanche spp*) control strategies such as soil solarization (Sahile *et al.,* 2005), soil fumigation with methyl bromide and methamso diumanddazomet (Foy *et al.,* 1989), the use of sulfonyl-urea and imidazolinone herbicides (Parker and Riches, 1993; Garcia-Torresetal, 1994) and development of tolerant faba bean varieties(Teklay *et al.,* 2013a). Use of site-specific weed control strategies have been successfully applied in various crops, resulting in a significant reduction of herbicide use and weed control costs (Dammer *et al.,* 1999; Christensen, 2009). To be able to apply site-specific weed control methodologies, weed spatial distribution have to be quantified and determined in well defined-manner, which is currently missing in the Ethiopian context.

Information on genetic diversity among and within parasitic weed populations, population dynamics, mating system, gene flow and virulence genetics in *O. crenata* are limited in our country. Particularly concerning molecular based diversity analysis, despite the importance of such studies for the development of control strategies for this parasitic weed and also control involving genetic resistance in legume crops. Moreover, understanding of the molecular genetic variation and genetic similarity between and within *O. crenata* populations is an important step to develop management strategy directed towards the control and diagnosis of parasite weeds with valuable traits. However, no attempt was made before to compile such relevant information on geographic distribution and diversity for this parasitic weed on faba bean in Ethiopia. Hence, the present study aimed at assessing the genetic diversity and relationships among *O. crenata* samples collected from Northern part of Ethiopia.

1.2. Research questions and Hypotheses tested

The major hypothesis was to test if inter-regional genetic distance of *O. crenata* follows the geographical pattern and populations from extensive legume producing regions exhibit higher diversity.

The research question of this study was: what is the genetic variation within and among of Ethiopian *O. crenata* population using SSR markers?

1.3. Objectives:

General Objective:

- To assess and determine the extent of molecular genetic diversity and patterns of distribution within and among Ethiopian *Orobanche crenata* populations.

Specific Objective:

- To identify transferable SSR markers from *O. cumana* affecting sunflower to *O. crenata* affecting faba bean
- To see the genetic distance and similarity among different origins of *O. crenata* populations
- To determine the genetic structure of *O. crenata* populations from Ethiopia.

2. Literature Review

2.1. Description of *Orobanche ssp*

Orobanchaceae (Broomrapes) is a morphologically diverse family of predominantly herbaceous, parasitic plants. There are several species of angiosperms utilizing a parasitic mode of nutrition but not much has been studied on all these species. These parasitic flowering plants could be separated into the two broad groups of holoparasites and hemiparasites (Diego, 2006; Kemal *et al.,* 2008; Habimana *et al.,* 2014). Holoparasitic species are always obligate parasites which lack chlorophyll and have little independent capacity to assimilate or fix carbon and/or inorganic nitrogen (Stewart and Press, 1990). Hemiparasitic species on the other hand, may be facultative or obligate i.e. contain chlorophyll, and are generally thought to rely on their host only for water and minerals. It has been suggested that the extent to which parasitic plants depend on their hosts must somehow be related to their own photosynthesizing abilities (Tuohy, *et al.,* 1986).

Broomrapes (*Orobanche spp.)* are belongs to the family *Orobanchaceae* in the obligate parasitic group. *Orobanche spp* produce large number of seeds that are widely distributed in harvested products as well as in the fields. Because of their size, they are easily distributed by water and wind (Jurado-Exposito, *et al.,* 1996).

Orobanche spp commonly parasitize *Solanaceae* and *Fabaceae (Leguminoceae)* hosts such as tomato, pepper, bean, pea and tobacco, reducing crop yields or totally destroying the crop. *Orobanche spp* are usually not specific to a single host with most species parasitizing a range of hosts, although among *Orobanche* species host ranges may differ (Dor *et al.,* 2014).

O. crenata produces leafless flowering stems, up to 100 cm high, usually un-branched, bearing alternate scales, less than 2 cm long. The plant is pale, completely lacking any chlorophyll. The base of the stem, below ground, is normally swollen and tuberous. Flowers are distinctly fragrant. Filaments are inserted in the corolla tube, 2-4 mm above the base. A capsule develops up to 10-12 mm long and may contain several hundred seeds, each about 0.2 x 0.4 mm. A single plant carries ten to several hundred flowers and hence may produce up to a quarter of a million seeds (Linke *et al.,* 1992; Rubiales, 2002; Habimana *et al.,* 2014)**.**

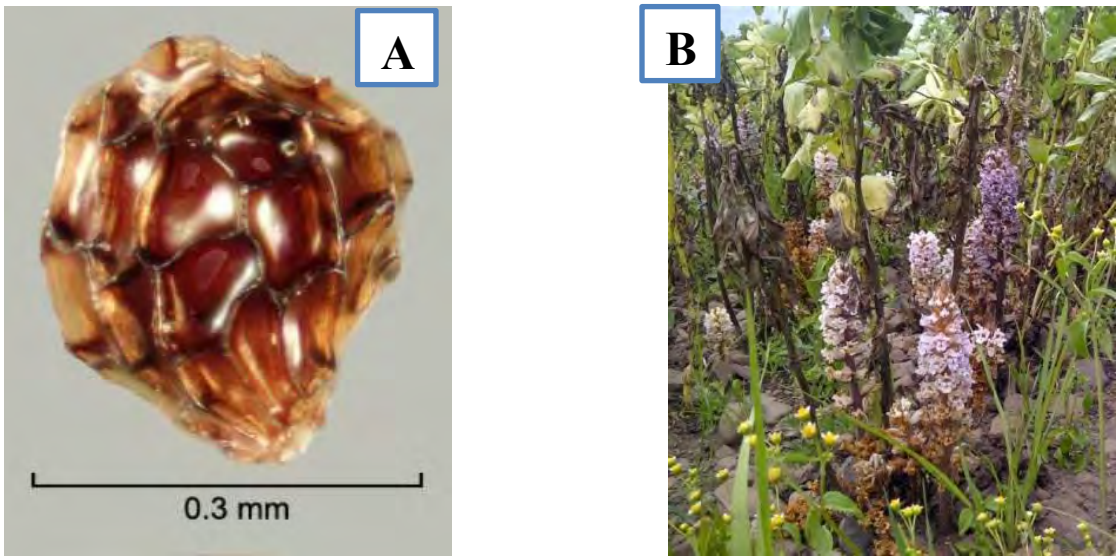


Figure 1: Orobanche Crenata description: **(A)** *Orobanche crenata* seed ***Source*:** photograph is copied from the website www.lucidcentral.org of Federal Noxious weed disseminates of the U.S. by Julia Scher. **(B)** Mature *Orobanche crenata* at Faba Bean field; ***Source*:** Photo taken by Gashaw during sample collection at South Wollo 2014.

2.2. Origin and distribution of *Orobanche*

Orobanche like other parasitic weeds have evolved specificity to crops and plants in the natural vegetation. Some of them however have more or less abandoned their natural hosts and become very noxious parasitic weeds causing serious losses in yields of economically important crops (Roman *et al.,* 2007). The Mediterranean region is considered to be one of the centers of origin of *Orobanche* species. The species are distributed worldwide from temperate climates to the semi-arid tropics except *Orobanche crenata* whose distribution is restricted to the Mediterranean regions, the Middle East and East Africa (Parker and Riches, 1993). Of the 100 or more species in the genus *Orobanche* only a few are of economic importance as weeds in cropping systems. These parasitic plants vary greatly with respect to host range and parasitize a wide range of plant families *Asteraceae*, *Fabaceae*, *Solanaceae*, *Apiaceae*, and *Cucurbitaceae* (Kroschel, 2001).

Table 1. Economic important *Orobanche* species and their main hosts

Orobanche Species	Main Host
O. crenata	Faba bean, pea, lentil and chickpea
O. ramosa	Tomato, tobacco, hemp, eggplant, lentil and cucurbit
O. aegyptiaca	Tomato, tobacco, hemp, eggplant, lentil and cucurbit
O. cumana	Sunflower, tobacco, tomato and potato
O. cernua	Sunflower, tobacco, tomato and potato
O. minor	Alfalfa, clover and trefoil (lotus)
O. foetida	Faba bean, alfalfa and trefoil (lotus)

O. crenata is common in countries where adjacent to the Mediterranean region. It extends sporadically east wards as far as Pakistan and India, and northward into northern Europe but is

rarely a significant problem away from the immediate Mediterranean region (Kemal *et al.,* 2008). *Orobanche* surveys made by (Besufekad *et al.,* 1999) indicated that several districts are plagued with *Orobanche* (Gojam and Gondar,Tatch Gayint, south Welo, Kedijo and Flagober). Farmers in Kedijo area indicated that the weed appeared for the first time in 1983 in Dehit area (Goshi-locality). The first collection and identification of *Orobanche crenata* was in 1993 in Amhara district - in one site (10ha), presently infesting 19 locations in two districts. Currently, the suspected occurrence in Ethiopia is an alarming development in regions where faba bean is a major crop grown (Teklay *et al.,* 2013b).

2.3. Taxonomic classification of *Orobanche crenata*

Orobanche crenata is occasionally known by the synonym *Orobanche speciosa* but there is no other nomenclatural confusion with closely related species. *O. crenata* is an outcrossing species showing much genetic diversity, but no clearly defined local races (Paran *et al.,* 1997).

Taxonomic Tree of *Orobanche crenata*

Domain: Eukaryota

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Scrophulariales

Family: *Orobanchaceae*

Genus: *Orobanche*

Species: *Orobanche crenata* Source: **http://www.cabi.org/isc**

As *O. crenata* is grown worldwide; areas with diverse socio-demographic characteristics it is obvious there is different naming for the same individuals. Therefore *O. crenata* has different common name in international as well as national communities. International common name: English: scalloped broomrape; Spanish: jopo (Colombia); French: Orobanche chevelue; Portuguese: penachos; National (Ethiopian) common name: North wollo and South Gondar: Gelmite, Daymerech; South Wollo: Daymerech, Kitegn; South Tigray: Akemchira.

2.4. Biology of Orobanche crenata

The host-parasite relationship of *Orobanche crenata* is of a highly specialized nature. *O. crenata* is an obligate parasite, needing to establish a connection to a host root within a few days of germination. The seed is minute (approximately 0.2 x 0.4 mm) from which only the radicle emerges and it can grow only a few mm long. A chemical stimulus is needed to trigger *Orobanche* germination; this stimulus normally comes from host roots. Therefore, *Orobanche* normally germinates only when a host root is nearby. However, a moist environment is required (for several days) together with suitable temperatures before the mature seed is responsive to germination stimulants. This preparatory period is known as conditioning or preconditioning. Conditioned seeds remain responsive to germination stimulants for several months (Joel *et al.,* 1995).

Years of investigation have uncovered a sophisticated system of chemical signaling by which root parasitic plants *(Striga* and *Orobanche)* recognize a potential host plant and regulate their development in order to optimize their chances for survival (Parker and Riches, 1993; Fernandez

et al., 2009). Several mechanisms exist that insure tighter coordination between developmental stages of parasite life cycle and the one of the host plants.

Orobanche spp totally depends on their hosts for all nutrition and also draws most of their water from the host root. Effects on the host are generally proportional to the biomass of the parasite, such that the mass of the parasite is reflected in a very similar loss in mass of the host crop (Manschadi *et al.,* 1996). The growth of the parasite occurs at the expense of water, mineral and organic compounds from the host. The tubercle and underground shoot accumulate carbohydrates and thereby become a strong sink for all plant nutrients. Effect on host plant growth becomes noticeable when the parasite emerges from the soil. Growth of *Orobanche* shoots is most rapid during that period and it induces a lack of carbohydrate in the host roots. Depending on environmental conditions the underground phase of the life-cycle of *O. crenata* ranges from 30 to over 100 days. The whole life cycle from seed germination to seed production requires about 3-5 months (Kroschel, 2001).

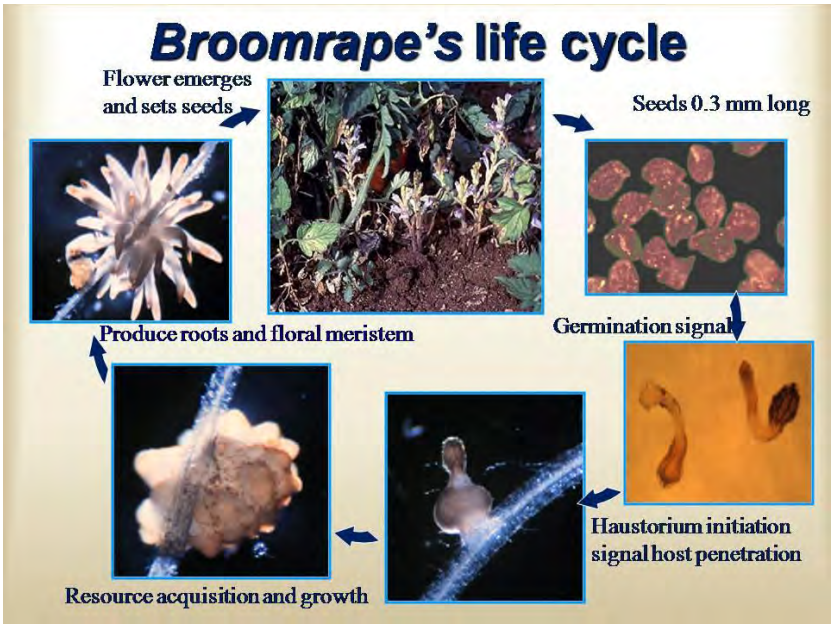


Figure 2. *Life cycle of Orobanche spp. Source:* http://www.jbb.uni-plovdiv.bg

2.5. Germination and attachment mechanism of *Orobnache*

Seed germination occurs when ripened seeds are preconditioned by exposure to warm moist conditions for several days followed by exogenous chemical signals produced by host roots and some non-hosts (Worsham, 1987). Strigolactones have been identified as growth stimulates chemicals for *Orobanche spp*. Optimum temperatures for conditioning and germination of *O. crenata* are in the region of 15-20°C but prolonged exposure to these temperatures in the absence of stimulant leads to secondary dormancy. Following germination, the seedlings attach to the host roots by the production of specialized feeding structures, described as haustoria that form a functional bridge into their hosts (Alessia, 2009).

Conditioning, germination, parasitic contact (attachment) and penetration are mediated by elegant systems of chemical communication between host and parasite. Broomrapes spend most of their life cycle underground where they undergo processes of germination, haustorial differentiation from the radicle, haustorial penetration of the host, formation of vascular connection with the host, acquisition of host nutrients, and storage of resources in a parasite organ called the tubercle or nodule (Fernández-Aparicio *et al*., 2011). Haustoria penetrate the host tissues until they reach the vascular system for uptake of water, nutrients, assimilate, and grow at the expense of the host plant's resources (Joel *et al*., 2007).

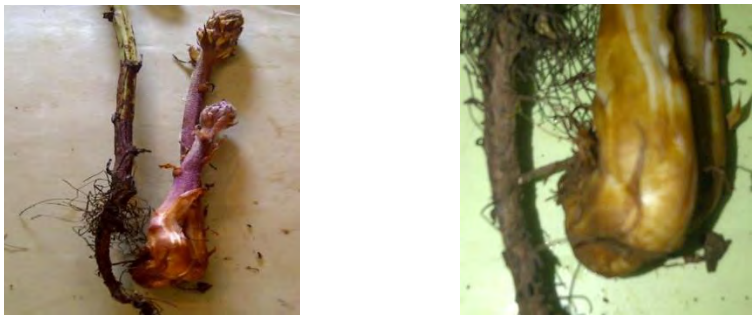


Figure 3. *Orobanche crenata* attachement to faba bean; Photo taken by *Gashaw,* 2014 at faba bean field.

2.6. *Orobanche crenata* infestation and its effect on crop

A considerable loss in growth and yield of many food and feed crops is caused by root parasitic flowering plants. Specifically, weeds of *Orobanche* species in particular cause great loss to crop farmers worldwide. Yield reduction is dependent on the timing and severity of infestation. These weeds compete with crop plants for nutrition, water and space and may serve as alternate hosts for insect pests or disease agents (Habimana *et al.,* 2014).

Legume crops represent an important component of agricultural food crops consumed in developing countries. Faba bean is familiar in Ethiopian feeding culture, the majority of the seed produced would be consumed domestically and a smaller percentage of the crop is delivered to the export market. In Ethiopia Faba Bean occupies near to 574,060 ha of land with annual production about 943, 964 tones (CSA, 2013). An average national productivity is 1.5 tons ha^{-1}, while world average grain yield of faba bean is around 1.8 t ha^{-1} (ICARDA, 2008). However, the productivity of faba bean in Ethiopia is still far below its potential due to abiotic and biotic factors of faba bean (Endale *et al.,* 2014). Among which *Orobacnhe* are the most important biotic factors causing significant faba bean yield reduction and take lions share (Teklay *et al.,* 2013). Globally, the damage caused by *Orobanche* on field and vegetable crops is significant it reaches yield losses ranging from 50-100% (Linke *et al.,* 1989, Teklay *et al.,* 2015).

Due to the complete devastation of faba bean by *Orobanche*, farmers are forced to replace faba bean by wheat and oil crops production in Northern Part of Ethiopia. The replacement of faba bean by other crops will pose multi impact problem (Besufekad *et al.,* 1999) of which depletion of soil nutrient due to lack of Pulse-Cereal crop rotation can be cited as major one.

2.7. Prevention and control attempt of *Orobanche crenata*

Since *Orobanche* has not yet infested all potential cultivation fields and regions of the world, the spread of this economic weed could be avoided. The seeds of *Orobanche* are disseminated by wind, water, animals, machines and contaminated crop seeds. The best control method is preventive by sowing clean seeds (Grenz and Sauerborn, 2007).

Several control methods have been tried and recommended for the control of *Orobanche*; these include cultural and mechanical (crop rotation, trap and catch cropping, fallowing, hand-pulling, nitrogen fertilization, time and method of planting, intercropping and mixed cropping), physical (solarisation, mulching), chemical (herbicides and artificial seed germination stimulants), use of resistant varieties, and biological control methods (Parker and Riches , 1993; Vurro and Gressel, 2007, Teklay *et al.,* 2015).

Overall, there is no single method controlling *O. crenata* because of its ability to produce numerous seeds even in adverse environmental conditions; its ease of seed dissemination; the ability of seeds to remain viable for a long time and to require only one external stimulant for germination; the close parasite-host connection; the lack of economical, practical, efficient, selective and eco-compatible means of control so suggestion were made on developing of integrated control methods for different ecological and socio-economic conditions (Alessia, 2009). Similarly, successful control of *O. crenata* in faba bean can only be achieved by integration of a range of options; like a combination of slightly delayed planting date, with low applications of imazethapyr pre-emergence, glyphosate or imazaquin post-emergence. Moreover hand-pulling and the use of less susceptible varieties may also be important components. Most countries also apply prohibiting entry of major parasitic weed species, including *O. crenata* (Suh, 2011).

2.8. Genetic markers for diversity studies in crops

The differences that distinguish one plant from another are encoded in the plant's genetic material, the deoxyribonucleic acid (DNA).

A genetic marker can be defined in one of the following ways: (a) a chromosomal landmark or allele that allows for the tracing of a specific region of DNA; (b) a specific piece of DNA with a known position on the genome (http://en.wikipedia.org/wiki/Genetic_marker); or (c) a gene whose phenotypic expression is usually easily distinguished, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, chromosomes, or locus (King and Stansfield, 1990).

Molecular markers are identifiable DNA sequences, found at specific locations of the genome and transmitted by the standard laws of inheritance from one generation to the next. The existence of various molecular techniques and differences in their principles and methodologies require careful consideration in choosing one or more of such marker types for diversity studies (Rajeev *et al.,* 2007).

Molecular genetic techniques both on their own and in combination with other biotechnological approaches are beginning to have a significant impact on plant genetic resources conservation and use (Carlos *et al.,* 2014). Initially the molecular techniques were used largely for the analysis of specific genes for understanding gene action, gene mapping and the development of gene transfer technologies. More recently, the techniques have been applied to problems of direct relevance for understanding the distribution and extent of genetic variation within and between species (Maheswaran, 2004).

Generally genetic markers fall into one of the three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers).

2.8.1. Morphological Marker

Morphological markers generally represent genetic polymorphisms which are visible as differences in appearance, such as the relative difference in plant height and colour, distinct differences in response to abiotic and biotic stresses, and the presence/absence of other specific morphological characteristics (Gökbayrak *et al.,* 2010).

Morphological traits were among the traditional markers used in germplasm management and they are the strongest determinants of the agronomic value and taxonomic classification of plants; especially if the traits are highly heritable morphological markers are one of the choices for diversity studies because the inheritance of the marker can be monitored visually (Yoseph *et al.,* 2005). However, morphological features have a number of limitations including low polymorphism, low heritability, late expression, vulnerability to environmental influences, time consuming, labor intensive (Yoseph *et al.,* 2005).

2.8.2. Biochemical (Isozyme) marker

Isozymes are structural variants of an enzyme and while they differ from the original enzyme in molecular weight and mobility in an electric field, they have the same catalytic activity (Kumar *et al.,* 2009). Isozyme markers are based on their biochemistry & thus are also known as biochemical or protein markers. The difference in enzyme mobility is caused by point mutations

resulting from amino acid substitution such that isozymes reflect the products of different alleles rather than different genes (Karaca, 2013).

Allozymes, the first molecular markers used for studies of natural variation, are limited by the number of loci, low levels of polymorphism compared with DNA-based markers due to their lower effective mutation rates, and lack of neutrality, as proteins are targets of natural selection (Elena *et al.,* 2010). Each isozyme can only be identified with a specific stain which also limits their use in practice and open challenges for the development of other accurate and convenient DNA based molecular markers.

Isozymes in the protein extract migrate to different positions on the gel depending on the electrical charge and size of the isozyme. Isozymes with different amino acid composition generally have a different charge and/or size, so it is these genetic differences that are revealed as mobility differences on the gel (Karaca, 2013). The location of an isozyme on a gel following electrophoresis is visualized by placing the gel in a solution that contains the enzyme substrate, appropriate cofactors and a dye. The colored bands on the gel are the products of the enzymatic reactions linked to the dye.

2.8.3. Molecular markers

A molecular marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. Recent developments in molecular biology have opened the possibility of employing various types of molecular tools to identify and use genomic variation improvement of several organisms (Francesco *et al.,* 2013).

The various molecular markers can be classified into different groups based on: a) Mode of transmission (biparental nuclear inheritance, maternal nuclear inheritance, maternal organelle inheritance, or paternal organelle inheritance); b) Mode of gene action (dominant or codominant markers); c) Method of analysis (hybridization-based or PCR based markers). These molecular markers include: (i) hybridization-based markers such as restriction fragment length polymorphism (RFLP), (ii) PCR-based markers: random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP): inter simple sequence repeat (ISSR), and microsatellite or simple sequence repeat (SSR), and (iii) sequence-based markers: single nucleotide polymorphism (SNP). For plant breeding applications, SSR markers, among different classes of the existing markers, have been proven and recommended as markers of choice (Gupta and Varshney, 2000).

Desirable molecular markers should meet the following character: (i) High level of genetic polymorphism; (ii) Co- dominance (so that heterozygotes can be distinguished from homozygotes); (iii) Clear distinct allele features (so that different alleles can be identified easily); (iv) Even distribution on the entire genome; (v) Neutral selection (without pleiotropic effect); (vi) Easy detection, so that the whole process can be automated; (vii) Low cost of marker development and genotyping; and (viii) High duplicability; so that the data can be accumulated and shared between laboratories; yet still no markers available having all characteristics(Ruvolo *et al.,* 2013).

Advance of biological science results molecular marker revolution. Currently wide ranges of molecular marker technologies are available for genetic diversity studies. Among these, RFLP, RAPD, AFLP, ISSR, SSR and SNP marker systems are widely used and have broader agricultural application including diversity analysis.

2.8.3.1 Restriction Fragment Length Polymorphism (RFLP)

The development of restriction fragment length polymorphism (RFLP) molecular markers (Botstein *et al.,* 1980) has facilitated the mapping of plant and animal genomes (Lark *et al.,* 1993). The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms. Although two individuals of the same species have almost identical genomes, they will always differ at a few nucleotides due to one or more of the following causes: point mutation, insertion/deletion, translocation, inversion and duplication. Some of the differences in DNA sequences at the restriction sites can result in the gain, loss, or relocation of a restriction site. Hence, digestion of DNA with restriction enzymes results in fragments whose number and size can vary among individuals, populations, and species (Semagn, 2006).

The major strength of RFLP markers are high reproducibility, codominant inheritance, good transferability between laboratories, provide locus specific markers that allow synteny (conserved order of genes between related organisms) studies, no sequence information required, and relatively easy to score due to large size difference between fragments. However, several limitations for RFLP analysis such as: it requires the presence of high quantity and quality of DNA (Roy et al., 1992). It depends on the development of specific probe libraries for the species; the technique is not amenable for automation; the level of polymorphism is low and few loci are detected per assay; it is time consuming, laborious, and expensive (Ahmed, 2012). Therefore currently RFLP markers have been replaced by other suitable markers.

2.8.3.2. Random Amplification of Polymorphic DNA (RAPD)

RAPD was the first PCR based molecular marker technique developed and it is by far the simplest (Williams *et al.,* 1990). Short PCR primers (approximately 10 bases) are randomly and arbitrarily selected to amplify random DNA segments throughout the genome. The resulting amplification product is generated at the region flanking a part of the 10 bp priming sites in the appropriate orientation. RAPD products are usually visualized on agarose gels stained with ethedium bromide and/or other nucleic acid staining chemicals.

A RAPD marker doesn't need prior genome information, relatively inexpensive, easily developed and because they are based on PCR amplification followed by agarose gel electrophoresis; they are also quickly and readily detected. RAPD technique was used extensively in studying genetic diversity between plant species (Ilan *et al.,* 1997).

RAPD markers are dominant and therefore heterozygous individuals cannot be distinguished from homozygotes; another disadvantage of using RAPD technique is the reproducibility between different runs which is due to the short primer length and low annealing temperature (Ahmed, 2012).

2.8.3.3. Amplified Fragment Length Polymorphism (AFLP)

AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer recognition sequences (adaptors) to the restricted DNA (Semagn *et al.,* 2006).

The first step in AFLP analysis involves restriction digestion of genomic DNA (about 500 ng) with a combination of rare cutter (EcoRI or PstI) and frequent cutter (MseI or TaqI) restriction enzymes. Double-stranded oligonucleotide adaptors are then designed in such a way that the initial restriction site is not restored after ligation. Such adaptors are ligated to both ends of the fragments to provide known sequences for PCR amplification(Saeed *et al.,* 2009). Complexity of the bands can be reduced by adding selective bases to the primers during PCR amplification. It was also used in studying genetic diversity of *Orobanche spp*. (Vaz *et al.,* 2008).

The advantages of AFLP include: it is highly reliable and reproducible; it does not require any DNA sequence information; it is information-rich due to its ability to analyze a large number of polymorphic loci simultaneously (effective multiplex ratio) with a single primer combination on a single gel as compared to RAPDs, RFLPs and Microsatellite's (Das and Swati, 2013). Beside the limitations of AFLP include, it requires more number of steps to produce the result; it requires pure DNA; the technique requires the use of polyacrylamide gel in combination with AgNO3 staining, radioactivity, or fluore-scent methods of detection, which will be more expensive and laborious than agarose gels; it involves additional cost to purchase both restriction and ligation enzymes as well as adapters; it is dominant. This reduces the accuracy of AFLP markers in population genetic analysis, genetic mapping and marker assisted selection (Marko *et al.,* 2008).

2.8.3.4. Inter-simple sequence Repeat (ISSR)

ISSR involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. ISSR are semi-arbitrary markers amplified by polymerase chain reaction (PCR) in the presence of one primer complementary to a target microsatellite. Each band corresponds to a DNA sequence bordered by two inverted microsatellites (Tsumara *et al.,* 1996).

ISSRs use longer primers (15–30 mers) as compared to RAPD primers (10 mers), which permit the subsequent use of high annealing temperature leading to higher stringency. The amplified products are usually 200–2000 bp long and amenable to detection by both agarose and polyacrylamide gel electrophoresis.

ISSRs have been used in genetic diversity studies in different crop plants (Brenner, 2011), including, Coffee (Tesfaye *et al,* 2014), Rice (Girma *et al.,* 2010). It does not require genome sequence information; it leads to multilocus, highly polymorphous patterns and produces dominant markers (Mishra *et al.,* 2003).

ISSRs exhibit the specificity of microsatellite markers, but need no sequence information for primer synthesis enjoying the advantage of random markers (Joshi *et al.,* 2000). The technique is simple, quick, and the use of radioactivity is not essential. ISSR markers usually show high polymorphism. However, like RAPDs, whereas reproducibility, dominant inheritance and homology of co-migrating amplification products are the main limitations of ISSRs.

2.8.3.5. Simple Sequence Repeats (SSR)

Microsatellites, Simple Sequence Repeats (SSRs), or Short Tandem Repeats (STRs), are repeating sequences of 1-6 base pairs of DNA. SSR allelic differences are, therefore, the results of variable numbers of repeat units within the microsatellite structure (Reddy *et al.,* 2002).

SSR are becoming the most important molecular markers in both plants and animals. SSR are very polymorphic due to the high mutation rate affecting the number of repeat units (Rajeev, et al., 2005). PCR reactions for SSRs is run in the presence of forward and reverse primers that anneal at the 5` and 3` ends of the template DNA, respectively.

SSR markers are highly informative due to co-dominance, multiallelism, heritability, abundance and wide coverage of the genome. Another advantage is the conservation of flanking regions across generations, which allows repeated use of the technique (Francesco *et al.,* 2013).

Generally, SSRs have several advantages over other molecular markers. For example, (i) microsatellites allow the identification of many alleles at a single locus, (ii) they are evenly distributed all over the genome, (iii) microsatellites can offer more detailed population genetic insight than maternally inherited mitochondrial DNA (mtDNA) because of the high mutation rate and bi-parental inheritance (iv) they are co-dominant, (v) highly polymorphic and specific (Jones et al., 1997). (vi) very repeatable (vii) little DNA is required and (viii) need a small amount of medium quality DNA and (ix) the analysis can be semiautomated and performed without the need of radioactivity (Gianfranceschi *et al.,* 1998; Guilford et al., 1997; Carla *et al.,* 2015), (xi) SSRs are typically codominant and multiallelic, allowing precise discrimination even if closely related individuals (Carla *et al.,* 2015).

Although SSR markers are developed for use in a single species, it is possible to extend known markers for use in related species. This is possible because the flanking regions are conserved and the number of duplications is variable (Hendre and Aggarwal 2007; Wanget *et al.,* 2009). Therefore, once an SSR marker is available in a related species, attempting to transfer known markers can be advantageous for the individual who does not have original developed SSR markers (Sudheer *et al.,* 2011).

The availability of new microsatellite markers is important to effectively contribute to genetic analysis of *Orobanche spp* (Pendia *et al.,* 2013). However it needed to design specific primers; the development of microsatellite markers involves the development of SSR-enriched libraries. This process is still time-consuming and expensive and also requires much discovery and optimization for each species before use (Cubry *et al.,* 2014).

2.8.3.6. Single Nucleotide Polymorphisms (SNP)

A Single Nucleotide Polymorphisms (SNP) is a genetic variation when a single nucleotide (i.e., A, T, C, or G) is altered and kept through heredity. SNP's, represent sites in the genome where DNA sequence differs by a single base when two or more individuals are compared. They may be individually responsible for specific traits or phenotypes, or may represent neutral variation that is useful for evaluating diversity in the context of evolution. SNPs are the most widespread type of sequence variation in genomes discovered so far (Carla *et al.,* 2015)

In plants, 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). Generally, SNPs are the most abundant form of genetic variation in eukaryotic genomes and occur in both coding and noncoding regions of nuclear and plastid DNA (Kwok *et al.,* 1996, Francesco *et al.,* 2013).

SNPs are widely used in breeding programs for several applications such as a) marker assisted and genomic selection, b) association and QTL mapping, positional cloning, c) haplotype and pedigree analysis, d) seed purity testing and d) variety identification e) monitoring the combinations of alleles that perform well in target environments (Bernardo, 2008; Jannink *et al.,* 2010; Kim *et al.,* 2010; McCouch *et al.,* 2010).

3. Materials and Methods

3.1. Site selection and field sampling

Sampling technique of this study was multi stage purposive sampling until uniform potential legume growing site found and followed by random sampling after potential site identified in respective administrative zones. The sites were selected systematically based on the following criteria: Faba bean production area coverage; prevalence and high level of infestation with the parasite weed; potential of the area to bean production; accessibility for sampling; economic importance of yield loss caused by the parasitic weeds

Based on the above criteria representative districts were selected by conducting preliminary key informant interview and/or by using secondary data. A total of 96 samples (30 samples each from South Tigray, and South Gondar, 31 samples from South Wollo, and 5 samples from North Wollo administrative zones) of *O. crenata* populations were used from selected districts for further molecular work. Moreover, additional *O. crenata* samples were collected from areas where farmers and local development agents reported to have the parasitic weed in first time spotting on their field.

3.2. Description of the study area

This study was conducted in the Amhara and Tigray Regional States of Ethiopia. Due to preliminary report, emphasis were given to highlands of Southern Tigray; North Wollo, South Wollo, and South Gonder administrative zones of the Amhara region; where the problem is increasing from time to time and many farmers have been abandoned to grow faba bean and other legumes due to *O. crenata* sever infestation. The collection sites were characterized by an altitude ranging from 1986 – 3226 m.a.s.l

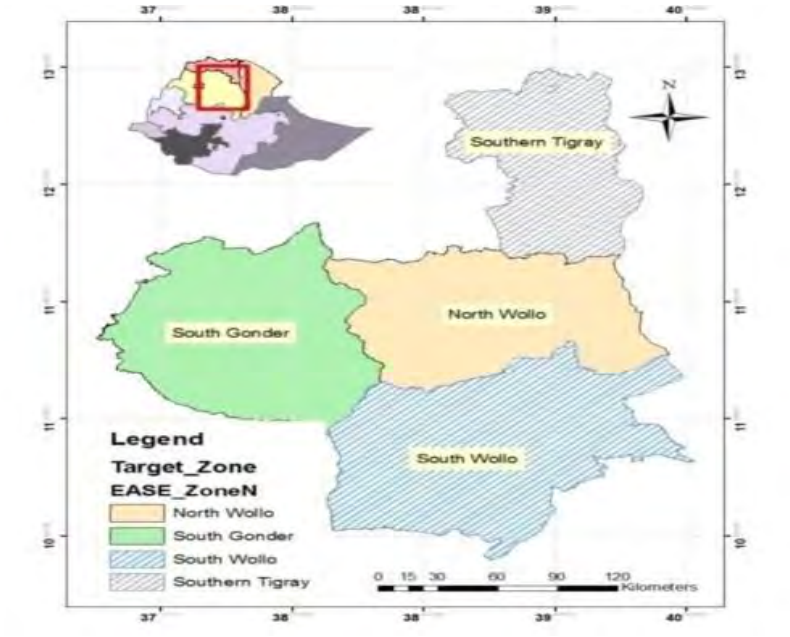


Figure 4. Location map of *O. crenata* study area by zone level

3.3. Plant material

A total of 96 broomrape (*O. crenata*) plants were collected from naturally infected cultivated food legume field in different location of the study area, Northern part of Ethiopia (Appendix 1). In addition to food legumes O. crenata samples were collected from other hosts such as weeds and oil crops. Individual samples of *O. crenata* (2-3cm long shoot tips) were collected from each farm land using height and color variation as criteria (Figure 2). To hasten dehydration the shoot tips, samples was sliced with blade and then 30g of Silca gel added to each sample on labeled zip locked plastic bag; thereby the samples were getting dried and ready for DNA extraction within a week.

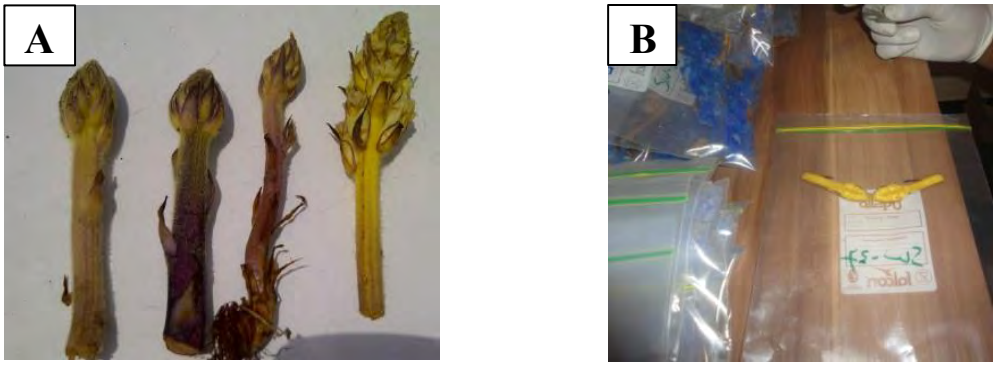


Figure 5. *Orobanche crenata* sample variability in height and color, and sample preparation, A= variability at field; B= Sliced sample treated with silca gel

3.4. Genomic DNA extraction

In order to assess the molecular genetic diversity within and among *O. crebata* populations; totally 96 samples of genomic DNA was isolated from Silca gel dried shoot tips of *O. crenata* samples using CTAB methods describe by Borsch *et al.,* (2003) (Appendix 2). DNA isolation was performed at Plant Genetics Research Laboratory of Addis Ababa University.

Approximately equal amounts (0.15g) of the dried single shoot tip samples were grounded with Mix and Mill grinding machine MM 400 and ready for downstream DNA extraction process as described in Borsch *et al.,* (2003). For detail procedures and protocol of DNA extraction (Appendix 2).

3.5. Genomic DNA quality and quantity detection

Detection and visualization of isolated genomic DNA was tested on 0.8% (w/v) agarose gel electrophoresis techniques. To prepare 0.8% agarose gel; 0.4g agarose was dissolved with 50ml TE buffer then after heat to melt completely. To detect genomic DNA on UV light Redsafe nucleic acid stain were added instead of ethdium bromid. The melted agarose was poured in to the gel Cast tray on leveled place and then allow the gel to solidify for 1 hour. Lambda DNA(50 and 100 ng/ µl) was used as ladder to compare with genomic DNA. A total of 1µl isolated genomic DNA were mixed with 5µl 1x loading dye and apply electric field with controlled power supply at 100V for 45 minute. Visualization and image capturing were done by using Bio-Rad gel doc system. Form the gel images all samples were shown good bands which indicates the presence of good quality and amounts of genomic DNA.

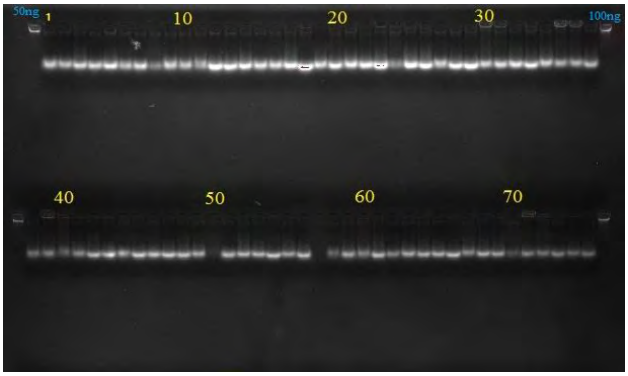


Figure 6. *Orobanche crenata* genomic DNA using agarose gel: where first lane 50ng and last lane 100ng =lambda DNA; lane number 1- 70 refers tested DNA samples of *O. crenata*

Furthermore the concentration and purity of the isolated genomic DNA were quantified by using NanoDrop (NanoDrop™2000/2000c) spectrophotometer at absorbance of 260 nm, 230 nm and 280 nm. OD ratios A260/A280, A260/A230 were calculated automatically to evaluate the quality and quantity of genomic DNA.

Overall the concentration of DNA was within a range of 162 - 2946(ng/µl). The ratio of OD (260/280) absorbance was also within a range of 1.8 to 2.19 which is considered as a good quality for further PCR analysis (Appendix 3).

Finally DNA samples were adjusted to concentration of 50 ng μL^{-1} by diluting with distilled sterilized water and later it was stored at –20°C until it used for SSR PCR analysis.

3.6. Marker transferability and Screening

The first set of 79 SSR markers designed for *O. cumana* (which affect sunflower) publicly available by Spanish researcher (Pineda-Martos *et al.,* 2013b, Appendix 4) were initially used to screened for *Orobanche crenata*. Being *O. cumana* and *O.crenata* belongs to the same genera, there is high possibility to found conserved and transferable SSR markers; thereby preliminary experiment were done to select transferable and functional SSR markers for *O. crenata* genetic diversity analysis. Out of 79 SSR markers 30 SSR markers were selected based on PIC for cross amplification test on eight samples of *O. crenata* collected from diverse geographic locations in Ethiopia. The polymorphic information content (PIC) values of tested markers ranges from 0.37–0.80. Finally 11 SSR markers were selected for this study.

Table 2. Details of 30 *Orobanche cumana* SSR markers used for screening test in this study

No.	Locus	Core sequence	Primer sequence (5'-3') Forward	Primer sequence (5'-3') Reverse	Expected size	PIC value
1	Ocum-003	$(GAT)_8$	CTCGAACGCAAACTTTTGAA	CAAAGATGGTGGTTTTGCG	94	0.51

2	Ocum-006	$(CT)_8$	CTTATGTATGTTGT TCTTCTCTGCC	CATACATCCAA TTAACATACAAG CA	90	0.53
3	Ocum-011	$(CA)_8$	GCCGTGAACTCCA CTACCAC	GAGTTAGGGTC AGTCTTGCGA	274	0.50
4	Ocum-023	$(AG)_9$	CATCACCTCGAGTT TTCCGT	CGCAAGTTCAC GAATTGAA	157	0.42
5	Ocum-031	$(AC)_9$	AGGTACAAGCACG GAAGCTG	TCTAGCCTGAA CCAACTCCA	193	0.37
6	Ocum-040	$(ATAC)_{10}$	AACAGAATCCATC TCAGGGC	ATGTTGGCATT CTCAAGCCT	105	0.45
7	Ocum-043	$(AGG)_{10}$	AGGTGCACTTAAC CTTGACCTT	CTGCAGGTGGT CATGCTAGA	104	0.37
8	Ocum-052	$(AG)_{10}$	CATGTCTAAGCTTT TGGCTCG	CAAGACTTGGA ACAAGCAAATC	108	0.44
9	Ocum-056	$(CT)_{11}$	CACCCTGCATGTTT CAAAAG	CAAGGGTATTT TCCCTATCTCAA	111	0.40
10	Ocum-059	$(TC)_{11}$	TCTTGATTTGTATA TGTCTGATGCAAT	ATGCTACAATA GAAATACACAAC GAAC	90	0.55
11	Ocum-063	$(AG)_{11}$	AACCAAGTTGATG CATCCGT	TCCCTCGGCAT TCAGACTTA	90	0.57
12	Ocum-070	$(TG)_{11}$	AAGCTGTAAACAA TGCCTGAA	CCTCCTCCAGT ACCACTAGGC	96	0.67
13	Ocum-074	$(GA)_{12}$	CCTAAAATTGAAA CCTTAAGGAAA	ACTTTCCGTGA GACGGAGTC	99	0.71
14	Ocum-075	$(CA)_{12}$	TGTGGATAGAGTA TAAGCTACCAGTTC	TTCCCGTAGCT TGGAGAATG	110	0.45
15	Ocum-081	$(CA)_{13}$	TTACAAGGTGAAA CCACCCA	CAGCTACTGTC CGCAAGAAA	90	0.54
16	Ocum-083	$(AG)_{13}$	GAGAATGCGGATT TCCTGA	GCGACTAGCAA CTTTAGTCCG	243	0.56

17	Ocum-085	$(GA)_{13}$	TCACAAGGAAGTAATGCGGC	CCAGACCCAGCTCTGTGATA	137	0.70
18	Ocum-087	$(TTC)_{13}$	TTCTCGACAGCTTTGGGTAAA	ATGCCAACTTCGAGTGATCC	134	0.64
19	Ocum-091	$(TTC)_{15}$	CACATAGTGCACCCCTGCTA	TCCCCTTCTTCATATGCCAC	173	0.55
20	Ocum-092	$(GT)_{15}$	GTCAACCTTGTTAAGGGGAGT	TGCAATCTGATGAGAGTAGAGGA	165	0.72
21	Ocum-094	$(GT)_{15}$	TGGGAGCTTTGTACAGACACTG	GTTTTCTATTAAACCGTAACAAACTCT	141	0.37
22	Ocum-108	$(GTAT)_6$	TCGTTAATAAGTGGTTCACGAAAA	TGACTAAAAATAAAATGTACGGGTG	143	0.49
23	Ocum-122	$(AGTGTG)_6$	GGAATACATCATTAAAGTAGTTGTCCG	GAAGGAGTCATTAAACTCCGTGA	241	0.55
24	Ocum-136	(GTT)6	ACGCTGGATCATCGGATTAC	ACGACCGCTACAAGTCCAAT	126	0.37
25	Ocum-141	$(CTT)_6$	CAGCAACTGTTTCTTCCATAGAG	TCCAAGAAGAGGAAAAGAAGTGA	191	0.62
26	Ocum-151	$(GGA)_6$	ATCTCCGGAGGAAGAGGAAG	CCATCACTCCCCAAGAGTTC	186	0.80
27	Ocum-156	$(GA)_7$	CAACGTTTGAGTGTGCTGCT	TGAAGCTAATGGCCAACTCA	124	0.37
28	Ocum-160	$(AG)_7$	TGAGGGTTTGTAAAGTGGGC	CGTACCTTATCCCTCCGTCA	136	0.68
29	Ocum-174	$(AAG)_7$	CAACCAACAAACAAGTAGTGACG	TCTTGCGGCAAAACCATT	190	0.66
30	Ocum-196	$(GT)_7$	GTATGTGCGCCCGTCTTG	GGGGATGACTGTGTTTCGAT	192	0.53

3.7. SSR polymerase chain reaction (PCR)

The SSR PCR was carried out in Agricultural Genetic Engineering Research Institute, ICARDA, Biotechnology laboratory, Cairo, Egypt.

SSR PCR amplifications were carried out in 10µl reaction volumes containing 1µl template DNA, 1x buffer, 0.075 units Taq DNA polymerase, 0.25 mM dNTPs and 0.5pM each primers.

Amplification was performed by using touchdown PCR program on Applied Biosystems: Veriti96 well Thermal Cycler, which consisted of an initial denaturation of 94°C for 2 min, followed by 1 cycle of 94°C for 30 s, final annealing temperature (TA) + 10°C for 30 s, and 72°C for 30 s, nine cycles in which the annealing temperature was decreased 1°C, and 32 cycles at 94°C for 30 s, TA for 30 s, and 72°C for 30 s, with a final extension of 20 min at 72°C with holding at 4°C.

For thirty SSR markers, PCR amplification was optimized by adjusting reaction mixtures to varying concentrations of $MgCl_2$, primers and DNA, and/or using a non-touchdown PCR program.

3.8. PCR product gel test

Amplified PCR products were separated on 2% agarose gels in 1x TAE buffer with RedSafe Nucleic Acid Stain incorporated in the gel and 50bp DNA Ladder was used as a standard molecular weight marker to compare amplicons with their expected size. Microsatellite alleles were detected for their amplification and correctness, hence true amplicons was subjected for QIAxcel capillary electrophoresis analysis.

3.9. QIAxcel capillary gel electrophoresis

Principle and procedures (QIAGEN hand book, 2014)

The QIAxcel system uses capillary gel electrophoresis to enable fast separation of nucleic acids based on size. Unlike agarose gel electrophoresis, separation is performed in a capillary of a precast gel cartridge. As with agarose gel electrophoresis, low-molecular-weight molecules migrate faster than high-molecular-weight molecules. As the molecules migrate through the capillary, they pass a detector which detects and measures the fluorescent signal. A photomultiplier detector converts the emission signal into electronic data, which are then transferred to the computer for further processing using QIAxcel ScreenGel Software or BioCalculator Software. After processing, the data are displayed as an electropherogram or gel image.

Fiture 7. Image of QIAGEN capillary gel electrophoresis machin

The QIAxcel system offers a number of advantages over traditional agarose gel electrophoresis, including: higher detection sensitivity, less sample wastage (minimal sample input volumes), fast analysis of up to 96 samples, automated loading and analysis, fast processing 12 samples in 3-10 minutes, up to 96 samples per run, sample input amount <0.1 µl, and digital data output.

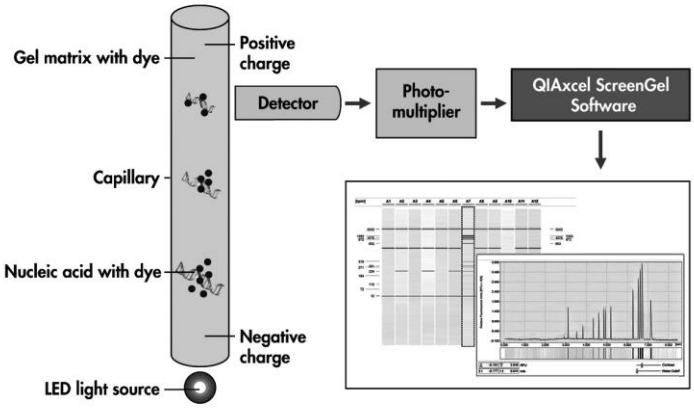


Figure 8. Sample separation process using the QIAxcel system. Nucleic acid molecules are separated according to size by applying an electrical current to a gel-filled capillary. A photomultiplier detector in the instrument detects the nucleic acid molecules as they migrate towards the positively charged end of the capillary. The data are converted to an electropherogram and a gel image by the QIAxcel ScreenGel Software

PCR amplification confirmed samples on 2% agrose gel electrophoresis were processed to QIAxcel electrophoresis system. Being amplicon size was laid between 90-300bp; high resolutions alignment marker (15bp - 1kb) were used for this study.

3.10. Allele scoring and genetic data analysis

Digital data from QIAxcel process output was analyzed by using QIAxcel ScreenGel Software and raw data was exported to Microsoft excel. Locus analysis and allele calling were done on excel sheet. Finally molecular data matrix were constructed for 96 samples and 11 markers based on allele size (bp) of the loci.

Different diversity and phylogenetic software were used for molecular data analysis. Allelic data were used for computation of different genetic analysis such as polymorphic information content

(PIC), observed heterozygosity, number of alleles, numbers of genotypes, allele frequency, and gene diversity. The analyses were conducted using PowerMarker V: 3.25 software (Liu and Muse, 2005). To examine genetic relationships among individual samples the unweighted pair group method with arithmetic average (UPGMA) analysis were conducted with the same software by using Nie's genetic distance.

To examine Population's genetic pattern; percentage polymorphism, allele number, observed and expected heterozygosity, allelic range, gene number and diversity, and Garza- Williamson index Arlequin software were used.

To examine and visualize the patterns of variation among 96 individuals of *O. crenata* on two dimensional plots, a principal coordinate (PCO) analysis was performed using PAlaeontological Statistics (PAST) software V2.17 (Hammer *et al.,* 2001).

The 96 Ethiopian *O. crenata* samples with a set of 11 SSR loci were used to understand the genetic structure and number of sub-populations in the collection used in this study. STRUCTURE version 2.3.1 (Pritchard *et al.,* 2000) was employed. For this, the number of subpopulations (K) was presumed as 1 to 12. For each run, burn-in and iterations were set to 1,000 and 2,000 respectively, and admixture and correlated allele frequencies were used. The run with maximum likelihood was used to assign individual genotypes into sub-population.

To see variation among and within the studied populations, analysis of molecular variance (AMOVA) was calculated using Genalex V6 (Peakall R and Smouse PE, 2006).

4. Results

4.1. Cross-Amplification and Transferability of SSR markers

A total of 79 SSR markers were developed and publicly available for *Orobanche cumana* , sunflower flower parasitic weed by Pineda-Martos *et al.,* (2013b) (Appendix 3). Based on PIC values a total of 30 SSR markers were selected for cross amplification and screening (Table 2).

Marker screening test were done on eight Ethiopian *O. crenata* samples collected from divers geographical origin and 11 SSR markers (Table 3) were identified and determined as functional and transferable markers, hence applied for studying of genetic diversity of entire Ethiopian *O. crenata* materials.

Moderate marker transferability from *O. cumana* to *O. crenata* was detected with11 (36%) of the 30 SSRs tested yielding fragments of expected size with sharp band. The selection of markers which were amplified and those which failed to be amplified were done based on expected size and specificity of band for each primers (Appendix 5).

All selected markers were exhibited above 0.5 PIC values ranging from 0.59 - 0.92 which are believed to be informative markers to study variability. The highest PIC value was recorded on locus Ocum-059 and the lowest PIC value also recorded on locus Ocum-063. The expected size (bp) of the loci ranges from 94bp - 274bp. The lowest expected size, 94bp were detected by locus Ocum-003 with tri-nucleotide repeated motif (GAT)8;while the highest expected size of 274bp was detected by locus Ocum-011 having di-nucleotide repeated motif $(CA)_{8.}$ All core SSR sequence repeat motif used in this study are di-nucleotide, except locus ocum-003 and Ocum-043 which are tri-nucleotide with core sequence repeated motif.

Table 3. Transferred and functional *O.cumana* SSR primers screened for *O.crenata* diversity study

Locus	Core sequence	Primer sequence(5'-3') Forward	Primer sequence(5'-3') Reverse	Average TA(°C)	Expected size(bp)	PIC value
Ocum-003	$(GAT)_8$	CAAAGATGGTGGTTTTGCG	CTCGAACGCAAACTTTGAA	55	94	0.75
Ocum-006	$(CT)_8$	CTTATGTATGTTGTTCTTCTCTGCC	CATACATCCAATTAACATACAAGCA	57	90	0.77
Ocum-011	$(CA)_8$	GCCGTGAACTCCACTACCAC	GAGTTAGGGTCAGTCTTGCGA	60	274	0.87
Ocum-023	$(AG)_9$	CATCACCTCGAGTTTTCCGT	CGCAAGTTCACGAATTGAA	55	157	0.82
Ocum-043	$(AGG)_{10}$	AGGTGCACTTAACCTTGACCTT	CTGCAGGTGGTCATGCTAGA	59	104	0.87
Ocum-052	$(AG)_{10}$	CATGTCTAAGCTTTTGGCTCG	CAAGACTTGGAACAAGCAAATC	57	108	0.80
Ocum-059	$(TC)_{11}$	TCTTGATTTGTATATGTCTGATGCAAT	ATGCTACAATAGAAATACACAACGAAC	56	90	0.92
Ocum-063	$(AG)_{11}$	AACCAAGTTGATGCATCCGT	TCCCTCGGCATTCAGACTTA	57	90	0.59
Ocum-075	$(CA)_{12}$	TGTGGATAGAGTATAAGCTACCAGTTC	TTCCCGTAGCTTGGAGAATG	60	110	0.82
Ocum-081	$(CA)_{13}$	TTACAAGGTGAAACCACCCA	CAGCTACTGTCCGCAAGAAA	56	90	0.87
Ocum-094	$(GT)_{15}$	TGGGAGCTTTGTACAGACACTG	GTTTTCTATTAAACCGTAACAAACTCT	58	141	0.73

4.2. SSR markers characterization and levels of diversity

The eleven SSR markers generated a total of 106 alleles which were used to estimate the genetic diversity among the 96 *O. crenata* genotypes. The number of alleles revealed by each marker ranged from four for locus Ocum-063 to seventeen for locus Ocum-059 with an average of 9.6 per marker (Table 4). The PIC value for the SSR loci ranged from 0.59 for locus Ocum-063 to 0.92 for Ocum-059 with a mean of 0.81. The mean level of heterozygosity per SSR marker was 0.38 ranging from 0.00 (homozygous) for marker Ocum-094 and Ocum-063 to 0.72 for locus Ocum-003; QIAGEN GelScreen software analysis revealed the homozygous and heterozygocity nature of the samples(Figure 9). Moreover, marker Ocum-059 had the highest gene diversity with 0.93, while Ocum-063 detects the lowest value of gene diversity with 0.64.

Table 4. Summary evaluation of 11 SSR loci on major allele frequency, number of alleles identified, gene diversity, heterozygosity and polymorphism information content (PIC) on: Ethiopian *O. crenata* population

Marker	Major Allele Frequency	Number of Allele	Gene Diversity	Heterozygosity	PIC
Ocum-003	0.4043	7	0.7709	0.7234	0.7461
Ocum-006	0.3351	7	0.7958	0.6277	0.7699
Ocum-011	0.2181	13	0.8880	0.4362	0.8785
Ocum-023	0.3471	13	0.8347	0.3529	0.8221
Ocum-043	0.1538	11	0.8858	0.3956	0.8746
Ocum-052	0.2111	7	0.8310	0.4222	0.8084
Ocum-059	0.1264	17	0.9253	0.4286	0.9204
Ocum-063	0.5313	4	0.6400	0.0000	0.5924
Ocum-075	0.2159	10	0.8456	0.3182	0.8270
Ocum-081	0.1649	11	0.8899	0.4681	0.8797
Ocum-094	0.3295	6	0.7689	0.0000	0.7344
Mean	0.2761	9.6	0.8251	0.3793	0.8048

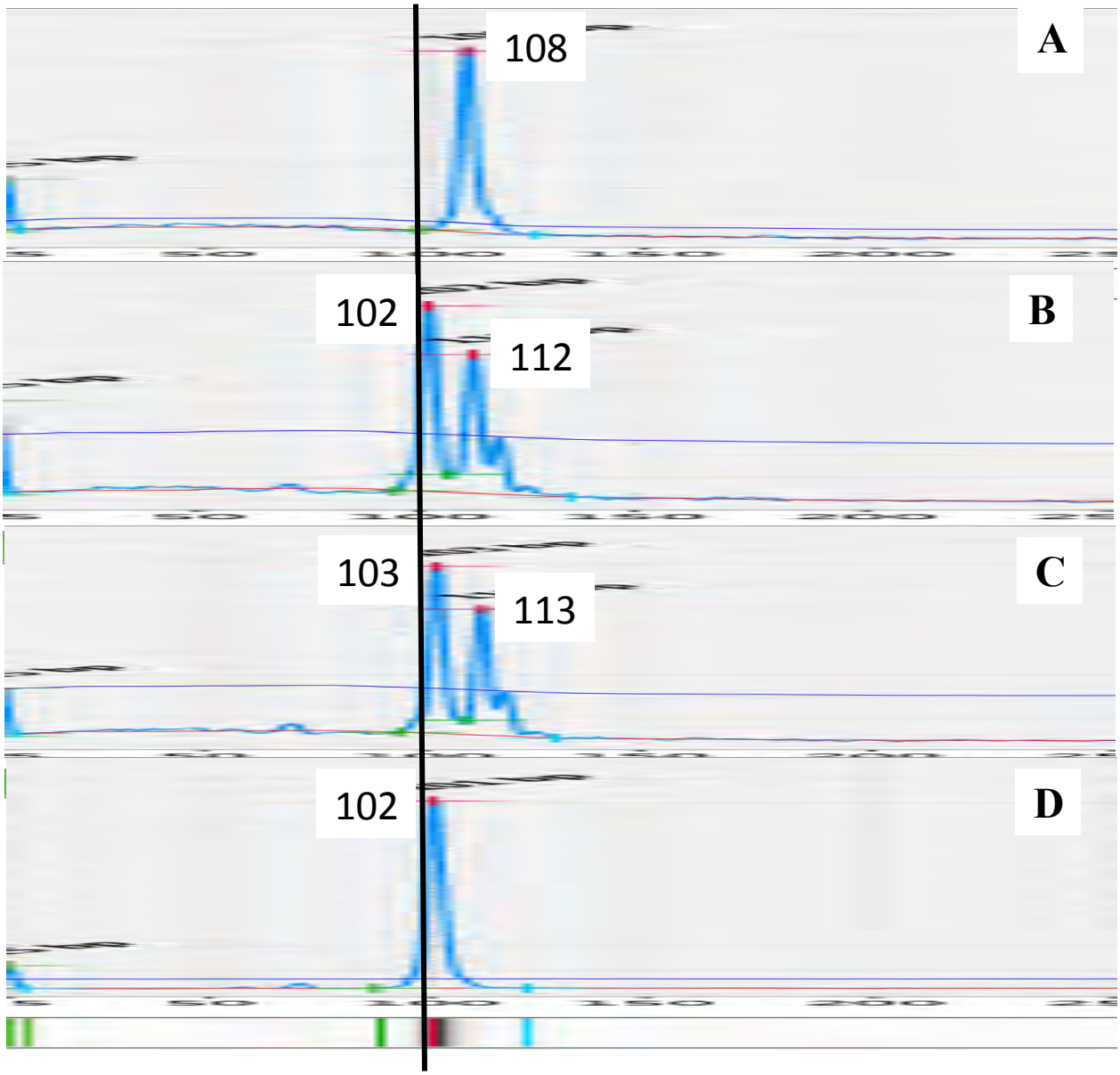


Figure 9. Elecropherogram image revealed by capillary gel electrophoresis which represent five alleles (102, 103, 108, 112, 113) of locus Ocum-052 with homozygous (A and D) and heterozygous (B and C) manner, the horizontal axis represent the peak size in base pair and the vertical axis correspond to the relative fluorescence unit (RFU). A, B, C and D represents samples.

4.3. Magnitude of genetic diversity analysis by geographical populations

In this study the percentage of polymorphic loci indicated that the populations of South Wollo had the highest percentage of polymorphic loci with 72.73% while the lowest was observed in South Tigray populations with 45.45%. The lower number of allele recorded in North Wollo population whereas the other population had nearly comparable number of alleles. The data in general revealed that the observed and expected hetrozygosity, allelic range, number of gene copies were similar in the three geographical population except North Wollo in which lower values were recorded for the above parameter. In addition, the average gene diversity over loci with the geographical populations was assessed, as a result the highest value recorded in South Wollo 0.82, while the lowest value again detected in North Wollo 0.74 closely followed by south Gonder 0.75 (Table 5).

Table 5. Population's genetic pattern estimates on percentage polymorphism, allele number, observed and expected heterozygosity, allelic range, gene number and diversity, and Garza-Williamson index.

Population	**N**	**%PL**	**A^n**	**Ho**	**He**	**A^r**	**N^g**	**D^g**	**I**
South Tigray	30	45.45	8.82	0.39	0.82	24.73	60	0.79	0.46
South Wollo	31	72.73	8.54	0.37	0.82	22.37	62	0.82	0.49
North Wollo	5	63.63	3.45	0.34	0.69	15.54	10	0.74	0.41
South Gondar	30	54.54	8.18	0.39	0.80	24.09	60	0.75	0.45
Mean	24	59.09	7.25	0.37	0.78	21.68	48	0.77	0.45

Where: N= population size, %PL= percent of polymorphic loci, A^n= number of allele, Ho=Observed hetrozygocity, He=expected hetrozygosity, A^r = Alleic range, N^g= number of gene copies, D^g= Average gene diversity over loci, I= Garza-Williamson index

4.4. Cluster Analysis

The Pairwise Nei's genetic distances between geographical populations of *O. crenata* was assessed and shown in Table 6. The analysis resulted in low to moderate genetic distance that ranges from 0.12 distance (between South Wolli and south Gondar) to 0.48 distance (between North Wollo and South Gondar). Based on such data the geographic location based population cluster were constructed and illustrated in Figure 10. Overall North Wollo was more divergent from others whereas South Wollo and South Gondar were observed to be closely related.

Table 6. Pairwise Nei's genetic distances between geographical origins of O. crenata populations

Populations	South Tigray	South wollo	North wollo	South Gondar
South Tigray	0.0000			
South wollo	0.1248	0.0000		
North wollo	0.3963	0.4449	0.0000	
South Gondar	0.1308	0.1172	0.4776	0.0000

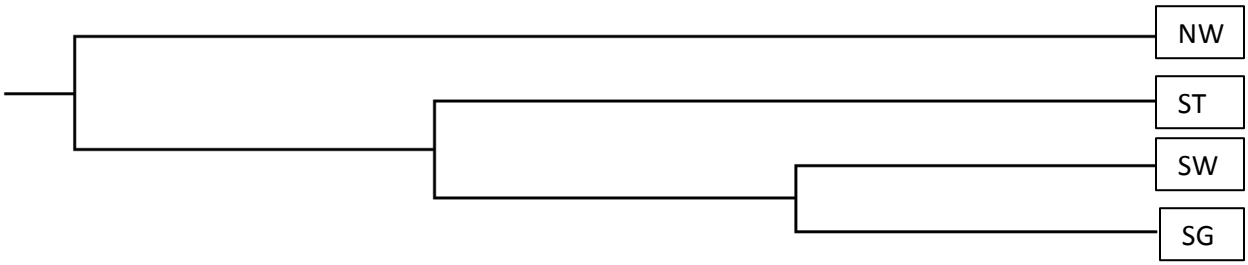


Figure 10. Dendogram of four location of O. crenata populations based on Nei's genetic distance, where: NW= North Wollo, ST= South Tigray, SW= South Wollo, SG= South Gondar

Unweighted Pair-Group Methods Using Arithmetic Averages (UPGMA) cluster analysis was also carried out for 96 individual samples of Ethiopian *O. crenata*. Based on this cluster analysis the 96 Ethiopian O. crenata samples were grouped into two major clusters, 'Group-1' and 'Group-2' (Figure 11).

Cluster group-1 was consisted totally 69 samples; of 21, 22, 4, and 22 samples from South Tigray, South Wollo, North Wollo, and South Gondar, respectively. Cluster group 1 was further subdivided into four sub clusters A, B, C and D. Three samples were grouped in sub cluster 1-A that comprised genotypes from South Tigray and South Wollo, while the rest clusters were composed of individual from various geographical populations and zones.

Cluster group-2 was consisted totally 27 samples; of 9, 9, 1, and 8 samples from South Tigray, South Wollo, North Wollo, and South Gondar, respectively. Cluster group 1 was also further subdivided into two sub-clusters A and B. Sub cluster 2-A was comprised 16 genotypes from all locations, while sub-cluster 1-B comprised 11 genotypes from South Tigray, South Wollo, and South Gondar.

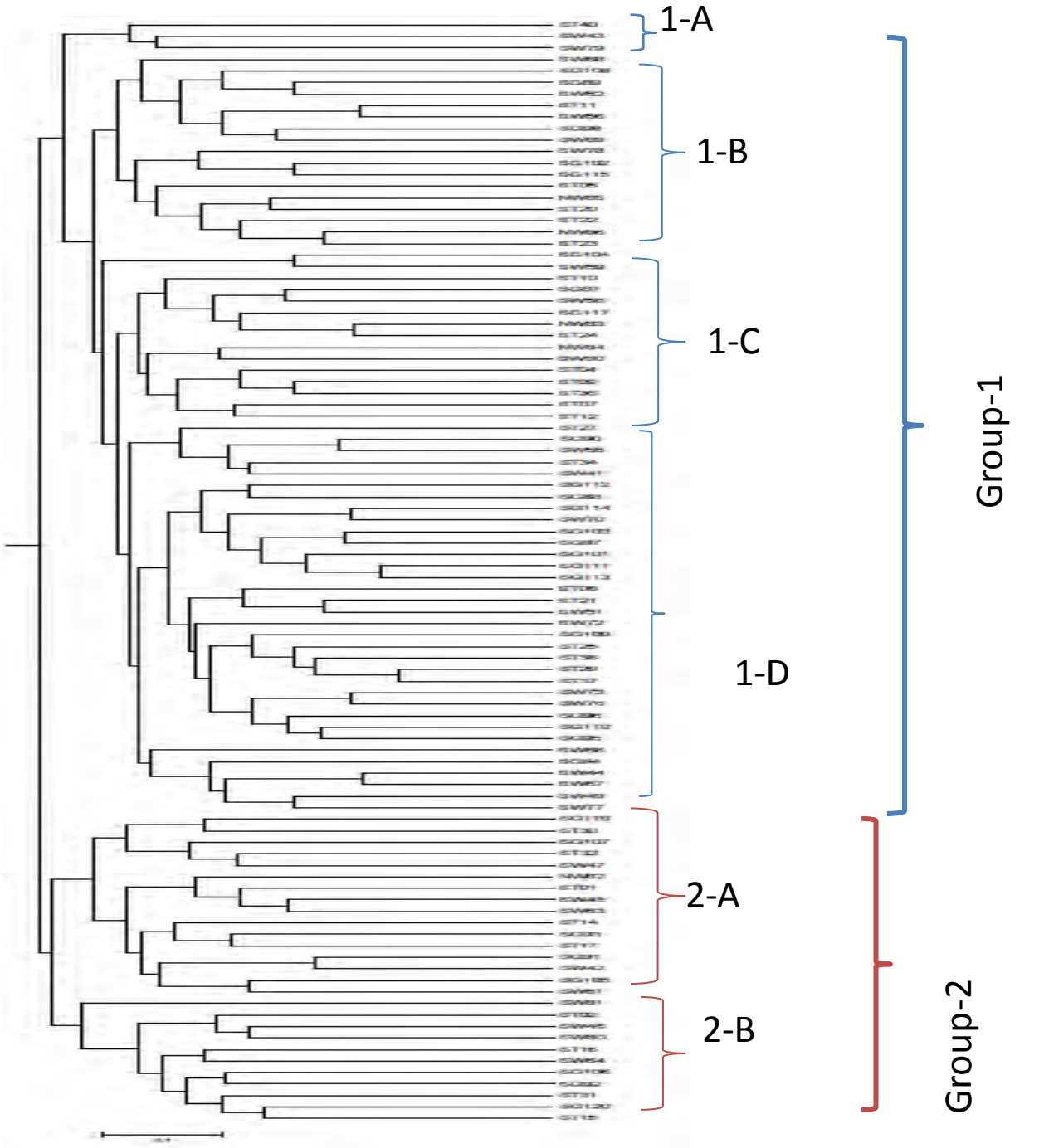


Figure 11. UPGMA dendrogram derived from microsatellite marker (SSR) analysis of 96 samples of *Orobanche crenata* from Ethiopia

4.5. Principal Coordinate Analysis (PCO)

The genetic relatedness among the Ethiopian *O. crenata* samples were further investigated using principal coordinate (PCoA) analysis (Figure 12).

The first two coordinates explained 73.6% of the total variation; however the samples were not grouped based on their origin of location.

The PCO analysis classified the 96 samples into three major groups (I, II, III) regardless of their geographic origin. However, Samples from South Gondar form their own group (Group III) and distant itself from other groups indicating uniqueness of their gene pool.

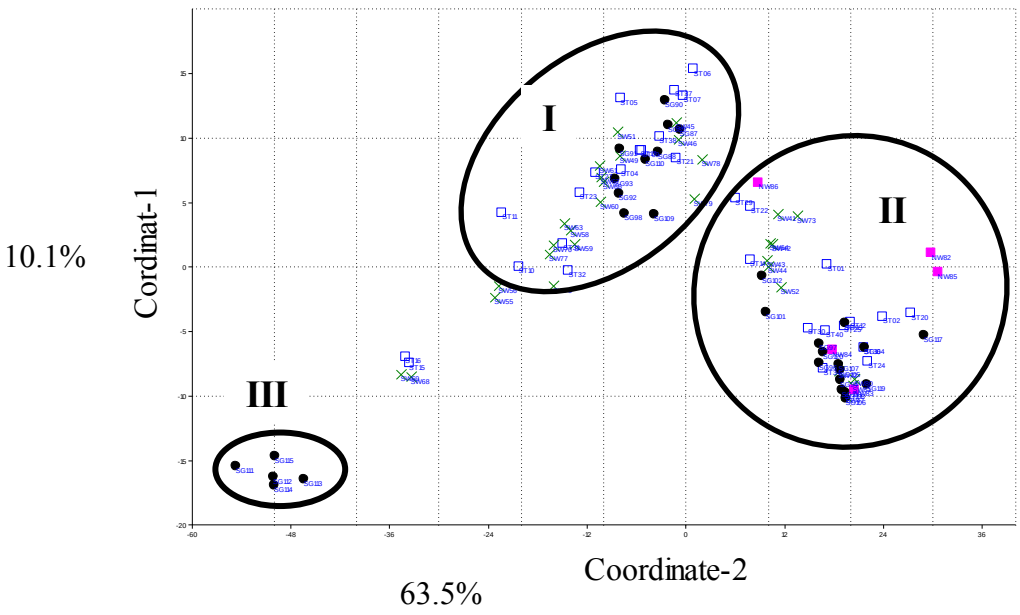


Figure 12. Principal coordinate analysis of Ethiopian *O. crenata* samples from different location

4.6. Structure analysis

To examine the genetic structure of Ethiopian O. crenata based on 96 samples, the genotypic data for 11 SSRs were analyzed using a model-based approach implemented in STRUCTURE. The LnP(D) for each given *k* increased with the increase of *k* and the most significant change and sharp peak was observed when *k* increased from 2 to 3 (Figure 13).

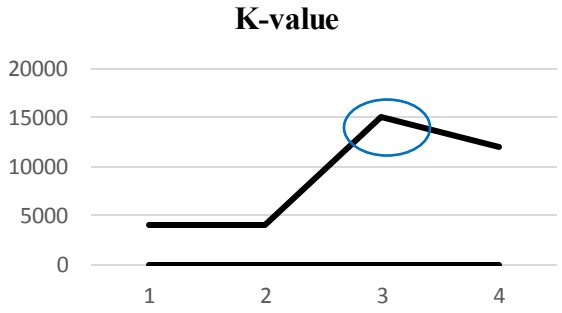


Figure 13. Structure estimation of the number of subpopulations for K ranging from 1 to 12

Accordingly, the total panel could be divided into three sub-population (SP1, SP2, and SP3) as suggested by the STRUCTURE analysis (Figure 14). The SP1 and SP3 included 37 and 23 genotypes collected from all locations. Whereas the SP2 included 36 genotypes originated from all locations except North Wollo.

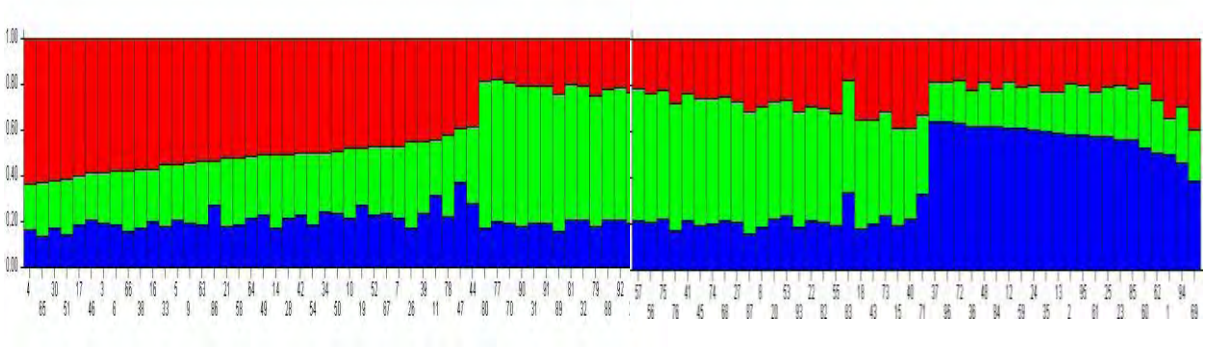


Figure 14. Three sub-population (SP) inferred from structure analysis. The vertical value of each sub-population indicates the membership coefficients for each individual, and the digits on the horizontal coordinate represent the samples corresponding to Appendix 1. Red:SP1; Green: SP2; Blue: SP3

4.7. Analysis of Molecular Variance (AMOVA)

The Analysis of Molecular Variation (AMOVA) revealed that 3 % of the variation resulted from the difference among populations, 97% variation from within populations (Table 7). This implies that there is high genetic diversity within population and gene flow among these populations..

Table 7. AMOVA showing the distribution of genetic diversity within and among populations of Ethiopian *O. crenata* from different sources of origins

Source of Variation	Df	SS	MS	Variance	
				Estimated	%
Among Population	3	74.845	24.948	0.480	3%
Within Population	92	1308.884	14.227	14.227	97%
Total	95	1383.729	39.175	14.707	

Where: Df =degrees of freedom; SS= sum of squares and MS=mean squares

5. Discussions

5.1. Transferability of SSR markers in *Orobanche*

The ability to transfer SSRs from one species to another depends on the primer sites flanking SSR motifs being conserved between the taxa (Phillip *et al.,* 2010). Thus the possibility of cross-species transferability of SSRs from closely related non source species becomes advantageous and minimizes the cost of SSR marker development.

Closely related species are more likely to share SSR priming sites than distantly related species and when there is cross-species transferability with high levels of polymorphism exist (Pineda *et al.,* 2013a).

In this study we evaluated the transferability of SSR markers developed for *O. cumana* species by Pineda *et al.,* (2013b) to *O. crenata* species that belongs to the same genera; and uses the shared 11 SSR markers to study the diversity of *O. crenata* from northern and north western Ethiopia. Marker transferability to *O. crenata* was moderate, with 36% (11) of the 30 SSRs tested yielding fragments of moderate to high quality for Ethiopian *O. crenata* population. Similarly, Pineda *et al.,* (2013b) conducted SSR marker transferability test from *O. cumana* to *O. cernua* it is close relatives of *O. cumana* and reported high marker transferability with 92.4% (145) of the 157 SSRs tested as compared to study.

One of the advantages of SSR markers is their high level of polymorphism, even between closely related lines (Madesis *et al.,* 2013). In this study all cross amplified markers showed PIC value more than 0.5 indicating their high resolution power to apply for further in-depth study on *O. crenata* populations.

Transferability of SSR loci isolated from one species to the other species has great advantage and it provides evidence that primer sites and flanking regions are conserved across species Several other authors were also conducted similar studies on other plants, such as Phillip *et al.,* (2010) on *Cornus* (Cornaceae) Species; Pineda *et al.,* (2013b) on *Orobanche cernua* species; Ludymila, *et al.,* (2014) on *Coffea canephora*. These results showed the successfully transferability of SSR marker from one species to other closely related and can be applied to investigate diversity and other genomic study without spending time and cost on SSR marker development.

5.2. Extents of SSR variability within population of *O. crenata*

In this study 11 cross amplified SSR markers have been used to detect variability in *O. crenata* genotypes and evaluate their genetic diversity and relatedness. The mean number of alleles per SSR locus (9.6) detected among the 96 *Orobanche crenata* in the current study was higher than those reported on *Orobanche cumana* by Pineda *et al.* (2013a), with mean allele per locus (2.2).

The mean gene diversity observed in current studied populations was 0.82 with the maximum gene diversity record (0.93), high PIC value (0.92), and high number of allele (17) in marker Ocum-059. In contrast, lower gene diversity was recorded in marker Ocum-063 (0.64) with least number of allele (4) and PIC values (0.59) as compared to other loci. The high levels of gene diversity of SSR markers observed in this study was probably due to the presence of an extensive genetic diversity in these *O. crenata* genotypes that represented different geographic origins and linages.

SSR markers have the power to resolve hetrozygociy and homozygocity nature of tested individuals **(**Carla *et al.,* 2015**).** In this study except markers Ocum-063 and Ocum-094 other

markers revealed hetrozygocity of studied individuals with the mean hetrozygocity (0.38), the higher hetrozygocity of O. crenata was revealed by marker Ocum-003 (0.72).

The PIC value of SSR markers could be varying by different species and technique apply to investigate genetic diversity of individuals. SSR markers originally developed on *O. cumana* by Penida *et al.,* (2013b) the PIC value in their study was 0.52 in average which is a bit less than the present study 0.75. This difference may arose due to species and difference technique used to SSR genotyping, i.e. agarose gel electrophoresis versus the high resolution capillary gel electrophoresis used in this study.

The 11 SSR markers used in this study amplified a total of 106 alleles over 96 genotypes of *O. crenata* which revealed high mean values of number of allele, gene diversity, heterozygosity, and PIC were recorded 7, 0.77, 0.72, and 0.75 respectively. These points towards the usefulness of most of these SSR markers for future *O. crenata* germplasm characterization and evaluation and generate relevant data on biology and genetics of this noxious weed. Hence; this study gave an evidence for occurrence of high degree of genetic variability among Ethiopian *O. crenata* genotypes in north and north western parts of Ethiopia.

5.3. Magnitude of genetic diversity among and within population of *O. crenata*

Understanding the variability within and between *Orobanche* populations is important for determining crop breeding strategies for *Orobanche* effective control and diagnosis approach (Pineda *et al.,* 2013a).

Analysis on the basis of geographical origins, South Tigray, South Wollo, North Wollo and South Gondar exhibited similarly higher values on percentage polymorphism, allele number, observed and expected heterozygosity, allelic range, gene number and diversity, and Garza-Williamson index except population from South Wollo which is relatively lower values recorded, it shows the presence of high within geographical origins genetic diversity. Moreover, among the four geographic populations, South Wollo accumulated more genetic diversity as compared to others, on the contrary populations from North Wollo showed the least genetic diversity, which could be explained by the effect of population size on genetic diversity.

Pairwise Nei's genetic distances between geographical origins of *O. crenata* populations showed narrow genetic distance among each pair. As a result populations from South Wollo and South Gondar have shown the least genetic distance to each other with 88% similarity. Moreover, Nei's genetic distance cluster analysis for populations revealed that populations form North Wollo was more divergent from other populations; it implies the uniqueness of North Wollo *O. crenata* genotypes.

For comparison of the geographical origins *of O. crenata* populations and to assess their distinctiveness, AMOVA was utilized. In this study AMOVA also revealed high genetic diversity within population than diversity among populations.

These higher within population variation could be associated with the nature of the parasitic weeds. For instance, Teklay *et al.,* (2013b) explains that the distribution mechanisms of *O. crenata* in Ethiopia were through different mechanism like via contaminated seed exchange, free grazing; farm equipment, transport and wind were identified as the major seed dispersion agent. These could be cited as the major dispatch mechanisms that resulted in observed higher gene flow and this suggests that there could be high gene flow among regions which causes less diversity among them compared to the within variation.

In abroad little previous study on genetic diversity in *O. crenata* populations from Israel, using RAPD marker (Ilan *et al.,* 1996); from Egypt using RAPD (Zeid *et al.,* 1997); from Spain and Israel using ISSR (Belén *et al.,* 2002) were reported that there is high genetic diversity of *O. crenata* in their respective countries. So far in Ethiopia the present study is the pioneer for *O. crenata* diversity analysis thereby SSR marker reveals high within population variability of studied genotypes.

5.4. Population structure and pattern of genetic differentiation on *O. crenata*

Knowledge about the patterns of population structure is essential for efficient germplasm organization (Tesfamichael *et al.,* 2014). In order to visualize the genetic structure and relationship among all studied *O. crenata* samples from different geographical origins; UPGMA clustering, PCo analysis, STRUCTURE analysis were performed.

In this study UPGMA cluster analysis demonstrated that the 96 samples clearly divided into two major group and six sub-groups. Principal coordinate analysis (PCO) also separated the 96 samples into three major groups. Using the maximum membership probability in STRUCTURE,

by using 11 SSR loci 96 samples were assigned to three sub-population. In the present study all the variation components confirmed by AMOVA shows that, there is high genetic diversity among individual samples within the population (97%) however low among populations (3%) genetic diversity.

From this study UPGMA analysis showed that most of the group comprise samples from different location (ST=South Tigray; SW=South Wollo; NW=North Wollo, SG=South Gondar) and all sub-cluster also contain almost equal proportion of samples from different geographical sources. The two dimensional plot (PCO) also confirmed that the same pattern like UGMA dendogram, except five isolated sample in group III which is entirely from South Gondar.

In this study like UPGMA and PCO analysis; STRUCTURE analysis result also confirmed that the members of each subpopulation were from different source of origin. Hence, this study showed that there is no strong correlation between geographic distance and genetic relatedness and/or diversity of individual's genotypes. The grouping obtained through UPGMA tree was found to be comparable to the clusters obtained by STRUCTURE analysis. However, clear distinction cannot be made about the distribution of genotypes in different groups based on their origins.

Clustering of populations was not associated with geographical origin of individuals; it could be justified by, being *O. crenata* is an outcrossing species it leads high gene flows among population showing much genetic diversity within populations (Paran *et al.,* 1997). This occurrence supported by AMOVA result which revealed high within population diversity. Gene flow increases genetic variation within populations because it brings in new alleles and reduces genetic differences among populations because alleles are being exchanged and shared among the different geographical population.

6. Conclusion

This study confirmed that previously developed SSR loci from one species can be potentially used to closely related species for further genetic experiments. This study established and identifies that informative and functional set of good quality SSR marker. It was found that 11SSR primers were functionally capable of cross amplification in *O. crenata*; even if it was originally developed for *O. cumana* species.

The SSR markers used in this study were revealed significant genetic variation within and among *O. crenata* populations and able to structure the Ethiopian *O. crenata* genotype. However, based on the 11 SSR markers used, it would be difficult to distinguish studied genotype based on their original populations. Hence, there is a need to further investigate the diversity with more SSR markers and samples from Ethiopia.

In this study even if most geographical population has similarly high genetic diversity and hetrozygosity, South Wollo genotypes have high genetic diversity and hetrozygosity as compared to others; in contrast North Wollo genotypes also have relatively low genetic diversity and low hetrozygocity as compared to others.

UPGMA, PCO, and STRUCTURE analysis showed that grouping of most of the *O. crenata* samples were not associated with their respective geographical origins. Almost all clusters comprise samples from all locations. It is due to high gene flow among geographical origins via various seed dissension mechanisms.

Eventually high level of genetic diversity achieved from this study could be used as prior information for *O. creanta* control strategies and diagnosis mechanism. Furthermore the finding could be applied for the improvement of faba bean breeding and enhance its productivity among small holders framer

7. Recommendation

The findings of this study provided available and validated primers for *Orobanche crenata*. These primers will facilitate further studies on the species diversity, genetic mapping, and establishing parasite weed control strategies.

Further studies on *O. crenata* genetic diversity using more number of molecular markers in its current distribution area are required for further understanding of national genetic variability, dispersal routes and evolutionary characteristics.

Molecular host parasitic weed interaction studies would have positive impact on the prevention and control of *O. crenata*, there by attention should be given in this regard.

In addition to this study output, to hasten the effective control and diagnosis of *O. crenata* association mapping of important *orobanche* agronomic trait with faba bean genotype should be conducted with strong work on resistance breeding.

8. Reference lists

Ahmed, L. and Abdel, M. (2012). DNA Based Techniques for Studying Genetic Diversity, genetic diversity in microorganisms, ISBN: 978-953-51-0064-5

Alessia (2009). Review on Biological Characteristics and Control of *Orobanche Crenata* Forsk. *Ital. J. Agron.* **1:** 53-68.

Assefa Admasu (2008). Integrated *orobanche* management in food legumes (faba bean): Experience of farmers' field school (FFS) in Dessie-zuria District, Ethiopia**. In:** Progress on farmer training in parasitic weed management, 45-50.

Barakat E. and Abu I. (2008). Integrated *Orobanche* management. **In:** Progress on farmer training in parasitic weed management, 17-29.

Belen, R., Satovic, Z., Rubiales, D., Torres, A.M., Cubero, J.I., Katzir, N. and Joel, D. M. (2002). Variation among and within populations of the parasitic weed *Orobanche crenata* from Spain and Israel revealed by inter simple sequence repeat markers. *Phytopathology* **92:** 1262-1266.

Besufekad Tadesse, Admassu Tadesse, Rezene Fessehaie, 1999. Orobanche problem in south Wollo. *Research review,* EARO.

Borsch, T., Hilu, K.W., Quandt, D., Wilde, V., Neinhuis, C. and Barthlott, W. (2003). Noncoding plastid *trnT-trnF* sequences reveal a well resolved phylogeny of basal angiosperms. *J. Evol. Biol.* **16**: 558–576.

Botstein, D., White, R., Skolnick, M. and Davis, R. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Genet*. **32:** 314-331.

Brenner, B.M., Swede, H., Jones, B.A., Anderson, G.R. and Stoler, D.L. (2011). Genomic Instability Measured by Inter-(Simple Sequence Repeat) PCR and High-Resolution

Microsatellite Instability are Prognostic of Colorectal Carcinoma Survival After Surgical Resection. *Ann Surg Oncol.* (http://www.annsurgoncol.org/journal/10434/0/0/1708/0/).

Carla,V.F.**,** Natalia, A.**,** Juan, G.R.**,** Jeremias, Z.**,,** Andrea, P., Diego, C.**,** Maria, V. M.**,** Corina, M. F.**,** Daniel, A.**,** Ruth, A. H.**,** Horacio, E.H.**,** Norma, B. P. and Veronica, V.L. (2015). Population structure and genetic diversity characterization of a sunflower association mapping population using SSR and SNP markers. *BMC Plant Biology* **15:** 52-67

Carlos, B.M., Cerqueira, S., Onildo, N.J., Elisa S.L., Santos, R.X. and Anete, P.S. (2014). Genetic breeding and diversity of the genus *passiflora*: progress and perspectives in molecular and genetic studies. *Int. J. Mol. Sci.* **15:** 14122–14152.

Castejon, M. (1989). Parasitism of broomrape (Orobanche cernua, Loefl.; O. cumana Wallr.) in sunflower (Helianthus annuus L.), its response to glyphosate and intraspecific variability isoenzyme . PhD thesis, School of Biology, University of Sevilla, Spain.

Castejon-Munoz, M., Suso, MJ, Romero-Munoz, F. and Garciatorres, L. (1991). Iso- enzymatic study of broomrape (*Orobanche cernua*) populations infesting sunflower. (Helianthus annuus).**In:** Proceedings of the 5th International Symposium on Parasitic Weeds, 313–319. International Maize and Wheat ImprovementCenter(CIMMYT). Nairobi, Kenya.

Central Statistical Agency (CSA) (2013). Report on area and production of major crops: private peasant holdings, meher season). *Statistical bulletin* **532**:10-14.

Christensen. S. (2009). Site-specific weed control technologies. *Weed Res.* **49:** 233–241.

Cubero J.I., Moreno M.T. and Martin A., (1979). Meiotic behavior in *Orobanche crenata* Forsk. Proceedings of Second International Symposium on Parasitic Weeds. *North Carolina State University, Raleigh*, 73–78.

Cubry, P., Pujade, V., Garcia, D., Espeout, S., Leguen, V., Granet, F. and Seguin, M. (2014) Development and characterization of a new set of 164 polymorphic EST-SSR markers for diversity and breeding studies in rubber tree. *Plant Breeding* 1-8.

Dammer, K.H., Schweigert, T. and Wittmann, C.H. (1999). Probability maps for risk assessment in a patchy weed control. *Precis Agr.* **1:** 185–198.

Das Gupta and Swati Sen Mandi (2013). Species Specific AFLP Markers for authentication of Zanthoxylum acanthopodium & Zanthoxylum oxyphyllum Debmalya. *Journal of Medicinal Plants Studies* **1:** 1-9.

Diego Rubiales, Alejandro P´erez-de-Luque, M´onica Fern´andez-Aparico, Josefina C. Sillero, Bel´en Rom´an, Mohamed Kharrat, Shaban Khalil, Daniel M. Joel and Charlie Riches, (2006). Screening techniques and sources of resistance against parasitic weeds in grain legumes**.** *Springer* **147:** 187–199.

Dor, E., Aly, R. and Hershenhorn, J. (2014). Pomegranate (Punica granatum) as host of the broomrapes. Phelipanche aegyptiaca and Orobanche crenata in Israel. Plant Disease, **98:** 859.

Duran, C.N., Appleby, T., Clark, D., Wood, M., Batley, I.J. and Edwards, D. (2009). Auto SNPdb: an annotated single nucleotide polymorphism database for crop plants. *Nucleic Acids Res.* **37:** 951–953.

Edwards, D. and Batley, J. (2010). Plant genome sequencing: applications for plant improvement. *Plant Biotech. J.* **8:** 2–9.

Elena, A., Miguel, E. and Roger, T. 2010. Chetelat Genetic diversity and population structure in the tomato-like nightshades*Solanum lycopersicoides* and *S. sitiens. Ann Bot.* **105:** 535–554.

Francesco, E., Silvia, L., Lukasz, G., Valentina, C., Marco, S., Michela, T., Sean, M., José, M. M., Eva, Z., Flavia, M. and Stella, G. (2013). Genetic diversity and population structure assessed by SSR. and SNP markers in a large germplasm collection of grape. *Plant Biol*. 13: 39.

Fernandez-Aparicio, M., Flores, F. and Rubiales D. (2009). Recognition of root exudates by seeds of broomrape (Orobanche and Phelipanche) species. *Annals of Botany* **103:** 423–431.

Fernández, A., M., Yoneyama, K. And Rubiales, D. (2011). The role of strigolactones in host specificity of *Orobanche* and Phelipanche seed germination. *Seed Sci. Res.* **21:** 55–61.

Foy, C.L., Jain R. and Jacobsohn R. (1989). Recent approaches for chemical control of broomrape (*Orobanche spp*.). *Rev.Weed Sci.* **4:**123–152.

Gagne, G., Roeckel-Drevet, P. and Grezes-Besset B. (1998). Study of the variability and evolution of *Orobanche cumana* populations infesting sunflower in different European countries. *Theoretical and Applied Genetics* **96:**1216–1222.

Garcia-Torres L., Lopez-Granados F. and Castejon-Munoz M., (1994). Pre-emergence herbicides for the control of broomrape (*Orobanch ecernua*) in sunflower . *Weed Res*. **34:** 395–402.

Gerald, M.S., Teresa, P., Alison, E.C. and Hanna, W.S. (2014). Chromosome Numbers and Karyotype Evolution in Holoparasitic Orobanche (Orobanchaceae)and Related Genera. *American Journal of Botany* **91:** 439–448.

Girma, G., Tesfaye, K. and Bekele E. (2010). Inter Simple Sequence Repeat (ISSR) analysis of wild and cultivated rice species from Ethiopia. *Afr. J. of Biot*. ***9:*** *5048-5059.*

Gökbayrak, Z., Özer, C. and Söylemezoğlu, G. (2010). Use of morphological markers to identify foliar disease resistance in grapevine. *The Journal of Animal & Plant Sciences* **20:** 243-247.

Grenz, J.H. and Sauerborn, J. (2007). Mechanisms limiting the geographical range of the parasitic weed Orobanche crenata. *Agriculture, Ecosystems and Environment* **122:** 275–281.

Gupta, P.K. and Varshney, R.K. (2000). The development and use of microsatellite markers for genetics and plant breeding with emphasis on bread wheat. *Euphytica* **113:** 163–185.

Habimana, S., Nduwumuremyi, A. and Chinama, R.J. (2014). Management of *orobanche* in field crops-A review. *Journal of Soil Science and Plant Nutrition*. **14:** 43-62.

Hailu, E., Getaneh, G., Sefera, T., Tadesse, N. and Bitew B. (2014). Faba Bean Gall; a New Threat for Faba Bean (*Vicia faba*) Production in Ethiopia. Adv Crop Sci Tech **2:** 144.

Hammer, O., Harper, D.A. and Ryan, P.D. (2001). PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontologia Electronica* **4:** 9.

Hocine, B.T, Christian, B., Catherine, T. and Patrick, T. (2004). Virulence diversity among branched broomrape (*O. ramosa* L.) populations in France. *Agron. Sustain. Dev*. **25:** 123–128.

Ilan, P., David, G. and Reuven, J. (1997). Variation between and within broomrape (Orobanche) species revealed by RAPD markers. *Heredity* **78:** 68-74.

Joel, D.M., Hershenhorn, J., Eizenberg, H., Aly, R., Ejeta, G., Rich, P.J., Ransom, J.K., Sauerborn, J. and Rubiales, D. (2007). Biology and management of weedy root parasites. *Hort. Rev*. **33:** 267–349.

Joel, D.M., Steffens, J.C., and Matthews, D.E., (1995). Germination of Weedy Root Parasites. Marcel Dekker: New York, USA *Inc.,* 567-598.

Joshi, S.P., Gupta, V.S., Aggarwal, R.K., Ranjekar, P.K., and Brar, D.S. (2000). Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. *Theor. Appl. Genet*. **100:** 1311–1320.

Jurado, M., Castejon, M. and Garcia-Torres, L. (1997). Broad bean and lentil seed treatments with imidazolinones for the control of broomrape (*Orobanche crenata*). *J. Agricultural Science* **129:** 307-314.

Kamal, Mohamed, I. and Lytton J.M.(2008). Taxonomy of Agronomically Important Striga and Orobanche Species. **In:** Progress on farmer training in parasitic weed management, 7-14.

Karaca, M. (2013). Isozymes as Biochemical Markers in Plant Genetics. *International Journal of Agri. Science* **3:** 851-861.

Kebreab, E. and Murdoch, A.J. (1999). Effect of moisture and temperature on the longevity of Orobanche seeds. *Weed Res*. **39:** 199–211.

King, R.C. and Stansfield, W.D. (1990). A dictionary of genetics. 4th ed., Oxford University Press, New York-Oxford, 188.

Kroschel, J. (2001). A Technical Manual for Parasitic Weed Res. and Extension. Kluwer Academic Publishers, Dordrecht, the Netherlands, 256.

Kumar, P., Gupta, V.K., Misra, A.K.,. Modi, D.R. and Pandey, B.K. (2009). Potential of Molecular Markers in Plant Biotechnology. *Plant Omics Journal Southern Cross Journals* **2:** 141-162.

Kwok, P.Y., Deng, Q., Zakeri, H., Taylor, S.L. and Nickerson, D. (1996). Increasing the information content of STS-based genome maps: identifying polymorphisms in mapped STSs. *Genomics* **31:** 123–126.

Lark, K.G., Weisemann, J.M., Matthews, B.F., Palmer, R., Chase, K. and Macalma, T. (1993) . A genetic map of soybean *(Glycine max* L.) using an intraspecific cross of two cultivars: 'Minosy' and 'Noir 1'. *Theor Appl Genet* **6:** 901 906.

Linke, K.H. (1998). Status quo of *Orobanche* control: preventive, cultural and physical control. Joint Action to control *Orobanche* in the wana-region: Experiences from Morocco. Rabat , Morocco.

Liu, K. and Muse, S.V. (2005). Power marker: Integrated analysis environment for genetic molecular data. *Bioinformatics* **21:** 2128-2129.

Ludymila, B.M., Taís, C.B., Maria, A.G., Eveline, T.C., Ronald, M.P. and Rodrigo, M.L. (2014). Transferability of microsatellite loci in *Coffea canephora. American Jornal of crop science **8:** 987*-991.

Madesis, P., Ganopoulos, I. and Tsaftaris, A. (2013) Microsatellites: Evolution and contribution. In: Microsatellites: Methods and Protocols. Methods in Molecular Biology. *Humana Press* **106**: 1–13.

Maheswaran, M. (2004). Molecular Markers: History, Features and Applications. *Advanced Biotech*. 17-24.

Manschadi, A.M., Kroschel, J. and Sauerborn, J. (1996). Dry matter production and partitioning in the host-parasite association Vicia faba- *Orobanche crenata*. *Angewandte Botanik* **70:** 224-229.

Marko, M., Jelka, Š., Branka, J. and Vladimir, M. (2008). The efficiency of AFLP and SSR markers in genetic diversity estimation and gene pool classification of common bean (*Phaseolus vulgaris L.*). *Acta agriculturae Slovenica*, 87-96.

Mathieu, P., Andre, J., Gerald, M., Schneeweiss, J., Macas, Petr, N.,, Heidrun, G., Eva M. and Susanne, S.R. (2012). Next-Generation Sequencing Reveals the Impact of Repetitive DNA Across Phylogenetically Closely Related Genomes of *Orobanchaceae*. *Mol. Biol. Evol.* **29:** 3601–3611.

Mesa-Garcia J. and Garcia-Torres L., (1984). A comparison index for *Orobanche crenata* Forsk effects on broad bean (Vicia faba L). *Weed Res.* **24:** 379–382.

Mishra, P.K., Fox, R.T.V. and Culhamm, A. (2003). Inter-simple sequence repeat and aggressiveness analyses revealed high genetic diversity, recombination and longrange dispersal in Fusarium culmorum. School of Plant Sciences, the University of Reading, Whiteknights, *Association of Applied Biologists*.

Moliero, R.L., Garcia, A.B., Collado, M., Raranciuc, S., Dominguez, J. and Melero, J.M. (2013). Pathogenic and molecular diversity in highly virulent populations of the parasitic weed Orobanche Cumana (sunflower broomrape) from Europe. *International Journal of weed Biology, Ecology and Vegitation Management.* DOI: 10.1111/wre.12056

Oveisi, M., Yousefi A.R. and Gonzalez-Andujar, J.L. (2010). Spatial distribution and temporal stability of crenate broomrape (Orobanche crenata Forsk) in faba bean(Viciafaba L.): A long-term study at two localities. *Crop Protection* **9:** 717–720.

Paran, I, Gidoni, D. and Jacobsohn, R. (1997). Variation between and within broomrape (Orobanche) species revealed by RAPD markers. *Heredity* **78:** 68-74.

Parker, C. (1994). The present state of the Orobanche problem. **In:** Proceedings of the Third International Workshop on Orobanche and Related Striga Research, 17–26.

Parker, C. and Riches, C.R. (1993). Parasitic Weeds of the World. CAB International, Wallingford.

Peakall, R. and Smouse, P.E. (2006). GENEALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288-295.

Phillip, A.W., John, K.M., Stan, C.H. and John A.S. (2010). Transfer of *Cornus florida* and *C. kousa* Simple Sequence Repeats to Selected Cornus (Cornaceae) Species. *J. Amer. Soc. Hort. Sci.* **135**:279–288.

Pineda, M.R., Velasco, L., Fern, A.J., Fern, A.M. and Erez-Vich, B.P. (2013a). Genetic diversity of *Orobanche cumana* populations from Spain assessed using SSR markers. *Weed Research* **53:** 279–289.

Pineda, M.R., Velasco, L. and Erez-Vich, B.P. (2013b). Identification, characterization and discriminatory power of microsatellite markers in the parasitic weed *Orobanche cumana*. *Weed Research* **54**: 120–132.

Pritchard, J.K., Stephens, M. and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Rajeev, K., Varshney, Andreas, G. and Mark, E.S. (2005). Genic microsatellite markers in plants: features and applications. *Trends in Biotechnology* **23**: 49-55.

Rajeev, K., Varshney, Thudi, M., Ramesh, K.A. and Andreas, B. (2007). Genic Molecular Markers In Plants: Development And Applications. *Genomics Approaches and Platforms* **1:** 13–29.

Reddy, M.P., Sarla, N. and Siddiq, E.A. (2002). Inter simple sequence repeat polymorphism and its application in plant breeding. *Euphytica* **128:** 9–17.

Rezene Fissihaye and Gerba Leta (2003). Weed research in high land food legumes of ethiopia. Holeta Agricultural Research Center, EIAR, Ethiopia. Research Review, 1-36.

Rogers, J.S. (1972). Measures of genetic similarity and genetic distance. In: Studies in Genetics, *University of Texas Publication Austin* **2:** 145-153.

Roman, B., Alfaro, C., Torres, A.M., Moreno, M.T., Satovic, Z., Pujadas, A. and Rubiales, D. (2003). Genetic Relation Among Orobanche Species as Reviled by RAPD Analysis. *Annals of Botany* **91:** 637-642.

Román, B., Rubiales, D., Torres, A.M., Cubero, J.I. and Satovic, Z. (2001) Genetic diversity in *Orobanche crenata* populations from southern Spain. *Theor Appl Genet*. **103:**1108–1114.

Román, B., Satovic, Z., Alfaro, C., Moreno, M.T., Kharrat, M., Pérez-de-Luque, A. and Rubiales, D., (2007). Host differentiation in *Orobanche foetida* Poir Flora-Morphology, Distribution. *Functional Ecology of Plant* **202:** 201-208.

Roy, A., Frascaria, N., Mackay, J. and Bonsquet, J. (1992). Segregating random amplified polymorphic DNA (RAPDs) in Betula alleghaniensis. *Theor. Appl. Genet*. **85:** 173-180.

Rubiales, D., Verkleij, J., Batchvarova, R. and Joel D. (2002). Proceedings of the meeting Broomrape: biology and resistance. Parasitic Plant Management in Sustainable Agriculture, Joint Meeting of WG , Sofia,Bulgaria.

Ruvolo, G., Fattouh, R.R., Bosco, L., Brucculeri, A.M. and Cittadini, E. (2013). New molecular markers for the evaluation of gamete quality. *J. Assist. Reprod.Genet*. **30:** 207–212.

Saeed, T., Esfahani, B.S. and Ghlolamreza, B. (2009). AFLP markers for the assessment of genetic diversity in european and North American potato varieties cultivated in Iran. *Crop Breeding and Applied Biotechnology* **9:** 75-86.

Sahile, G., Abebe, G. and Tawaha, A.l. (2005). Effect of soil solarizationon *Orobanche* soil seed bank and tomato yield in central rift valley of Ethiopia. *World J. Agr.Sci.* **1:** 143–147.

Semagn, K., Bjørnstad, A. and Ndjiondjop, M.N., (2006). An overview of molecular marker methods for plants. *African Journal of Biotechnology* **5:** 2540-2568.

Shu, C. (2011). Evaluation of Bioactivity of Phytotoxins from Pathogenic Fungi of Orobanche Spp. PhD Dissertation, University of Athens, Grecee.

Sonja, G., Snje, B., Svetislav, P., Tihomir, U., Marijana, T. And Vinko, K. (2008). Comparison of morphological and RAPD markers in evaluation of red clover (*Trifolium pratense L.)* changes caused by natural selection. Periodicum Biologorum **3:** 237–242.

Stewart, G.R. (1990). The physiology and biochemistry of parasitic angiosperm, annual review plant physiology. *Plant Mol. Biol*. **41:** 127-151.

Teklay Abebe, Hadas Beyene and Yemane Nega, (2013a). Distribution and economic importance of broomrape (*orobanche crenata)* in food legumes production of south tigray, Ethiopia. *ESci J. Crop Prod.* **2:** 101-106.

Teklay Abebe, Kiros Meles, Yemane Nega, Hadas Beyene and Abrha Kebede (2013b). Interaction between broomrape (*Orobanche crenata*) and resistance faba bean genotypes (*Vicia faba* L.) in Tigray region of Ethiopia. *Canadian Journal of Plant Protection* **1:** 104-109.

Teklay Abebe, Yemane Nega, Muez Mehari, Adhiena Mesele, Assefa Workineh and Hadas Beyene, (2015). Genotype by environment interaction of some faba bean genotypes under diverse broomrape environments of Tigray, Ethiopia. *Journal of plant Breeding and crop science* **7:** 79-86.

Teryokhin, E. (1997). Weed Broomrapes -Systematics, Ontogenesis, Biology and Evolution. Aufstieg-Verlag, Germany.

Tesfamichael, Githiri, R.M., Kasili, W., Skilton, R.A., Solomon, M. and Nyende, A.B. (2014). Genetic Diversity Analysis of Eritrean Sorghum (*Sorghum bicolor* L.*)* Germplasm using SSR Markers. *Molecular Plant Breeding* **5:** 1-12.

Tesfaye, K., Govers, K., Bekele, E. and Borsch, T. (2014). ISSR fingerprinting of *Coffea arabica* throughout Ethiopia reveals high variability in wild populations and distinguishes them from farmer's varieties. *Plant Systematics and Evolution* **300:** 881-897.

Tsumura, Y., Ohba, K. and Strauss, S.H. (1996). Diversity and inheritance of inter-simple sequence repeat polymorphisms in Douglas-fir (*Pseudotsuga menziesii*) and sugi (*Cryptomeria japonica*). *Theor. Appl. Genet*. **92:** 40–45.

Tuohy, J., Smith, E.A. and Stewart, G.R. (1986).The parasitic habit: Trends in morphological and ultrasonic reductionism. **In:** Proceedings of a Workshop on the Biology and Control of Orobanche, Wageningen, the Netherlands 86-95.

Vaz, P.M., Di´Az-Ruiz, R., Satovic, Z., Roma,´ N.B., Pujadas-Salva,` A.J. and Rubiales, D. (2008). Genetic diversity of Moroccan populations of Orobanche foetida: evolving from parasitising wild hosts to crop plants. *Weed Research* **48:** 179–186.

Vurro, M. and Gressel J., (2007).Novel biotechnologies for biocontrol agent enhancement and management, springer, dordrecht, the Netherlands, 384.

Yoseph Beyene, Anna-Maria, B. and Alexander, M.A. (2005). A comparative study of molecular and morphological methods of describing genetic relationships in traditional Ethiopian highland maize. *African Journal of Biotechnology* **4:** 586-595.

Zeid, M., Madkour, M., Koraiem, Y., Nawar, A., Soliman, M. and Zarroun F. (1997). Molecular Studies on *Orobanche. J. Phytopathology* **145:** 351-355.

9. Appendices

Appendix 1. Ethiopian *O.crenata* samples profile used for this study

Samples	Location/Zone	Worreda	Host	Latitude	Longitude	Altitude (m.a.s.l)
ST01	Southern Tigray	Ofla	FABA BEAN	12 30 51.3	039 15 43.6	2077
ST02	Southern Tigray	Ofla	FABA BEAN	12 30 51.3	039 16 29.8	2138
ST04	Southern Tigray	Ofla	FABA BEAN	12 30 15.4	039 24 42.3	2560
ST05	Southern Tigray	Ofla	FABA BEAN	12 30 15.4	039 24 42.3	2560
ST06	Southern Tigray	Ofla	PEA	12 30 14.9	039 24 42.2	2556
ST07	Southern Tigray	Ofla	PEA	12 30 14.9	039 24 42.2	2556
ST09	Southern Tigray	Ofla	PEA	12 30 15.8	039 24 43.6	2568
ST10	Southern Tigray	Ofla	LENTIL	12 30 39.6	039 25 84.6	2728
ST11	Southern Tigray	Ofla	LENTIL	12 30 39.6	039 25 84.6	2728
ST12	Southern Tigray	Ofla	FABA BEAN	12 31 03.4	039 29 32.4	2497
ST14	Southern Tigray	Ofla	FABA BEAN	12 34 41.8	039 31 48.6	2483
ST15	Southern Tigray	Ofla	FABA BEAN	12 34 30.1	039 31 37.8	2481
ST16	Southern Tigray	Ofla	FABA BEAN	12 34 30.1	039 31 37.8	2481
ST17	Southern Tigray	Ofla	FABA BEAN	12 32 40.5	039 30 52.8	2508
ST20	Southern Tigray	Alaje	FABA BEAN	12 56 43.8	039 31 16.4	2571
ST21	Southern Tigray	Ofla	FABA BEAN	12 37 00.9	039 31 21.8	2496
ST22	Southern Tigray	Ofla	FABA BEAN	12 37 03.0	039 31 20.4	2491
ST23	Southern Tigray	Ofla	PEA	12 37 02.4	039 31 19.9	2494
ST24	Southern Tigray	Ofla	FABA BEAN	12 36 58.9	039 31 24.9	2511
ST25	Southern Tigray	Ofla	FABA BEAN	12 36 55.8	039 31 19.7	2506
ST27	Southern Tigray	Ofla	FABA BEAN	12 32 57.6	039 31 00.0	2531
ST29	Southern Tigray	Ofla	FABA BEAN	12 31 57.2	039 30 58.1	2531
ST30	Southern Tigray	Ofla	FABA BEAN	12 32 56.3	039 30 52.0	2522
ST31	Southern Tigray	Ofla	FABA BEAN	12 32 56.3	039 30 52.0	2522
ST32	Southern Tigray	Ofla	FABA BEAN	12 32 56.3	039 30 52.0	2522
ST34	Southern Tigray	Ofla	LENTIL	12 36 42.7	039 26 42.5	2943
ST36	Southern Tigray	Ofla	FABA BEAN	12 36 49.8	039 26 45.1	2946
ST37	Southern Tigray	Ofla	FABA BEAN	12 36 58.5	039 26 44.0	2944
ST38	Southern Tigray	Ofla	FABA BEAN	12 36 58.5	039 26 44.0	2944
ST40	Southern Tigray	Ofla	FABA BEAN	12 34 33.7	039 28 48.0	2467
SW41	South Wollo	Dessie Zuria	FABA BEAN	11 08 24.0	039 29 48.1	2721
SW42	South Wollo	Dessie Zuria	FABA BEAN	11 08 24.0	039 29 48.1	2721
SW43	South Wollo	Dessie Zuria	FABA BEAN	11 08 24.0	039 29 48.1	2721
SW44	South Wollo	Dessie Zuria	FABA BEAN	11 08 24.0	039 29 48.1	2721
SW45	South Wollo	Dessie	FABA BEAN	11 08 01.5	039 29 51.3	2811

		Zuria				
SW46	South Wollo	Dessie Zuria	FABA BEAN	11 08 01.5	039 29 51.3	2811
SW47	South Wollo	Dessie Zuria	FABA BEAN	11 07 36.8	039 29 35.4	2791
SW49	South Wollo	Dessie Zuria	FABA BEAN	11 07 15.3	039 29 38.5	2834
SW51	South Wollo	Dessie Zuria	FABA BEAN	11 07 15.3	039 29 38.5	2834
SW52	South Wollo	Dessie Zuria	FABA BEAN	11 07 11.2	039 29 46.3	2866
SW55	South Wollo	Dessie Zuria	FABA BEAN	11 06 05.9	039 30 21.7	2821
SW56	South Wollo	Dessie Zuria	FABA BEAN	11 06 05.9	039 30 21.7	2821
SW58	South Wollo	Dessie Zuria	FABA BEAN	11 04 29.7	039 32 25.8	2591
SW59	South Wollo	Dessie Zuria	FABA BEAN	11 04 29.7	039 32 25.8	2591
SW60	South Wollo	Dessie Zuria	LENTIL	11 04 42.9	039 32 52.1	2461
SW61	South Wollo	Dessie Zuria	LENTIL	11 04 42.9	039 32 52.1	2461
SW63	South Wollo	KUTABER	PEA	11 18 56.6	039 27 19.0	2951
SW64	South Wollo	KUTABER	FABA BEAN	11 19 10.4	039 27 49.2	2873
SW66	South Wollo	KUTABER	FABA BEAN	11 18 25.8	039 28 37.2	2877
SW67	South Wollo	KUTABER	FABA BEAN	11 18 25.8	039 28 37.2	2877
SW68	South Wollo	KUTABER	FABA BEAN	11 15 42.5	039 30 39.1	2858
SW69	South Wollo	KUTABER	FABA BEAN	11 15 42.5	039 30 39.1	2858
SW70	South Wollo	KUTABER	LENTIL	11 15 42.5	039 30 39.1	2858
SW72	South Wollo	TENTA	FABA BEAN	11 21 31.9	039 14 22.2	2839
SW73	South Wollo	TENTA	FABA BEAN	11 22 54.0	039 15 05.7	2712
SW76	South Wollo	TENTA	FABA BEAN	11 22 22.1	039 14 38.8	2741
SW77	South Wollo	TENTA	FABA BEAN	11 22 22.1	039 14 38.8	2741
SW78	South Wollo	TENTA	FABA BEAN	11 20 45.9	039 14 43.6	2847
SW79	South Wollo	TENTA	FABA BEAN	11 20 45.9	039 14 43.6	2847
SW80	South Wollo	TENTA	Grass Pea	11 20 46.9	039 19 45.7	2855
SW81	South Wollo	TENTA	FABA BEAN	11 11 43.2	039 13 31.3	2987

NW82	North Wollo	MEKET	FABA BEAN	11 45 37.9	038 54 54.1	2206
NW83	North Wollo	LASTA	FABA BEAN	11 53 10.9	038 57 20.9	1986
NW84	North Wollo	MEKET	FABA BEAN	11 45 31.8	038 55 09.7	2341
NW85	North Wollo	WADLA	FABA BEAN	11 33 40.3	038 58 40.5	2581
NW86	North Wollo	MEKET	Lean sead	11 41 02.5	038 46 48.8	2871
SG87	South Gondar	T/GAYNT	FABA BEAN	11 37 09.1	038 32 53.1	2710
SG88	South Gondar	T/GAYNT	FABA BEAN	11 37 09.1	038 32 53.1	2710
SG89	South Gondar	T/GAYNT	FABA BEAN	11 37 09.1	038 32 53.1	2710
SG90	South Gondar	T/GAYNT	FABA BEAN	11 37 09.1	038 32 53.1	2710
SG91	South Gondar	T/GAYNT	FABA BEAN	11 37 10.9	038 32 02.5	2716
SG92	South Gondar	T/GAYNT	FABA BEAN	11 37 10.9	038 32 02.5	2716
SG93	South Gondar	T/GAYNT	FABA BEAN	11 37 10.9	038 32 02.5	2716
SG94	South Gondar	T/GAYNT	FABA BEAN	11 37 27.9	038 31 37.3	2742
SG95	South Gondar	T/GAYNT	FABA BEAN	11 37 43.5	038 31 23.6	2784
SG96	South Gondar	T/GAYNT	FABA BEAN	11 37 43.5	038 31 23.6	2784
SG97	South Gondar	T/GAYNT	FABA BEAN	11 37 43.5	038 31 23.6	2784
SG98	South Gondar	T/GAYNT	FABA BEAN	11 37 43.4	038 31 20.3	2789
SG101	South Gondar	T/GAYNT	LENTEL	11 38 37.7	038 30 46.4	2834
SG102	South Gondar	T/GAYNT	LENTEL	11 38 37.7	038 30 46.4	2834
SG103	South Gondar	T/GAYNT	FABA BEAN	11 38 50.7	038 30 36.8	2857
SG104	South Gondar	T/GAYNT	FABA BEAN	11 38 50.7	038 30 36.8	2857
SG105	South Gondar	T/GAYNT	FABA BEAN	11 38 50.7	038 30 36.8	2857
SG106	South Gondar	T/GAYNT	FABA BEAN	11 39 03.0	038 30 05.5	2913
SG107	South Gondar	T/GAYNT	FABA BEAN	11 39 03.0	038 30 05.5	2913
SG108	South Gondar	T/GAYNT	FABA BEAN	11 39 03.0	038 30 05.5	2913

SG109	South Gondar	T/GAYNT	FABA BEAN	11 39 53.1	038 29 59.7	2980
SG110	South Gondar	T/GAYNT	FABA BEAN	11 39 53.1	038 29 59.7	2980
SG111	South Gondar	T/GAYNT	FABA BEAN	11 40 57.7	038 28 03.5	3217
SG112	South Gondar	T/GAYNT	FABA BEAN	11 40 57.7	038 28 03.5	3217
SG113	South Gondar	T/GAYNT	FABA BEAN	11 40 57.7	038 28 03.5	3217
SG114	South Gondar	T/GAYNT	FABA BEAN	11 40 57.7	038 28 03.5	3217
SG115	South Gondar	T/GAYNT	FABA BEAN	11 40 57.7	038 28 03.5	3217
SG117	South Gondar	L/GAYNT	FABA BEAN	11 43 28.9	038 21 30.1	3132
SG119	South Gondar	FARTA	WEED	11 51 51.4	037 59 50.6	2604
SG120	South Gondar	FARTA	PEA	11 51 50.5	037 59 42.1	2622

Appendix 2. DNA extraction protocol

1. Pour CTAB solution (700 µl per sample) in a 15ml-tube and add 0.2 vol % Mercapto-ethanol (use fume hood!). Mercapto-ethanol is stored at 4

2. Aliquot CTAB in 1.5 ml eppendorf-caps and warm in water bath up to 65

3. Weigh in 100 mg fresh leave material (50mg dry material) per sample. Pulverize thoroughly using a clean mortar and pestle. For fresh material add liquid nitrogen or quartz sand for dry material. First grind down slightly, then more powerful (cells have to be crashed). Use safety goggles!

4. Transfer the powder into an Eppendorf cap with warm CTAB solution immediately (use a new, clean spatula for each sample)

5. Add 700 µl of warm CTAB solution to the powdered sample (open the caps carefully), dissolve the powder and incubate the sample for 30 minutes at 65

6. Centrifuge for 5 minutes at 15000 rpm.

7. Transfer the supernatant (only clear liquid) in a new eppendorf-cap. Use blue pipette tips which are cut.

8. Add new CTAB solution (700 µl) to the tissue pellet and stir slightly with a new 1000 µl pipette tip, incubate 30 min at 65 . Step 6 and 7 are repeated. The same is carried out for a third extraction. Each fraction proceeds with step 9 and is treated separately.

9. Add 600 µl chloroform to the cap with supernatant and shake carefully a few times upside down. This chloroform step should be carried out immediately.

10. Shake the samples thoroughly by turning inversing the eppendorf caps for approximately 5 minutes. (Longer incubation is possible)

11. Centrifuge for 5 min at 15000 rpm.

12. Transfer the supernatant (only clear liquid) in a new Eppendorf-cap. Use blue pipette tips which are cut. Work carefully; do not transfer suspended matter (normally the chloroform is covered by a thin layer of fine sediment material). Chloroform has to be disposed of in a special waste bottle.

13. Repeat the chloroform extraction (step 9-12) to make sure that all impurities are removed, and then proceed with step 14.

14. Add cooled iso-propanol (4), approximately 2/3 of the solution volume. Shake carefully by inversing the eppendorff cap. In most cases DNA becomes visible as white threads. Freeze for more than 2 h at -20. (BREAK POSSIBLE)

15. Centrifuge 10 min at 15000 rpm.

16. Aspirate liquid using yellow tips (without touching pellet!). If pellet is solid enough the larger part of the liquid may be poured out. (Alternatively add TE and proceed with qiagen kit)

17. Add 200 µl ethanol 70 % to the pellet. Rinse the inner cap surface by turning the cap.

18. Centrifuge for 10 min at 15000 rpm in a cooled centrifuge.

19. Aspirate ethanol using yellow tips. Dry the DNA-pellet at room temperature. (Usually 15 min are sufficient; after drying no liquid drops are to be seen)

20. Dissolve pellet in 100 µl TE (1x, p.a. grade) and store at 4°c. (BREAK POSSIBLE)

21. Add cooled 7.5 m nh4ac-solution (4°c, half of the solution volume). Mix carefully.

22. Add cool ethanol 100 % (double of the solution volume). Mix carefully. Freeze for more than 2 h at -20°c. (BREAK POSSIBLE)

23. Centrifuge 30 min at 15000 rpm. Aspirate fluid carefully.

24. Add 200 µl ethanol 70%. Rinse the inner cap surface by turning the cap.

25. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

26. Repeat steps 21 to 24 with 3 M NaAC solution (4°c, half the volume) then proceed with step

27. Centrifuge 10 min. at 15000 rpm. Aspirate liquid and dry pellet at room temperature. Dissolve the pellet in 100 µl TE (1x, p.a. grade)

- ✓ cleaning the mortar and pestle:
  - rinse the mortar and pestle with water
  - Clean the mortar and pestle in a 1:10 klorex-bath for 24 hours
  - rinse with ddH2o
  - Autoclave the mortar and pestle wrapped in aluminum foil at 134°c

Appendix 3. Genomic DNA NanoDrop test result of *Orobanche crenata* samples

Samples(S)	Concentration(C) (ng/µl)	OD:(D) 260/280	S	C	D	S	C	D
ST01	516.4	2.12	SW58	239.4	2.11	SG107	1312.5	1.8
ST02	631.9	2.09	SW59	679.4	2.06	SG108	2448.8	2.06
ST04	644.1	2.08	SW60	986.4	1.97	SG109	1083.1	2.11
ST05	1074.4	2.1	SW61	1455.9	2.07	SG110	1565.3	1.98
ST06	388.9	2.09	SW63	1736	2.1	SG111	1967.1	2.11
ST07	438.6	2.09	SW64	1943.1	2.05	SG112	1026.1	2.02
ST09	568.1	2.11	SW66	1858.6	1.94	SG113	1687.7	2.09
ST10	396	2.06	SW67	886.7	2.08	SG114	306.2	2.02
ST11	381.1	2.07	SW68	1561	2	SG115	1385.7	2.07
ST12	1108.8	2.11	SW69	2336.3	2.04	SG117	2139.4	2.1
ST14	1214.6	2.13	SW70	277.4	2.11	SG119	265.5	2.06
ST15	1330.5	2.14	SW72	1322.3	1.96	SG120	199.4	2.19
ST16	460.6	2.15	SW73	899.3	2.1			
ST17	1471.2	2.08	SW76	827.9	2.15			
ST20	880.6	2.05	SW77	565.3	2.13			
ST21	1203.5	2.05	SW78	2238	2.07			
ST22	1698.5	2.12	SW79	1261.5	2.08			
ST23	2069.4	2.12	SW80	820.9	2.06			
ST24	318.9	2.13	SW81	1400.5	2.09			
ST25	936.6	2.12	NW82	322.8	2.13			
ST27	556.7	2.13	NW83	356.9	2.04			
ST29	1751.9	2.11	NW84	691.8	2.08			
ST30	2904.8	1.85	NW85	162	2.13			
ST31	1258.3	1.83	NW86	688.1	2.07			
ST32	2066.9	2.08	SG87	1023.4	2.07			
ST34	788.6	2.06	SG88	1430.4	2.02			
ST36	1802.6	1.91	SG89	961.3	2.13			
ST37	208.4	1.8	SG90	929.1	2.08			
ST38	1647.8	1.91	SG91	1374.9	2.06			
ST40	893.3	1.81	SG92	2309.4	2.06			
SW41	1304.2	1.95	SG93	1241.8	2.04			
SW42	983.6	2.04	SG94	1395.9	2.15			
SW43	1295	2.14	SG95	2498.3	2.06			
SW44	543.9	2.13	SG96	1578.1	2.08			
SW45	2169.9	2.12	SG97	2227.2	2.05			
SW46	1014.2	2.07	SG98	1215	2.16			
SW47	2436.1	2.14	SG101	779.8	2.02			
SW49	1033.7	2.04	SG102	790.8	1.93			
SW51	1064.5	2.16	SG103	1928.8	2.1			
SW52	2946.9	2.07	SG104	897.7	2.08			
SW55	2489.7	2.08	SG105	2071.7	1.86			
SW56	2292.3	2.06	SG106	2576.2	2.11			

Appendix 4. Characteristics of 79 validated *Orobanche cumana* SSR markers

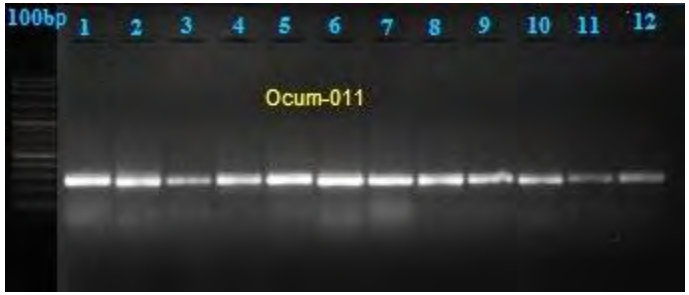
Locus	Core sequence	Primer sequence (5′–3′) Forward	Primer sequence (5′–3′) Reverse	Product length (bp)	Amplification quality in O. cumana screening panel (n = 4)*	Informative-ness in O. cumana char-acterisation panel (n = 18)	
						NA	PIC
Ocum-001	$(CTT)_8$	CGTGCACCATTTCAATTCTT	CATCACATACCGTTACAAGGGA	100	+++ (*)	2	0.362
Ocum-003	$(GAT)_8$	CTCGAACGCAAACTTTTGAA	CAAAGATGGTGGTTTTGCG	94	+++ (*)	4	0.513
Ocum-005	$(AG)_8$	TACAATATCTGCAGCCACGA	CGTGTATACATACGAAATATGTGAAGA	140	+++	2	0.099
Ocum-006	$(CT)_8$	CTTATGTATGTTGTTCTTCTCTGCC	CATACATCCAATTAACATACAAGCA	90	+++ (*)	6	0.530
Ocum-011	$(CA)_8$	GCCGTGAACTCCACTACCAC	GAGTTAGGGTCAGTCTTGCGA	274	+++ (*)	3	0.504
Ocum-013	$(TCT)_8$	TCTTGTGAAGATTATTTTGCAATC	GGAATTGTGTCTGTCTATGGTGTT	243	+++	3	0.269
Ocum-014	$(GAA)_8$	GACAGGCACTCTGAGCACAT	TTGCTTTTCATCTCCCTGCT	209	+++ (*)	3	0.294
Ocum-015	$(AG)_8$	AATGAAAGTGGTAAAGTAGTGTTGC	CATAACGATTTTGCTCTTGTACG	241	+++	2	0.105
Ocum-021	$(CTT)_9$	ACTCGTGAGGGTAACAACGTC	TTCAGAAAGTCAGTCAGGGGA	90	+++ (*)	3	0.194
Ocum-023	$(AG)_9$	CATCACCTCGAGTTTTCCGT	CGCAAGTTCACGAATTGAA	157	+++ (*)	4	0.421
Ocum-030	$(CTT)_9$	CAAGGGTTTTATCCAAATGGG	GAAACCCAGGAAGCAAACAA	106	+++	3	0.370
Ocum-031	$(AC)_9$	AGGTACAAGCACGGAAGCTG	TCTAGCCTGAACCAACTCCA	193	+++ (*)	2	0.372
Ocum-032	$(TG)_9$	CTGAGCACTTCCTTGAACCC	CCTGTAATACTAACAGAATGCCACA	156	+++ (*)	2	0.286
Ocum-033	$(CA)_9$	CTGATGAACCTAAAATATCCCCA	TGGGGTATTGAGTGACGAGA	153	+++ (*)	2	0.178
Ocum-036	$(TC)_9$	CTCTGTGGGCAGAGGCTTTA	TGTGAAGAGAAAAGGGTCGC	101	+++	2	0.321

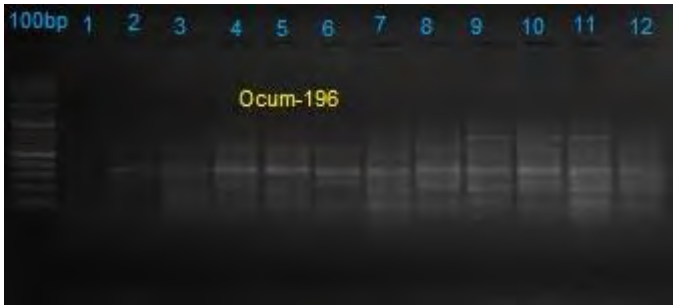
Ocum-037	$(GA)_9$	ACAATCTCCGGTCACAATCG	CCATGCTCTGCTTGTGAAAA	121	+++ (*)	2	0.346
Ocum-040	$(ATAC)_{10}$	AACAGAATCCATCTCAGGGC	ATGTTGGCATTCTCAAGCCT	105	+++ (*)	3	0.453
Ocum-041	$(AC)_{10}$	TGAAGATGTTGAAAAGCGCA	TTTTCCTTCCTCCCACACTT	92	++ (*)	3	0.371
Ocum-043	$(AGG)_{10}$	AGGTGCACTTAACCTTGACCT T	CTGCAGGTGGTCATGCTAGA	104	++ (*)	2	0.371
Ocum-044	$(TTC)_{10}$	TTCTGGTCTAACAAACCGCA	TTTGTGAGAATTTGATGGCG	128	+++ (*)	2	0.286
Ocum-045	$(AC)_{10}$	CGTCAAGGAGCGGAGAACTA	AAGGGAAGGTTCCTACGTGAA	183	+++	2	0.178
Ocum-047	$(GT)_{10}$	CGTTTTCCGGATCCAAAGT	CCTCACACCGCAGTACAAGA	152	+++ (*)	2	0.367
Ocum-048	$(TTC)_{10}$	AGGGCAAAACGTACTCTGGA	GCCCTTTATCCTCATTCTCTAT TG	127	+++	2	0.286
Ocum-052	$(AG)_{10}$	CATGTCTAAGCTTTTGGCTCG	CAAGACTTGGAACAAGCAAAT C	108	+++ (*)	3	0.449
Ocum-056	$(CT)_{11}$	CACCCTGCATGTTTCAAAAG	CAAGGGTATTTTCCCTATCTCA A	111	++ (*)	3	0.398
Ocum-059	$(TC)_{11}$	TCTTGATTTGTATATGTCTGAT GCAAT	ATGCTACAATAGAAATACACA ACGAAC	90	++ (*)	3	0.551
Ocum-063	$(AG)_{11}$	AACCAAGTTGATGCATCCGT	TCCCTCGGCATTCAGACTTA	90	+++ (*)	3	0.568
Ocum-064	$(TCT)_{11}$	GGGCTCTTCATTGAATTTGC	CCGTGGACCCATTTCATTAT	126	+++ (*)	2	0.286
Ocum-066	$(TC)_{11}$	AGGGCTTCATTAAACTGCCT	GGGGAGGAAACATTAGGACA	193	+++ (*)	2	0.105
Ocum-067	$(AC)_{11}$	TGTCCAGTCAATTTCAGAAAC G	CGCTCATTCCTGATGCAGA	241	+++	2	0.321
Ocum-069	$(AC)_{11}$	CTGTTGACAGATATCTTGAAG CGGT	AGGACAACTTAAATCGATAAG CA	240	+++	2	0.321
Ocum-070	$(TG)_{11}$	AAGCTGTAAACAATGCCTGAA	CCTCCTCCAGTACCACTAGGC	96	+++ (*)	5	0.672
Ocum-074	$(GA)_{12}$	CCTAAAATTGAAACCTTAAGG AAA	ACTTTCCGTGAGACGGAGTC	99	+++ (*)	6	0.712
Ocum-075	$(CA)_{12}$	TGTGGATAGAGTATAAGCTAC CAGTTC	TTCCCGTAGCTTGGAGAATG	110	++ (*)	4	0.450
Ocum-076	$(AC)_{12}$	TTATCAACCAAAGATATGCAT TTATCA	TGGCACTATAATCTTCATGCCA C	290	+++	2	0.110
Ocum-079	$(AG)_{12}$	AAATTGGTCTCAAAATCTACC CA	GATTTATAGCATATTTGTTCCA CAGA	241	+++	2	0.346
Ocum-080	$(AG)_{12}$	AGATCTCACGACCCATTTGC	TCTTCCAAGAAGAACCAAGAA A	115	+++	2	0.105
Ocum-081	$(CA)_{13}$	TTACAAGGTGAAACCACCCA	CAGCTACTGTCCGCAAGAAA	90	++ (*)	5	0.542
Ocum-083	$(AG)_{13}$	GAGAATGCGGATTTCCTGA	GCGACTAGCAACTTTAGTCCG	243	++ (*)	3	0.556
Ocum-084	$(TG)_{13}$	ATGGAATCAAGAGAATGACA ACTG	TTTACTAAGACGCGCACACC	140	+++	3	0.194

Ocum-085	$(GA)_{13}$	TCACAAGGAAGTAATGCGGC	CCAGACCCAGCTCTGTGATA	137	+++	5	0.704
Ocum-087	$(TTC)_{13}$	TTCTCGACAGCTTTGGGTAAA	ATGCCAACTTCGAGTGATCC	134	+++ (*)	5	0.642
Ocum-089	$(AC)_{14}$	CCTCACTCTCCTCTGACCCA	AGCTTACGAGCTTTTGCTGC	133	+++ (*)	2	0.239
Ocum-091	$(TTC)_{15}$	CACATAGTGCACCCCTGCTA	TCCCCTTCTTCATATGCCAC	173	+++ (*)	5	0.550
Ocum-092	$(GT)_{15}$	GTCAACCTTGTTAAGGGGAGT	TGCAATCTGATGAGAGTAGAGGA	165	+++ (*)	5	0.719
Ocum-093	$(CA)_{15}$	GGAAGGAAAAAGAAGAACAGGAA	GAATCCTGGAGTGGTGATGG	105	+++	2	0.305
Ocum-094	$(GT)_{15}$	TGGGAGCTTTGTACAGACACTG	GTTTTCTATTAAACCGTAACAAACTCT	141	++ (*)	2	0.371
Ocum-097	$(CA)_{16}$	CAGATATGTTGTTTCGCGGTT	ACGTCAGGTGGGAATGCTAA	159	+++ (*)	3	0.194
Ocum-099	$(AC)_{17}$	TCCAATTCACCAACATTTCG	TACTGCGTAGGTAGTCGCGT	121	+++	3	0.204
Ocum-108	$(GTAT)_{6}$	TCGTTAATAAGTGGTTCACGAAAA	TGACTAAAAATAAAATGTACGGGTG	143	+++ (*)	3	0.489
Ocum-110	$(TTC)_{6}$	CCACTTGATTGTTCTTGTAGATTGAA	GCAACTAATATCATCAATCATGGTGTA	90	+++ (*)	2	0.362
Ocum-113	$(CT)_{6}$	AACCTAATCTGCAGCCGACC	TCTGCATATTTGTCTTCGGC	95	+++	2	0.337
Ocum-115	$(AGA)_{6}$	AAATCTCGGGAAGCTGGAAT	GCAACATTATTTGCTTTTCCG	105	+++ (*)	2	0.346
Ocum-121	$(AT)_{6}$	CTTAGGATCCGTTTCCGGC	GGAGTTATATTTGGGGAATCGAA	142	+++ (*)	2	0.362
Ocum-122	$(AGTGTG)_{6}$	GGAATACATCATTAAAGTAGTTGTCCG	GAAGGAGTCATTAAACTCCGTGA	241	+++ (*)	3	0.555
Ocum-136	(GTT)6	ACGCTGGATCATCGGATTAC	ACGACCGCTACAAGTCCAAT	126	+++	3	0.371
Ocum-141	$(CTT)_{6}$	CAGCAACTGTTTCTTCCATAGAG	TCCAAGAAGAGGAAAAGAAGTGA	191	+++ (*)	4	0.625
Ocum-145	$(GAA)_{6}$	AAGATGGCTCATTGCGGTTA	ATCTCGGGCTGAGTTTTCCT	274	+++ (*)	2	0.178
Ocum-151	$(GGA)_{6}$	ATCTCCGGAGGAAGAGGAAG	CCATCACTCCCCAAGAGTTC	186	+++ (*)	10	0.800
Ocum-152	$(CTT)_{7}$	GCAGTAGGTTTTATACTTTATTTCTCG	AACATGGGTGAAGTGTGAACAG	141	+++	2	0.329
Ocum-156	$(GA)_{7}$	CAACGTTTGAGTGTGCTGCT	TGAAGCTAATGGCCAACTCA	124	+++ (*)	2	0.372
Ocum-160	$(AG)_{7}$	TGAGGGTTTGTAAAGTGGGC	CGTACCTTATCCCTCCGTCA	136	+++ (*)	4	0.685
Ocum-163	$(AC)_{7}$	AAGCATTTTCCATGCGTGAT	CGGGTTTATTCAATGGGTATG	140	+++	2	0.099
Ocum-166	$(GA)_{7}$	TGTTGTTATCAAGAGGAAGCGA	CGAAATAAATCAATGGGCTG	94	+++	2	0.359
Ocum-167	$(ACA)_{7}$	CGACAAGTTGGAAGAAGTGA	TTCATGTTAGCAGACCCCAA	139	+++	2	0.180

Ocum-174	$(AAG)_7$	ACAACCAACAAACAAGTAGTGACG	TCTTGCGGCAAAACCATT	190	+++ (*)	4	6	0.660
Ocum-176	$(TCGTTG)_7$	GCAATTGTCGACCAAGTACG	CGCCAGATCAACATGAGGT	239	+++	2	9	0.099
Ocum-184	$(AG)_7$	CCGTTCAATTTTCTCTGCAA	ACGGCGTCTTCCTTGTTGTA	107	+++	2	9	0.099
Ocum-189	$(CA)_7$	GCACGCATGTACACATACACA	TGCTATTTTCTAGTTTCTATTCATGG	95	++ (*)	2	4	0.124
Ocum-190	$(TC)_7$	GCCGTTTTCTTTCCTTGTCC	CCCGAAACAAAACACCACTC	98	+++	2	9	0.099
Ocum-196	$(GT)_7$	GTATGTGCGCCCGTCTTG	GGGGATGACTGTGTTTCGAT	192	+++ (*)	3	5	0.535
Ocum-197	$(GA)_7$	AGAGACGGCATCATCAATCA	GTGATCGTGCAGGCACCTA	95	+++ (*)	6		0.683
Ocum-199	$(TG)_7$	TTGGGTATTTGGTTTCCTGG	GTGTCTCGATCTTCACCCCT	91	+++ (*)	2		0.346
Ocum-206	$(TG)_8$	CCGATTGCTGTTTATGTTGTATT	TGTAGGAGATGCCCAGTTCA	119	+++ (*)	2	0.346	
Ocum-209	$(CT)_8$	AATTTTGCATTAATCCGCGA	TTCAGGATCCCCATCTTCAG	130	+++	2	0.286	
Ocum-213	$(GA)_8$	GGTACGGCATCCACTCTGAT	TTCGGGCCTCCTTACTTTCT	97	+++ (*)	2	0.321	
Ocum-215	$(TG)_8$	GCTACATTGGCACATTTGTATTTC	TTCAACTCTGCTGTATTGCCA	112	+++	2	0.105	
Ocum-216	$(TG)_8$	GCAATTGTTCATGTATCTTGCG	ACGCACATGACCATTACGAG	124	+++ (*)	2	0.346	
Ocum-217	$(CA)_8$	TTGTTCGACTGGATGAAAGG	CACTTGTGGGGCATCATTT	120	+++ (*)	3	0.371	

Appendix 5. Marker screening gel picture for worked and not worked markers





Worked 2% Agarose gel picture: not- worked 2% Agarose gel picture

Where: -100bp is the DNA ladder used as a marker
-lane1to 12 represented samples tested with each primer

-The selection of worked and not-worked marker was done based on expected size and specificity of band

- Gel pictures showed us locus **Ocum-011** gives specific band with expected size, whereas locus **Ocum-196** gives not clear many non-specific bands