ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

Microbial Cellular and Molecular Biology Program Unit

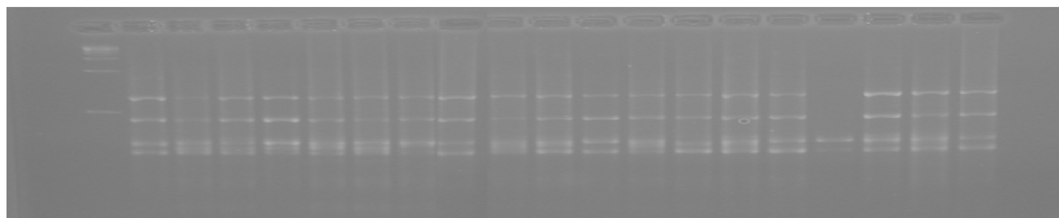
(Applied Genetics Stream)

Phenology and Genetic Diversity of *Boswellia papyrifera* (Del.) Hochst. Populations of Metema District as Revealed by ISSR Markers.

A Thesis submitted to the School of Graduate Studies, Addis Ababa University, in partial fulfillment of the requirements for the Degree of Master of Science in Biology (Applied Genetics)

By

Abush Zinaw



June, 2012

ACKNOWLEDGEMENTS

I am very grateful to my advisors Dr. Kifle Dagne, Dr. Kassahun Tesfaye and Dr. Wubalem Tadesse for their professional supports and due concerns from the very start of designing the research proposal up to thesis write up. I am very much indebted to them for the amount of work they put into this task, which made the study to be completed successfully.

I would like to thank Center for International Forestry Research (CIFOR) for sponsoring the reproductive biology part of this study without which the completion of this study would not have been possible in such a successful way. Grateful acknowledgements are extended to the enumerators for their co-operation and to the sample respondents and key informants who co-operated with me in supplying relevant information in addition to their hospitality during the period of data collection.

Ministry of Education is highly acknowledged and appreciated for offering me the opportunity to pursue this study, and Addis Ababa University for hosting me and Genetics research laboratory for providing laboratory facility and supplies. In addition, I am very indebted to Biruk Getnet and Shimekt Tadele for their impressive support especially during laboratory work.

Table of Content

Contents **pages**

ACKNOWLEDGEMENTS i

List of Figures iv

List of Tables v

LIST OF ABBREVIATION vi

ABSTRACT viii

1. INTRODUCTION 1

2. LITRATURE REVIEW 4

2.1 Botanical Description and Ecological Distribution 4

2.2 Reproductive Biology 5

2.3 Important Uses of *Boswellia papyrifera* 6

2.3.1 Frankincense Production 6

2.3. 2 Uses of frankincense 8

2.3.3 Uses of wood, leaves and flowers 10

2.3.4 Environmental functions 10

2.4 Important issues and ecological concerns 11

2.5 Genetic markers for diversity analysis 12

2.5.1 Morphological markers 14

2.5.2 Biochemical markers 14

2.5.3 Molecular markers 15

2.5.3.1. Non-PCR based techniques 16

2.5.3.2 PCR arbitrary priming 17

2.5.3.3 Targeted-PCR and Sequencing 20

2.6 Genetic diversity study in *Boswellia* 22

3. OBJECTIVES 23

3.1 General objective 23

3.2 Specific objectives 23

4. MATERIALS AND METHOD 24
4.1 Plant material 24
4.2 DNA extraction 24
4.3 Primer selection and optimization 26
4.4 PCR and gel electrophoresis 27
4.5 Data management and statistical analysis 27
4.6 Phenological observations 29
4.7 Pollinator activity 29
5. RESULTS 29
5.1 Banding patterns of the ISSR primers used 29
5.2 Polymorphism based percent polymorphism 31
5.3 Genetic diversity 32
5.4 Analysis of molecular variance 33
5.5 Clustering analysis 34
5.6 PCO analysis 38
5.7 Phenology 39
5.8 Pollinator activity 41
6. DISCUSSION 43
6.1. Molecular diversity 43
6.2 Phenology and pollinator activity 46
7. CONCLUSION 48
8. RCOMMENDATIONS 50
9. REFERENCES 51
10. APPENDICES 64

List of Figures

Figure 1. Administrative woredas of Metema Zone showing sites where the Boswellia population used in this study were collected.. 25

Figure 2ISSR fingerprint generated using primer 818 31

Figure 3. UPGMA based dendrogram for five Metema Zone *Boswellia papyrifera* populations using 5 ISSR primers 35

Figure 4. UPGMA based dendrogram for 64 individuals of *Boswellia papyrifera* using five ISSR primers. The UPGMA algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint. 36

Figure 5 Neighbor-joining analysis of 64 individuals based on 39 PCR bands amplified by five primers. The neighbor joining algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint. 37

Figure 6. Two dimensional representation of principal coordinate analysis of genetic relationships among 64 individuals of five Metema populations. 38

Figure 7. Three dimensional representation of principal coordinate analysis of genetic relationships among 64 individuals of five Metema populations. 39

Figure 8. Various phenoevents in *Boswellia papyrifera* 40

Figure 9 Figure showing flowers of B. papyrifera being visited by honey bees. 42

List of Tables

Table 1. Work force deployed by the Ethiopian Gum Processing and Marketing Enterprise (1996/97-1999/2000) 8

Table 2. List of primers, annealing temperature, primer sequence, amplification pattern and repeat motives used for optimization 26

Table 3. The five primers used, their repeat motifs, amplification quality and number of bands scored 30

Table 4. Number of scorable bands (NSB), number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (GD) and Shanon Index (I), with each primers (a). and for each population (b). 32

Table 5. Analysis of Molecular Variance (AMOVA) among populations of Boswellia without grouping popuplations 34

Table 6. DBH and height of the trees 40

LIST OF ABBREVIATION

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of molecular variance
ANRS	Amhara National Regional State
CTAB	Cetyltrimethyl Ammonium Bromide
DBH	Diameter at Breast Height
EDTA	Ethylene Diamine Tetra Acetic acid
FAO	Food and Agriculture Organization
GD	Gene diversity
IPGRI	International Plant Genetic Resource Institute
ISSR	Inter Simple Sequence Repeats
IUCN	International Union for Conservation of Nature
NJ	Neighbor Joining
NPL	Number of polymorphic loci
PCO	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PP	Percent Polymorphism

RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SSR	Simple Sequence Repeats
SSRP	Simple Sequence Repeat Polymorphisms
STMS	Sequence-Tagged Microsatellite Sites
TNRS	Tigray National Regional State
TRAFFIC	Trade Records Analysis of Flora and Fauna in Commerce
UBC	University of British Colombia
UPGMA	The Un-weighted Pair Group Method with Arithmetic mean
WWF	World Wide Fund for Nature

ABSTRACT

Boswellia papyrifera (Del.) Hochst. (family Burseraceae), is one of the key plant species in the drylands of the Horn of Africa. Unregulated extraction of gums and resins and land use change are among the main factors that threatened this dry land forest. This plant is a culturally and ecologically relevant species that is showing symptoms of decline due to anthropogenic factors. The study was done on five populations of *B. papyrifera* from Metema zone, Northern Ethiopia. Inter-Simple Sequence Repeat markers (ISSR) were used to estimate genetic diversity among and within five populations of *Boswellia papyrifera* collected from Zebach Bahir, Das Gundo, Gubay, Shinfa and Agam Wuha sites. Five selected ISSR primers yielded 39 reproducible bands from 64 individuals studied. All the loci were found to be polymorphic. The total genetic diversity (GD) and Shannon's diversity information index (I) for entire populations showed fairly high to medium values, 0.42, and 0.60, respectively. The individuals from Zebach Bahir site showed the highest level of gene diversity, 0.42; while the least variability showed by Agam Wuha populations with 0.25. Analysis of Molecular Variance showed that the within populations variation was higher (76%) than among population variation (24%). UPGMA analysis revealed one major group and two outliers (Gubay and Agam Wuha). This major cluster again forked into two sub-groups, one group containing Das Gundo while the second group contained Zebach Bahir and Shinfa populations. NJ analysis, based on individuals of *Boswellia*, showed three distinct clusters and two sub-clusters within the second and third major clusters. In 3D, most of Agam wuha and Gubay individuals tended to form their own separate grouping, while individuals from Zebach Bahir, Das Gundo and Shinfa were inter-mixed and formed a separate group from the other populations. Studies were carried out on the phenology, and pollination ecology of *B. papyrifera*. The trees remain leafless during the entire period of flowering and fruiting. The inflorescence is a terminal raceme and produces 8-15 bisexual flowers, but it might produce up to 60 flower buds. Moreover, during maximum flowering period on average 14.5 inflorescences per branch were recorded. Flowers offer nectar and pollen as rewards to floral visitors. The honey bee is found to be the effective and frequent pollinator. Each flower is visited by an average number of 6.35 insect visitors every 15 minutes and, on average, each insect visitor spents 5.8 seconds on a flower. High to moderate level of variation was observed with populations of Metema, this indicated the needs for urgent conservation attention.

Key words: *Boswellia papyrifera,* genetic diversity, ISSR, phenology, pollination ecology

1. INTRODUCTION

Boswellia papyrifera (Del.) Hochst. belongs to the Burseraceae family, which contains about 600 species in 17 genera (Fichtl and Admasu, 1994). Among the twenty species of the tropical genus *Boswellia* Roxb. (Burseraceae), *B. papyrifera* is known since antiquity for the production of fragrant gums and resins that are burned as incense (Coppi *et al*., 2010). *Boswellia papyrifera* is a small deciduous tree that grows in a savanna belt that stretches from northern Nigeria eastwards to the highlands of Eritrea and Ethiopia (Rigkers *et al.,* 2006).

Boswellia papyrifera is one of the key plant species in the drylands of the Horn of Africa. Its aromatic resin frankincense constitutes one of the export items providing considerable foreign currency for Ethiopia, Sudan and Eritrea. It is also known to ameliorate soils, produce nutritious and palatable livestock fodder, and provides various wood products for local uses and income for poor households through involvement in frankincense production (Abeje *et al.,* 2005). The frankincense is prepared from irregular lumps of yellowish brown gum exuded by the trunk of the trees and was already used medicinally and for fumigation by the ancient Egyptians. Since ancient times, Ethiopia has been involved in the production of this resin, and its trading flourished for many centuries (Kindeya *et al.*, 2003).

In Ethiopia, *B. papyrifera* occurs within the *Acacia – commiphora* woodlands in the northwest, north east and southeast parts of the country at elevations ranging from 300-1800 m. a. s. l. (Vollesen, 1989). It grows on degraded sites with very shallow soils, steep rocky slopes, lava flows or sandy river valleys, within the 950–1,800 m a.s.l. altitudinal range (Fitchl and Admasu, 1994). These lands are rapidly being degraded through population growth, overgrazing, cropping in marginal lands, inappropriate irrigation and deforestation. The populations of *B. papyrifera*

are dwindling and its natural regeneration is severely hampered due to a number of anthropogenic and natural factors. Several population assessments in Ethiopia, Eritrea and Sudan reported a serious lack of recruitment through natural regeneration even in areas protected against grazing and fire. Unless effective conservation measures are taken significant amounts of genetic erosion will occur mainly due to the destruction of ecosystems and habitats by human activities (Tilahun, 1997; Ogbazghi, 2001; Abeje, 2002).

The information on the amount of total genetic variation and its partitioning among and within populations is considered of crucial importance in modern conservation program and breeding of endangered or economically important species. The selection of the stands to be protected in the wild and/ or the germplasm to be collected and propagated *ex situ* are often based on genetic evidence (Frankel *et al.*,1995; Holsinger and Gottlieb, 1991). Accordingly, we performed a molecular study to analyze amount and distribution of inter- and intra-population genetic diversity and differentiation using Inter Simple Sequence Repeats markers (ISSRs). ISSR is a PCR-based fingerprinting technique that involves amplification of DNA segment between two inversely oriented and closely spaced microsatellites repeat regions (Bornet and Branchard, 2001; Reddy *et al.,* 2002), and is widely used in plant populations genetics thanks to the reliability and reproducibility of the results (Rakoczy Trojanowska and Bolibok, 2004). In particular, the technique has been applied to assess genetic variation and population differentiation in a number of endangered angiosperm or gymnosperm species from different areas and climates (Hatcher *et al.,* 2004; Ge *et al.,* 2005a, b; Pe´rez-Collazos and Catala´n, 2007; Li and Jin 2007; Luo *et al.,* 2007).

The phenological cycle of the species is largely controlled by the rainfall regime, which is characterized by a long dry season from mid-September to mid-June. After leaf fall in September,

floral buds develop at the end of the branches and flowering takes place in November and December. Fruits mature in January and February, and new leaves start to develop at the beginning of the small rains in late March. Tapping starts in the beginning of the dry season, before the emergence of floral buds, and coincides with the whole sexual reproductive cycle (Rijkers *et al*., 2006). Chromosome number and ploidy level of Ethiopian *Boswellia* populations are unknown. Likewise, no literature data are available on the reproductive biology and pollination systems of the species.

This study focuses on the genetic diversity and relationships in a geographically representative population sample of the frankincense tree, *B. papirifera,* from Metema District, which is one of the major location with large population of *B. papyrifera,* with the ultimate goal of contributing useful data that may assist with the identification and selection of the genetic material to be conserved and propagated for ecological and economic purposes, and also on the phenology and pollination ecology of *B. papyrifera*.

2. LITRATURE REVIEW

2.1 Botanical Description and Ecological Distribution

Boswellia papyrifera (Del.) Hochst. belongs to the Burseraceae family, which contains up to 600 species in 17 genera (Fichtl and Admasu, 1994). The family is distinguished by the presence of resin ducts in the bark (Groom, 1981) and production of aromatic oils or resins (Fichtl and Admasu, 1994). This aromatic and volatile oil had and still has great value in ceremonies of worship and in medicinal use (Van Beek, 1960; Groom, 1981; Tucker, 1986). *B. papyrifera* is one of the 20 species in the genus *Boswellia* Roxb. Two new species, i.e. *B. bullata* and *B. dioscoridis*, were recently identified in Yemen (Thulin, 2001). *B. papyrifera* is found in Ethiopia, Nigeria, Cameroon, Central African Republic, Chad, Sudan, Uganda and Eritrea (Vollesen, 1989, Hepper, 1969). It mainly occurs in the Sudanian regional centre of endemism and the Sahel regional transition zone (White, 1983). The centre of geographic distribution of the genus *Boswellia* is located in north-eastern parts of Africa where more than 75% of its species are endemic to the area (Vollesen, 1989).

Boswellia papyrifera can be as tall as 12 m with a rounded crown and a straight regular bole. The bark is whitish to pale brown, peeling off in large flakes; and the slash is red brown, exuding a fragrant resin. The bark contains schizogenous oleo-gumresin pockets (Verghese, 1988). Leaves are deciduous, large, compound, arranged on long stalks with 11 to 29 leaflets, which are narrowly ovate to oblong, and waved or toothed along the margin. *B. papyrifera* is a bi-sexual flower species with sweet scented flowers, which are white to pink, arranged on long red flower stalks, in loose panicles at the end of branches. Fruits are obtetrahedral with red capsules about 2 cm long, usually containing three tapered seeds (Vollsen, 1989).

In Ethiopia, *B. papyrifera* occurs in dry *Acacia-Commiphora* woodlands in Gonder, Gojam, Shewa and Tigray (Vollesen, 1989). It grows on degraded sites with very shallow soils, steep rocky slopes, lava flows or sandy river valleys, within the 950-1800 m a.s.l. altitudinal range (Fichtl and Admasu, 1994). Although no national inventory has been carried out, preliminary mapping and assessments show that the frankincense resource is found in eight Regional States of Ethiopia covering an area of about 2.9 million ha (Abeje *et al.*, 2005).

2.2 Reproductive Biology

B. papyrifera is a bisexual species with sweet scented flowers which are white to pink, arranged on long red flower stalks, in loose panicles at the end of branches. Fruits are obtetrahedral with red capsules about 2 cm long, usually containing three tapered seeds (Vollesen, 1989; Kindeya *et al.*, 2003).

The pink sweet smelling flowers of *B. papyrifera* are frequently visited by honeybees for pollen and nectar. The long flowering period, from October to February is helpful for bee colony maintenance (Kindeya *et al.,* 2003). Fitchl and Admasu (1994) recommended planting of the species to increase honey production in lowland areas. Areas where *B. papyrifera* grows are often known for their good quality honey (Kindeya *et al*., 2003).

Phenology is the study of the periodicity or timing of recurring biological events, in relation to short-term climatic change. In canopy trees, phenology and reproduction are expected to be particularly important since they provide a significant resource of leaves, flowers and fruits. Recent studies indicate that phenology patterns are selected to optimize pollination, seed dispersion and seedling survivorship (Kudo, 2006).

Chromosome number and ploidy level of Ethiopian *Boswellia* populations are unknown. Likewise, no literature data are available on the reproductive biology and pollination systems of the species

2.3 Important Uses of *Boswellia papyrifera*

2.3.1 Frankincense Production

The use of frankincense has a long history in human civilisation (Van Beek, 1960). The natural oil content and pleasant smell of frankincense made it desirable to burn as an incense in temple rituals and to use as a base for perfumes and for medicinal purposes since ancient times (Groom, 1981). The first recorded mention of frankincense was found on a 15th century B.C. tomb in Egypt (Abercrombie, 1985). Frankincense is mentioned 22 times in the Bible; 16 times for religious worship, twice as a tribute of honour, once as an article of merchandise, and three times as a product of the royal gardens of Solomon (Moldenke, 1952, cited in Tucker, 1986). These gifts were not only of the greatest value at the time but also symbolised Christ's life: gold for royalty, frankincense for divinity and myrrh for suffering.

Boswellia papyrifera is the main source of Ethiopian frankincense in the world market. When the bark of a *B. papyrifera* tree is incised, white oleo-gum-resin exudates is produced. This emulsion of aromatic oil and resin later dries into globular, pear or club shaped tears, known as frankincense. The amount of exudates varies depending on the diameter of the tree, site productivity and season. Annually, between 100 and 1000 grams of frankincense can be collected from a tree (Wubalem *et al.,* 2002). *B. papyrifera* can continue producing frankincense up to the age of 50-60 years (Tilahun, 1997).

The use of natural gums for commercial purpose is an age old activity in Ethiopia (Abeje *et a1.,* 2005). Ethiopia was, and still is, one of the major producers and exporters of natural gums from different indigenous tree species of *Boswellia, Commiphora* and *Acacia* that are found in different agro-ecological zones of the country (Vollesen, 1989; Mulugeta and Demel, 2003; Wubalem *et al.,* 2002). Virtually all the frankincense resource is found in and harvested from the natural woodlands.

In Ethiopia frankincense yield ranges from 0.1-1 kg/tree/year or 1-3 kg/tree/year. The annual production volume for the country, for the period 1992-1999, varied between about 248 and 3,215 tons. The total amount of oleo-gum resin, including frankincense, exported annually through official routes varied between 147 and 1925 tons, generating foreign currency earnings, from 451,000.00 to 2,603,000.00 US$ during the period 1992/93-1998/99 (Abeje *etal.,* 2005).

Incence collection offers off-farm employement for many farmers. In western Tigray alone, annually about 7000 seasonal labores are employed; among which 31% are women. A taper can collect about 10-15 quintals of incense per annum and receives a net income of US $ 100 to 150 (Aregawi, 1997). In Ethiopia, enterprises involved in frankincense international trade, are recently focusing more on ensuring quality by offering high price to collectors of export grades (Wubalem *et al.,* 2002).

Table 1. Work force deployed by the Ethiopian Gum Processing and Marketing Enterprise (1996/97-1999/2000) (Source: Ethiopian Gum Processing and Marketing Enterprise, unpub.).

Type of employment	Budget year			
	1996/97	1997/98	1998/99	1999/2000
Collectors	800	1700	1131	1634
Sorters	1000	1150	693	915
Full time employees	227	221	204	199
Contract workers	36	52	46	51

Moreover, a growing number of investors and full time workers are involved in the business. For instance, the Ethiopian Natural Gum Processing and Marketing Enterprise, one of the many enterprises involved in frankincense collection, one average, deploys a work force of 2515 people per annum.

2.3. 2 Uses of frankincense

Burning incense: Incense has been associated with religious ceremonies all over the world since time immemorial. The Greeks and Romans began to use frankincense as a substitute for sacrifice in the 6th and 2nd centuries B.C., respectively. Incense was also recognised as a means of propitiating the gods and as a purifier and practical fumigant, with which bad smells of primitive urban life could be disguised (Groom, 1981). The use of frankincense for ritual purposes in Ethiopia dates back at least to the Aksumite Empire, ca. 500 BC (Goldschimdt, 1970, cited in Goettsch, 1986). Frankincense was introduced into church ceremonies at the beginning of Christianity (Abercrombie, 1985). Ever since, the hardened resin burns in the Roman Catholic and Orthodox Churches throughout the world.

Perfume industry: Frankincense has been used in cosmetics since ancient times. Essential oil from frankincense is obtained from the crude resin by steam distillation and then used directly for flavouring and fragrance (Coppen, 1995). This oil from frankincense can take up to six hours to evaporate completely, making it an important ingredient in many perfumes. The essential oil of frankincense from *B. papyrifera* in Ethiopia consists of mainly n-Octyl acetate (56%), octanol (8%) and limonene (6.5%) (Aman *et al.,* 1999). The essential oil is used in the Oriental bases, "powder" perfumes, floral perfumes, citrus colognes, spice blends, violet perfumes, male fragrances, and other scented preparations. It blends well with spice oils, mimosa, neroli, muguet bases, woody notes, and other balsamic notes (Tucker, 1986). Hence, frankincense is included in the formulations of a number of modern perfumes. Examples include "*Replique"* by Colonia, "*Me!*" By Frances Denney, "*Mennen Millionaire*" by Mennen, "*Nino Cerruti Pour Homme*" by Uniperf, "*Onna*" by Gary Farn, "*Sculptura*" by Jovan, "*Volcan d'AmourI*" by Diane von Furstenburg, "*Paul Sebastian* V.S.O.P." by Sebastian, and "*Gambler*" by Jovan (Fragnance Foundation, 1983, cited in Tucker, 1986).

Medicinal uses: The traditional medicinal uses by local people indicate the potential and opportunity for the use of *B. papyrifera* for the pharmaceutical industry as well. China imports frankincense mainly for preparing traditional medicines (Coppen, 1995). In aromatherapy, frankincense oil, steam distilled or alcohol extracted, is believed to have effects on the respiratory system (Wilson, 1995), to reduce anxiety, asthma, bronchitis, extreme coughing, stress and stretch marks (Lawless, 1995). Boswellic acid (which constitutes about 50-70% of the oil) extracted from *B. serrata* in India is used as an anti-arthritic and anti-inflammatory pharmacological agent. Extracts from *B. serrata* was found to be more beneficial, less toxic, and

more potent than standard anti-inflammatory drugs (Bucco, 1998). It is used as a laxative ingredient in pharmaceutical industries (Tilahun, 1997).

2.3.3 Uses of wood, leaves and flowers

The wood of *B. papyrifera* is light and relatively soft. It is used for fencing, household furniture, matchboxes, splints, particleboard, pencils, plywood, picture frames and veneers (Tilahun, 1997). Various parts and products are used in traditional and modern medicine. The leaves and roots are used against lymphadenopathy while the resin is used as a febrifuge (Fichtl and Admasu, 1994). The bark of *B. papyrifera* is chewed to treat stomach disturbances. Frankincense is burnt as a mosquito repellent in the tropics and also chewed to quench thirst during hot days (Tilahun, 1997). In China, imported frankincense was once used as a medicine to treat leprosy (Tucker, 1986). The pink sweet smelling flowers of *B. papyrifera* the species are frequently visited by honeybees for pollen and nectar. The long flowering period from October to February is helpful for bee colony maintenance. *B. papyrifera* growing in lowland areas are often known for their good quality honey. Fichtl and Admasu, (1994) recommended the species for planting to increase honey production. The leaves and seeds of *B. papyrifera* are highly valued as fodder for goats, camels and other livestock. The succulent stem is also used as a fodder during the dry season.

2.3.4 Environmental functions

B. papyrifera grows in dry and rocky sites where other tree species often fail. In Northern Ethiopia, where the majority of the soils (60-80%) are only about 20 cm in depth (Hurni, 1988). *B. papyrifera* trees are found on these shallow soils and steep slopes with an average gradient of 30-40%. The species makes economic use of the marginal areas, on which other species could

not grow. In those sites, it provides plant cover and produces biomass and hence protects the soil and provides shade. The non-destructive extraction of frankincense maintains continued presence of trees to protect the land (Mulugeta *et al.,* 2003). If growing this species can be kept economically and socially attractive, it can increase the attention for the protection of degraded areas.

2.4 Important issues and ecological concerns

The decline in the population of *B. papyrifera* in eastern Africa has become an ecological concern (Tilahun, 1997; Marshall, 1998; Ogbazghi, 2001). In Tigray (northern Ethiopia) more than 177,000 ha of *B. papyrifera* forests were destroyed in the last 20 years. In the late 1970's, about 510,000 ha of land were covered by *B. papyrifera* in Tigray (Wilson, 1977) as compared to the existing 330,000 ha. Besides, the existing population consists of mainly mature trees, thus highlighting the problems of natural regeneration. For instance more than 76% of the existing *Boswellia* trees in western Tigray are greater than 30 cm DBH (Kindeya, *et al*. 2002). In Eritrea, frankincense export has dropped from 2000 tonnes in 1974 to 400 tonnes in 1998 (Ogbazghi, 2001). It is, therefore, appropriate that *B. papyrifera* is listed by TRAFFIC (wildlife trade monitoring program of WWF and IUCN) among the endangered species that need priority in conservation (Marshall, 1998). The decline in the population of *B. papyrifera* is related to several factors. As is the case in many tropical areas, there has been increased population pressure in the *B. papyrifera* growing parts of Eastern Africa, which has resulted in the conversion of *Boswellia* woodlands to agricultural lands. Unregulated free range grazing has damaged natural regeneration of *B. papyrifera* as its seeds and seedlings are preferred by livestock and wild life for grazing and browsing. They also suffer trampling while being grazed and browsed. Grazing on *B. papyrifera* seedlings result in the total removal of all vegetative parts, because of its

shallow root (Kindeya *et al.*, 2002). It is not uncommon to see over-tapping and use of inappropriate tapping methods by unskilled labourers. There is little supervision during tapping. More accessible trees are often tapped continuously with no resting periods. As a consequence, the natural regeneration and health of trees is affected. Seeds from tapped stands in Eritrea have a low germination rate (14% and 16%) when compared to seeds from un-tapped stands (94% and 80%) (Ogbazghi, 2001). According to Abeje (2002), the proportion of trees damaged by an unidentified worm was higher in tapped as compared to untapped stands in Gonder. To avoid such problems, some resting period is necessary for wound healing.

Genetic diversity is a key parameter in evaluating and planning the sustainable management of forest ecosystems. An understanding of the degree of diversity within and between the populations of a species is required to support the continuously rising plantation programs and to provide information for the conservation of the species' genetic resources, since geographically separated populations are expected to have different genetic compositions (Parasharami and Thengane, 2012). Within each tree species, amount and distribution of genetic variation is crucial for long-term stability of forest ecosystems (Sharma *et al.,* 2002). Genetic diversity is of prime importance for the species' persistence, since the evolutionary adaptive potential of populations depends on genetic variation patterns (Siregar, 2000; Finkeldey and Hattemer, 2007). Moreover the amount of genetic variation available within species also determines the potential for improving species through breeding programs.

2.5 Genetic markers for diversity analysis

A genetic marker is any visible character or otherwise assayable phenotype, for which alleles at individual loci segregate in a Mendelian manner (Weising *et al.,* 2005). Genetic markers can be

used to study the genetics of organisms, including trees, at the level of single genes (Spooner *et al.,* 2005). Genetic markers are used to study the genetics of natural and domesticated populations of trees and the forces that bring about change in these populations. Some of the more important applications of genetic markers include: (1) Describing mating systems, levels of inbreeding, and temporal and spatial patterns of genetic variation within stands (2) Describing geographic patterns of genetic variation (3) Inferring taxonomic and phylogenetic relationships among species (4) Evaluating the impacts of domestication practices, including forest management and tree improvement, on genetic diversity (5) Fingerprinting and germplasm identification in breeding and propagation of populations (6) Constructing genetic linkage maps and (7) Marker assisted breeding (Adams *et al.,* 2007).

Now a day's several genetic markers are available, each of which has different attributes that make it more or less desirable to use in certain applications (Karp *et al.,* 1997a&b). Some of the desirable attributes of a given type of genetic marker are that it be: (1) Inexpensive to develop and apply; (2) Unaffected by environmental and developmental variation; (3) Highly robust and repeatable across different tissue types and different laboratories; (4) Polymorphic, i.e. reveal high levels of allelic variability; and (5) Co dominant in its expression (spooner *et al.,* 2005). This 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) (Karp *et al.,* 1997a&b). Genetic markers are widely used by breeders and conservationists to study genetic diversity.

2.5.1 Morphological markers

Traditionally, diversity within and between populations was studied by assessing differences in the morphology, such as morphometric traits which are observable characters of the individual and are the result of genetic differences in loci distributed throughout the genome (Kermali, 1994). These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus they are available for immediate use. However, morphological determinations need to be taken by an expert in the species, they could be subject to changes due to environmental factors and may vary at different developmental stages; moreover, their number is limited (De Vicente and Fulton, 2003).

The detection of regional and local patterns of phenotypic variation offers an effective method of stratifying and sampling variation in germplasm collection (Peeters *et al.*, 1989) and assist in the formulation of conservation priorities (Maxted *et al.,* 1995). The results of these works can specifically be used in the planning of future collection missions (Bennett and Maxted, 1997), creating core collections (Tohme *et al.*, 1995) and identifying possible sources of parental material for breeding programs. Furthermore, the assessment of phenotypic variation is also useful for studying phylogeny and habitat adaptations of species and varieties (Hedenäs and Kooijman, 1996).

2.5.2 Biochemical markers

To overcome the limitations of morphological traits, other markers have been developed at both the protein level (phenotype) and the DNA level (Karp *et al.,* 1997a&b). Protein markers are usually named biochemical markers. These are also termed isozyme/allozyme markers or simply protein markers. Allozymes are allelic variants of enzymes encoded by structural genes (Adams

et al., 2007). Enzymes are proteins consisting of amino acids, some of which are electrically charged. As a result, enzymes have a net electric charge, depending on the stretch of amino acids comprising the protein (Karp *et al.,* 1997a). When a mutation in the DNA results in an amino acid being replaced, the net electric charge of the protein may be modified, and the overall shape (conformation) of the molecule can change. Because changes in electric charge and conformation can affect the migration rate of proteins in an electric field, allelic variation can be detected by gel electrophoresis and subsequent enzyme-specific stains that contain substrate for the enzyme, cofactors and an oxidized salt (Devine *et al.,* 2003).

Isozyme analysis is, in principle, a robust and reproducible method (Adams *et al.,* 2007). In addition, isozymes are codominant markers and are suitable for estimating all population genetics parameters and for genetic mapping. The major limitation of isozyme analysis is the low number of markers (Karp *et al.,* 1997a). Consequently, the percentage of genome coverage is inadequate for a thorough study of genetic diversity (De Vicente and Fulton, 2003). Another disadvantage of isozyme analysis lies in the markers being based on phenotype. As such, they may be influenced by environmental factors, with differences in expression confounding the results interpretation. Since differential expression of the genes may occur at different developmental stages, or in different tissues, the same type of material must be used for all experiments (De Vicente and Fulton, 2003).

2.5.3 Molecular markers

DNA polymorphisms can be detected in nuclear and organelles DNA, which is found in mitochondria and chloroplasts. Molecular markers concern the DNA molecule itself and, as such, are considered to be objective measures of variation. They are not subject to environmental

influences; tests can be carried out at any time during plant development; and, they have the potential of existing in unlimited numbers, covering the entire genome (De Vicente and Fulton, 2003; De Vienne *et al.*, 2003).

Due to the rapid developments, in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Whitkus *et al.,* 1994; Karp *et al.,* 1996, 1997a, b; Parker *et al.,* 1998; Schlötterer, 2004). These techniques may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications (Spooner *et al.*, 2005).

These Marker techniques could be divided into three broad categories with respect to basic strategy (Karp and Edwards, 1997):

I. Non-PCR based approaches**;**

II. PCR Arbitrary priming;

III. Targeted-PCR and sequencing.

These techniques are very fruitful tools in diversity assessment on the level of DNA sequences which became important method in studying plant genetics (Gebhardt *et al.*, 1991).

2.5.3.1. Non-PCR based techniques

a. Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis was one of the first molecular techniques used for enabled the detection of polymorphisms at the sequence level. The principle behind the technology rests on the possibility

of comparing band profiles generated after restriction enzyme digestion in DNA molecules of different individuals. These differences in fragment lengths can be seen after gel electrophoresis, blotting the fragments to a filter and hybridizing probes and visualization (Karp and Edwards, 1997).

RFLP analysis was used extensively in the construction of genetic maps and has been successfully applied to genetic diversity assessments, particularly in cultivated plants, but also in populations and wild accessions (Sinclair *et al.*, 1999). As a technique for diversity studies, there are three important advantages which should be considered (Karp and Edwards, 1997): RFLPs are highly reproducible between laboratories and the diversity profiles generated can be reliably transferred, RFLPs are co-dominant markers, enabling heterozygotes to be distinguished from homozygotes and no sequence-specific information is required and the approach can be applied immediately for diversity screening in any system. There are serious limitations in RFLP technique such as it needs large amount of DNA, can not be automated, needs a suitable probe library, and consumes time and costly.

2.5.3.2 PCR arbitrary priming

With the advent of PCR, a number of techniques became available for the screening of genetic diversity. These require no prior sequence-specific information and can, therefore, be applied directly to any organism. Three main techniques fall within the category of PCR-based markers using arbitrary primers: RAPD, DAF and AP-PCR (Caetano-Anollés *et al.,* 1992). The techniques are based on the use of a single 'arbitrary' primer, which may be purchased from commercial companies, in a PCR reaction on genomic DNA and result in the amplification of several discrete DNA products.

a. Randomly Amplified Polymorphic DNA (RAPD)

The most commonly used is RAPD analysis in which the primers are usually 10-mer or 20-mers and in which the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light (Williams *et al.*, 1990). Polymorphisms are detected as the presence or absence of bands and result from sequence differences in one or both of the primer binding sites. RAPD techniques played an important role in characterizing the plant genetic resources around the world (Lee *et al.*, 2001). This technique helped also in genetic relatedness assessment (Cabrita *et al.*, 2001).

b. Amplified Fragment Length Polymorphism (AFLP)

Vos *et al.* (1995) described the AFLP technique as being based on the detection of restriction fragments by PCR amplification and argued that the reliability of the RFLP technique is combined with the power of the PCR technique. AFLPs are DNA fragments (80–500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR. This technique is essentially involves both RFLPs and PCR, in that the first step is restriction digestion of the genomic DNA but this is then followed by selective rounds of PCR amplifications of the restricted fragments that are then separated by polyacrylamide gel electrophoresis. This technique can be analyzed on automatic sequencers using fluorescent labeled primers and, therefore, high throughput can be achieved (Winfield *et al.*, 1998).

The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of applications, and the fact that no sequence data for primer construction are required. AFLP is a widely valued technology

for gene mapping studies (Vos *et al.*, 1995). The key feature of AFLP is its capacity for the simultaneous screening of many different DNA regions distributed randomly throughout the genome (Mueller & Wolfenbarger, 1999). Disadvantages of AFLP include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible non-homology (Spooner *et al.*, 2005).

c. Inter Simple Sequence Repeats (ISSR)

ISSRs are DNA fragments of about 100–3000 bp located between adjacent, oppositely oriented microsatellite regions. The technique is based on PCR amplification of inter-microsatellite sequences. ISSRs are amplified by PCR using, as primers (16-18 bp), an oligonucleotide sequence typical of the microsatellite repeats ending with few selective nucleotides which anchors the primers into the adjacent non-repeat regions. About 10–60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as presence or absence of fragments of particular size. The ISSR primers are used to detect the variation in a given DNA sample include one of these highly variable microsatellite sequences and arbitrary pair of bases at the 3’end (Zietkiewicz *et al.*, 1994).

The main advantage of ISSRs are: (i) no sequence data for primer construction are needed, (ii) the analytical procedures include PCR, hence only low quantities of template DNA are required (5–50 ng per reaction), (iii) are randomly distributed throughout the genome, and (iv) the variation within unique regions of the genome may be found at several loci. Furthermore, it is very useful marker for DNA profiling, especially for closely related species. Since ISSR is a multilocus technique, disadvantages include the possible non-homology of similar sized fragments, and ISSR is dominant marker. Moreover, ISSR, like RAPD, can have reproducibility

problems but more reproducible than RAPD (Spooner *et al.*, 2005). ISSR analysis can be applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Zietkiewicz *et al.*, 1994).

2.5.3.3 Targeted-PCR and Sequencing

a. Microsatellites, Simple Sequence Repeats (SSRs)

Microsatellites, also called Simple Sequence Repeats (SSRs) and, occasionally, Sequence-Tagged Microsatellite Sites (STMS) or Simple Sequence Repeat Polymorphisms (SSRPs), represent short tandem repeats (1–10 base pairs) (Hearne *et al.*, 1992). If nucleotide sequences in the flanking regions of the microsatellite are known, specific primers (generally 20–25 bp) can be designed to amplify the microsatellite by PCR. Microsatellites and their flanking sequences can be identified by constructing a small-insert genomic library. This type of repeated DNA is common in eukaryotes, the number of repeated units varying widely among organisms (Spooner *et al.*, 2005, Weising *et al.*, 2005). These polymorphisms are identified by constructing PCR primers homologous to the DNA sequences flanking the microsatellite region. PCR product size variation is caused by differences in the number of microsatellite repeat units. SSR polymorphisms can be visualized by agarose or polyacrylamide gel electrophoresis. Microsatellite alleles can be detected, using various methods: ethidium bromide, silver staining, radioisotopes or fluorescence. Since microsatellite analysis is, in principle, a single-locus technique, multiple microsatellites may be multiplexed during PCR or gel electrophoresis if the size ranges of the alleles of different loci do not overlap (Dean *et al.*, 1999).

The advantages of microsatellites include their codominance, high genomic abundance in eukaryotes and random distribution throughout the genome, with preferential association in low-copy regions (Morgante *et al.*, 2002). Because the technique is PCR based, only low quantities (10–100 ng per reaction) and not necessarily high quality DNA are required. Due to the use of long PCR primers, the reproducibility of microsatellites is high. This decreases significantly the analytical costs. Furthermore, the screening of microsatellite variation can be automated, if the use of automatic sequencers is an option. One of the main microsatellite disadvantages is their high development costs if adequate primer sequences for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. A very common observation in microsatellite analysis is the appearance of stutter bands which are artifacts products that occur by DNA slippage during PCR amplification. These can complicate the interpretation of the band profiles because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes.

Simple sequence repeats (SSRs) have become important molecular markers for a broad range of applications, such as genome mapping and characterization, phenotype mapping, marker assisted selection of crop plants and a range of molecular ecology and diversity studies (Robinson *et al.*, 2004). Microsatellites are also considered ideal markers in gene mapping studies (Hearne *et al.*, 1992).

2.6 Genetic diversity study in *Boswellia*

The conservation and sustainable use of *Boswellia* genotypes is mainly depends on proper characterization of the *Boswellia* genetic resources. For aiding the breeding program in *Boswellia* it is imperative to study genetic diversity of the existing sesame germplasms through systematic evaluation and characterization and making the information available to the researchers. Only few studies have done on genetic diversity of *Boswellia* previously. Coppi *et al.,* 2010, used DNA sequences from the ITS region of the nuclear genome and Inter-Simple Sequence Repeat markers (ISSR) to estimate genetic diversity among and within populations of the Frankincense tree *Boswellia sacra* from Dhofar, Oman. They found 511 bp long ITS sequences with low (6.4%) variation among geographically different populations. In addition total genetic diversity and average heterozygosity within populations using four ISSR primers resulted fairly low (0.22 and 0.136, respectively). Moreover, analysis of Molecular Variance showed that differentiation among populations was relatively high (38.1%), possibly due to the reduced gene flow between the largely isolated stands of *Boswellia* (N_m= 0.39). Genetic distances and AMOVA analysis was suggested a clear differentiation between the eastern and western coastal populations (Copii *et al.,* 2010).

ISSR marker, in addition to its suitability to genetic diversity study, is highly polymorphic, reproducible,and cost effective and requires no prior information of the sequence. In economically important forest trees such as *Boswellia* this marker is used to study the patterns and level of diversity. Hence, the ISSR marker assay has been chosen to study the level of diversity and patterns of distribution of *Boswellia* genetic resources in Metema.

3. OBJECTIVES

3.1 General objective

- The general objective of the study was to investigate the reproductive biology and genetic variability among *Boswellia papyrifera* populations of Metema, Northern Ethiopia.

3.2 Specific objectives

- To assess molecular genetic diversity present among *B. papyrifera* populations found in Matema, Northern Ethiopia.
- To identify populations of Metema with higher diversity for *Boswellia* improvement and conservation.
- To determine the phonological pattern of *Boswellia* populations of Metema
- To study the pollination ecology of *Boswellia* populations of Metema

4. MATERIALS AND METHOD

4.1 Plant material

The study site was selected in relatively homogenous *B. papyrifera* natural stands of North Gondar, Metama Woreda, which is one of the major frankincense producing area in the country with coverage of 68, 000 ha. The altitude of Metema ranges from as low as 550 to 1608 m asl while the minimum annual temperature ranged between 22^{0}C and 28^{0}C. Daily temperature becomes very high during the months of March to May, where it may get to as high as 43^{0} C.The mean annual rainfall for the area ranges from 850 to 1100 mm (Report on Metema pilot learning site diagnosis and program design (unpb.), 2005).

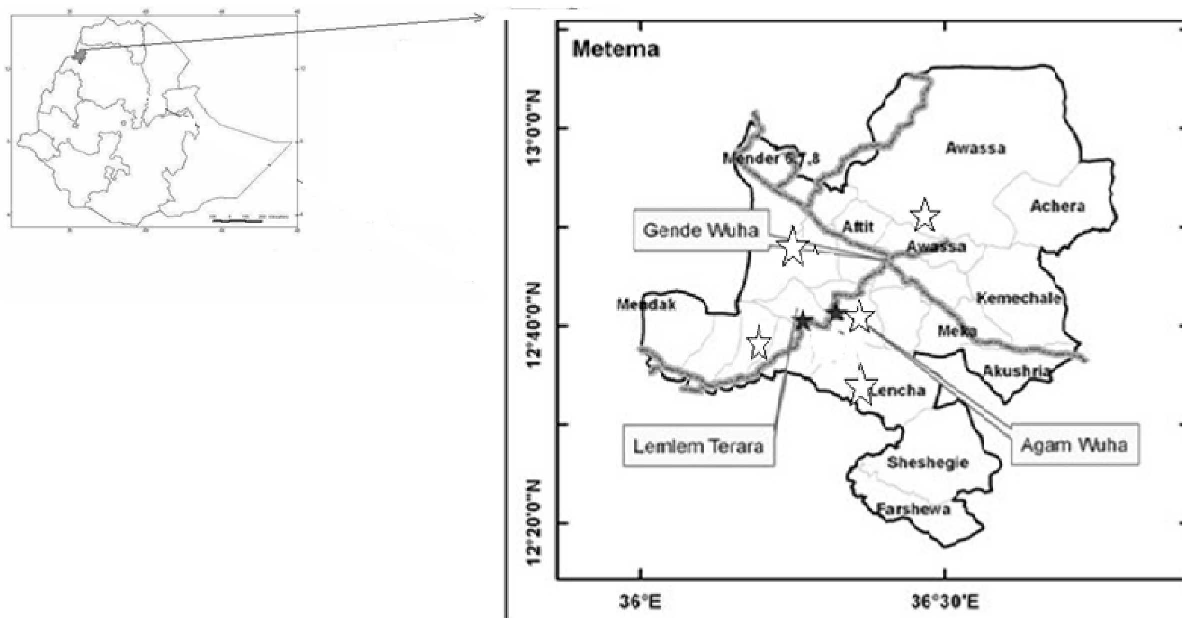
Five populations were selected based on their population size and relative distances with each other. Those are, Das Gundo (15,855 ha), Gubay (6,186 ha), Zebach Bahir (3,050 ha), Shinfa (2,088 ha) and Agam Wuha (1,932 ha), respectively (Report on Metema pilot learning site diagnosis and program design (unpb.), 2005). Except Zebach Bahir (12) each population were represented with 13 individual tree and totally 64 individuals were sampled. Four to five young leaves were collected from each tree and dried in silica gel.

4.2 DNA extraction

Total genomic DNA was extracted from silca-dried leaf-tissue by using Cetyl Trimethyl Ammonium Bromide (2% Cetyltrimethyl ammonium Bromide, 1% polyvimylpyrolidone, 100mMTris: PH=8, 20mM EDTA, 1.4M NaCl, 0.2% beta-Mercapto-ethanol) extraction protocol based on Borsch *et al.,* (2003) with minor modifications. Approximately equal amounts (ca. 0.2g)

of the dried leaf samples were ground with pistle and mortar. Total genomic DNA were isolated from about 0.2 g of the pulverized leaf sample.

For ISSR-PCR analysis, two extractions for each sample was run out on agarose gel at 0.98% concentration to check the presence and quality of genomic DNA and then to select between extraction on the basis of quality and concentration. From stock solution, 5µl of genomic DNA and 2µl of 6x loading dye was used and applied by adding 5 µ ethidium bromide on the gel in 1x TBE solution running buffer and then electrophoresed at 80 volt constant for 45 min. For comparisons, 100bp DNA ladder with known concentration was used. The gel picture was further examined and used to make selection of good quality DNA extract. Genomic DNA from the second extractions was found to be promising and was selected for ISSR-PCR analysis.



Key: ☆ - Locatons where the samples were collected

Figure 1. Administrative woredas of Metema Zone showing sites where the Boswellia population used in this study were collected. Source: Tefera, (2011).

4.3 Primer selection and optimization

The ISSR marker assay was conducted at Genetics Research Laboratory of the Microbial, Cellular and Molecular Biology Department, Addis Ababa University. A total of nine primers were used for the initial testing of primers variability and reproducibility. One individual was selected from each population to screen the primers with 1:10 dilutions. Same procedure described under section 4.4, next page, was followed in the screening process. A total of five polymorphic and reproducible ISSR primers were selected after testing and screenings. Table 2 shows the list of primers used and tested, their annealing temperature with respective sequences and other properties.

Table 2. List of primers, annealing temperature, primer sequence, amplification pattern and repeat motives used for optimization (Source: UBC).

Primer	Annealing temperature	Primer Sequence[1]	Amplification pattern	Repeat motives
810	45^0C	GAGAGAGAGAGAGAGAT	Good	Dinucleotide
812	45^0C	CTCTCTCTCTCTCTCTA	Good	Dinucleotide
818	48^0C	CACACA CAC ACA CAC AG	Good	Dinucleotide
824	45^0C	CTCTCTCTCTCTCTCTRA	Not reproducible	Dinucleotide
834	45^0C	AGAGAGAGAGAGAGAGYT	Good	Dinucleotide
844	45^0C	GAGAGAGAGAGAGAGAYT	Not polymorphic	Dinucleotide
873	45^0C	GACAGACAGACAGACA	Not polymorphic	Dinucleotide
878	45^0C	GGATGGATGGATGGAT	Not polymorphic	Tetranucleotide
880	48^0C	GGAGAGGAGAGGAGA	Good	Pentanucleotide

[1] *Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T)*

4.4 PCR and gel electrophoresis

The polymerase chain reaction was conducted in Biometra 2000 T3 Thermo cycler. PCR amplification was carried out in a 25 µl reaction mixture containing 1µl template DNA, 11.05µl H_20, 5.6µl dNTP (1.25mM), 2.6µl Taq buffer (10xThermopol reaction buffer), 1.25µl $MgCl_2$ (2mM), 2.4µl beitein, 0.6µl primer (20pmol/µl) and 0.2µl Taq Polymerase (5u/µl). The amplification program was 4 minutes preheating and initial denaturation at 94^0C, then 40 x 15 seconds at 94^0C, 1 minute primer annealing at (45^0C/ 48^0C) based on primers used, 1.30 minutes extension at 72^0C and the final extension for 7 minutes at 72^0C. The PCR reactions were stored at 4^0C until loading on gel for electrophoresis. The amplification products were differentiated by electrophoresis using an agarose gel (1.67% agarose with 100 ml 1xTBE) and 8µl amplification product of each sample with 2µl loading dye (6 times concentrated) was loaded on gel. DNA marker 100 bp was used to estimate molecular weight and size of the fragments. Electrophoreses were run for 3 hours at constant voltage of 100V. The DNA was stained with 30µl (10mg/ml) ethidum bromide which were mixed with 450 ml distilled water for 30 minutes and washed with distilled water for 30 minutes.

4.5 Data management and statistical analysis

ISSR fingerprint profiles were scored manually for each individual sample from the gel photograph. The bands were recorded as discrete characters, presence '1' or absence '0' and '?' for missing data. Based on recorded bands different software's were used for analysis. POPGENE version1.32 software (Yeh*e et al.*, 1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, gene diversity and Shannon–Weaver diversity index (H). Analysis of molecular variance (AMOVA) was used to

calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al.,* 2006).

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

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

Where,

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

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

'c' is the total number of bands present in individual j but not in individual i.

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

To further examine the patterns of variation among individual samples on 3D (three dimension), a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer *et al.,*2001). The first three axes were used to plot the three dimensional PCO with STATISTICA version 6.0 software (Hammer *et al.,* 2001; Sta. soft, Inc.2001).

4.6 Phenological observations

Ten trees were selected and tagged for diameter at breast height (DBH) and their height measurements were taken. Flushing, flowering and fruiting percentages of crown in each individual tree were estimated visually. The numbers of inflorescences per branch were counted from selected branches per tree. The number of flower buds/flowers per inflorescence were recorded for each selected tree (Sunnichan *et al*., 2005). Observations were carried out weekly from September 2010 to March 2011. Selected individuals were observed throughout the flowering and fruiting season and visual estimate were taken to assess the extent of flowering and fruiting. This was initiated in the flower bud stage and continued until the end of flowering season.

4.7 Pollinator activity

Observations on floral visitors were made between dawn and dusk for 15 min every hour (Sunnichan *et al*., 2005). The observations were made on a single flower on a single branch of a tree once at a time. Therefore, the number of floral visits made by an insect and the time spent on each flower were recorded using a stop watch.

5. RESULTS

5.1 Banding patterns of the ISSR primers used

Out of the nine primers tested initially, five which gave relatively clear banding pattern (four di-nucleotide and one penta-nucleotide) were selected and used in this study (Table 3). The molecular weight of the bands amplified using the primers were in the range of 300 bp to 700 bp.

A total of 39 clear and reliable bands were scored. The least polymorphic bands (six) were scored from primer 834 and the highest polymorphic bands (ten) were scored from primer 880. Both primers 810 and 818 show equal polymorphic bands (eight) and the remaining primer, 812, show seven polymorphic bands. Average number of both bands and polymorphic bands per primer were 7.8. Figure 2 shows the amplification pattern of primer 818.The other primers gel pictures used in this study along with tested non-polymorphic primers are presented in appendix 1.

Table 3. The five primers used, their repeat motifs, amplification quality and number of bands scored

Primers	**Repeat motif**[1]	**Amplification pattern**	**Number of scored bands**
810	$(CA)_8G$	Good	8
812	$(GA)_8$ A	Good	7
818	$(CA)_8G$	Good	8
834	$(AG)_8YT$	Good	6
880	(GGAGA)3	Good	10
Total			39

[1] *Single-letter abbreviations for mixed base positions: Y = (C,T)*

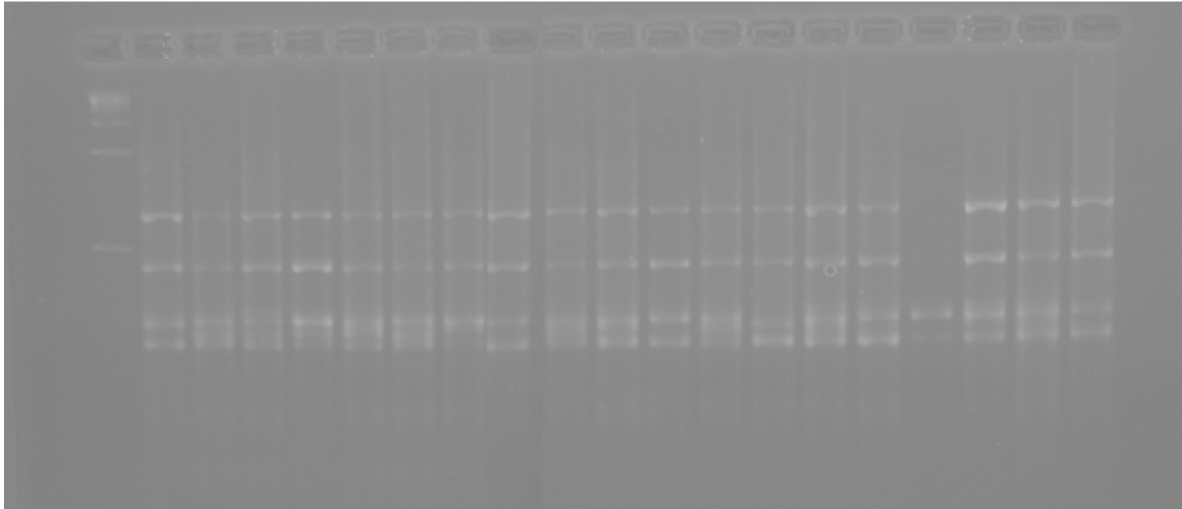


Figure 2ISSR fingerprint generated using primer 818

5.2 Polymorphism based percent polymorphism

In all populations or individuals, the number of polymorphic loci ranges from six for primer-834 to ten for primer 880. All 39 loci scored were observed to be polymorphic (100%) considering the entire populations as one group. The percent polymorphism for all the populations studied were ranged from 97.4% (Zebach Bahir) to 59% (Agam Wuha), respectively (Table5.2b). No unique bands were observed for either the individuals or the populations. As to the primers used, all the primers showed 100 per cent polymorphism considering the entire populations as one group.

Among the *Boswellia* populations evaluated the highest percentage polymorphic loci was obtained for samples from Zebach Bahir (*P* = 97.6%), followed by samples from Das Gundo (82.1), while samples from Agam Wuha (59%) showed the least percent polymorphic loci (Table 5.2b).

5.3 Genetic diversity

Genetic diversity expressed as gene diversity value showed higher variability for Zebach Bahir population with 0.42, followed by Das Gundo population with 0.33 values. Agam Wuha population was observed to be the least diverse with gene diversity value of 0.25. The total diversity index values for the total populations were found to be 0.60 (Table 4b).

The same diversity patterns were also observed for Shannon diversity index, whereby Zebach Bahir and Das Gundo populations showed the highest value with 0.60 and 0.48, respectively. The least variability was showed by Agam Wuha population.

Table 4. Number of scorable bands (NSB), number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (GD) and Shanon Index (I), with each primers (a). and for each population (b).

(a)

Primers	NSB	NPL	PP	GD+SD	I+SD
810	8	8	100.00	0.37+0.13	0.55+0.15
812	7	7	100.00	0.41+0.08	0.60+0.08
818	8	8	100.00	0.41+0.13	0.59+0.15
834	6	6	100.00	0.42+0.09	0.61+0.10
880	10	10	100.00	0.46+0.05	0.65+0.05

(b)

Populations	NPL	PP	GD+SD	I+SD
Zebach Bahir	38	97.44	0.42+ 0.10	0.60+ 0.13
Shinfa	29	74.36	0.30+ 0.20	0.44+0.28
Das Gundo	32	82.05	0.33+0.18	0.48+0.25
Gubay	29	74.36	0.26+0.19	0.38+0.26
Agam Wuha	23	58.97	0.25+ 0.22	0.36+0.31
Over All	39	100.00	0.42+0.10	0.60+0.11

5.4 Analysis of molecular variance

Analysis of molecular variance (AMOVA) was carried out on the overall ISSR data score of *Boswellia* populations without grouping (Table 5). AMOVA without grouping population revealed that higher percentage of variation (74%) is attributed to the within population variation while the remaining variation is due to the among population variation (26%). The variations was found to be highly significant at (P=0.00).

Table 5. Analysis of Molecular Variance (AMOVA) among populations of Boswellia without grouping popuplations

Source of variation	Sum of squares	Variance components	Percentage variation	Significance
Among populations	120.815	1.93572	26.00	0.00
Within populations	324.140	5.51064	74.00	
Total	444.954		7.44636	

5.5 Clustering analysis

UPGMA and neighbor joining analysis was used to construct dendrogram for the five populations and 64 individuals based on 39 PCR bands amplified by four di-nucleotides (810, 812, 818, 834) and one penta nucleotides (880) ISSR primers. The dendrogram derived from neighbor-joining analysis of the whole ISSR data showed three distinct clusters and two sub-clusters within the second and third major cluster (Figure 5). Most individuals from all populations were tended to form their own cluster while only few of the individuals distributed all over the tree. Among the five populations Gubay (major cluster **III**, sub-cluster **II**), Agam Wuha (major cluster **III**, sub-cluster **II**) and Das Gundo (major cluster **III**, sub-cluster **I**) showed clear grouping. UPGMA analysis of Metema *Boswellia* populations revealed one major cluster and two outliers (Gubay and Agam Whuha). The major cluster again forked into two sub groups

the first containing Das Gundo populations, while the second containing Zebach Bahir and Shinfa, which are highly related populations (Figure 3). On the other hand, individual based UPGMA clustering of an overall analysis showed strong clustering of individuals with respect to their populations except few intermixed individuals from other population (Figure 4). This result is similar to that of individual based NJ. Grouping based on the origins of individuals is the dominant pattern observed on NJ and UPGMA trees.

Key:

- Zb – Zebach Bahir
- Sh - Shinfa
- Dg – Das Gundo
- Aw – Agam Wuha
- Gu - Gubay

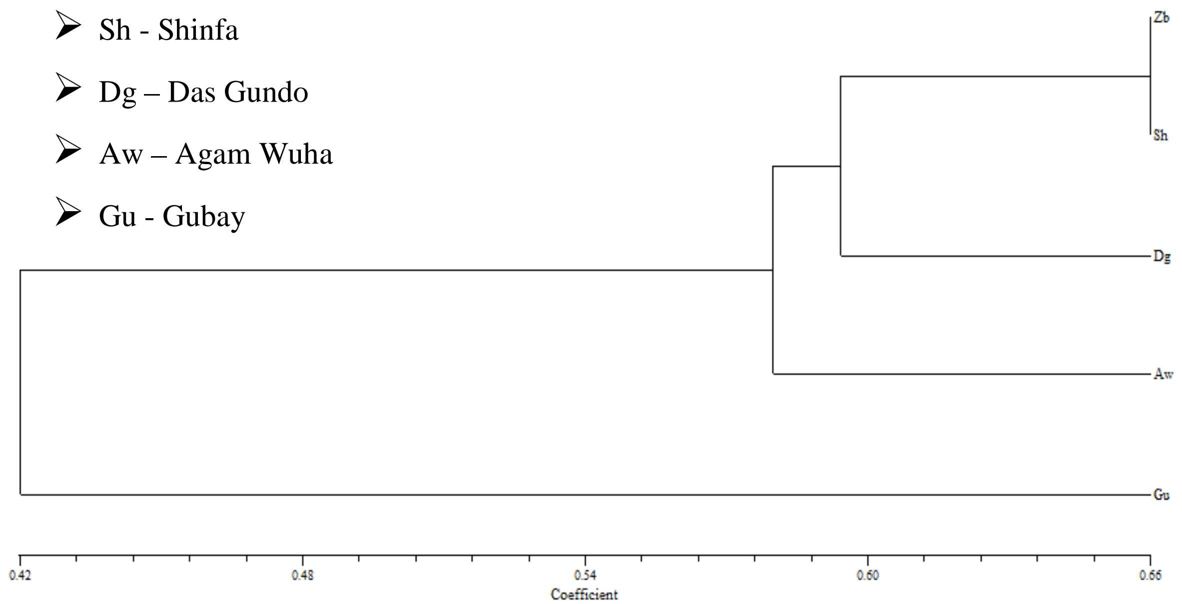


Figure 3. UPGMA based dendrogram for five Metema Zone *Boswellia papyrifera* populations using 5 ISSR primers

.

Key:

- Zb-1-12- Zebach Bagir
- Sh-1-13- Shinfa
- Dg- 1-13- Das Gundo
- Gu- 1-13- Gubay
- Aw- 1-13- Agam Wuha

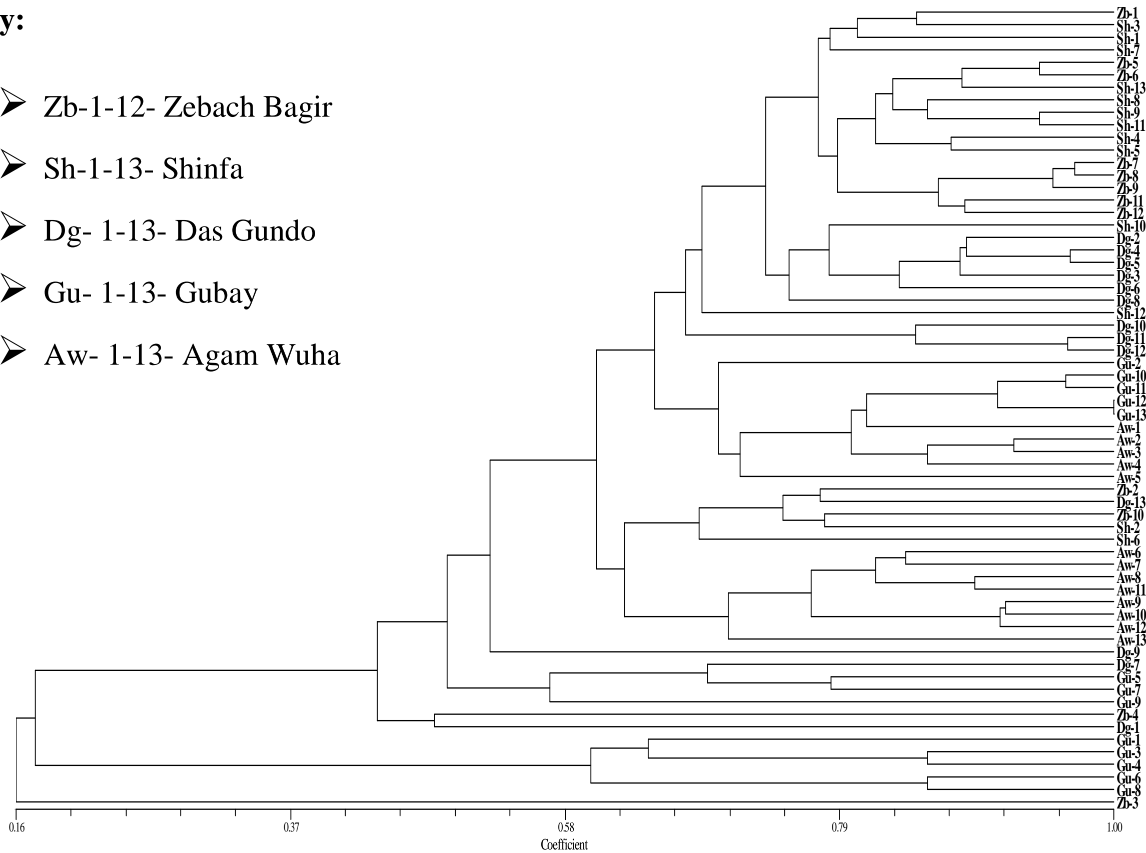


Figure 4. UPGMA based dendrogram for 64 individuals of *Boswellia papyrifera* using five ISSR primers. The UPGMA algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.

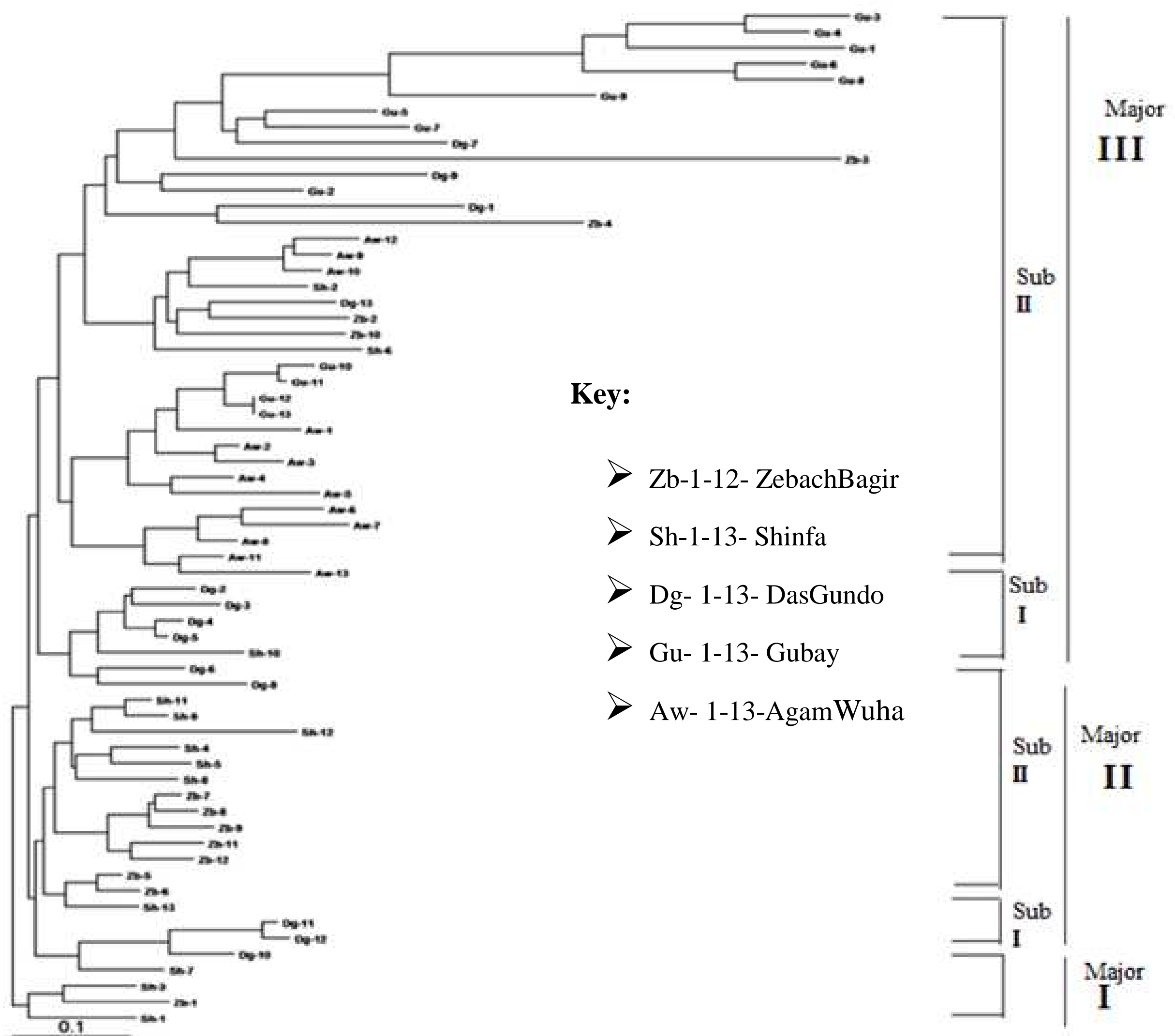
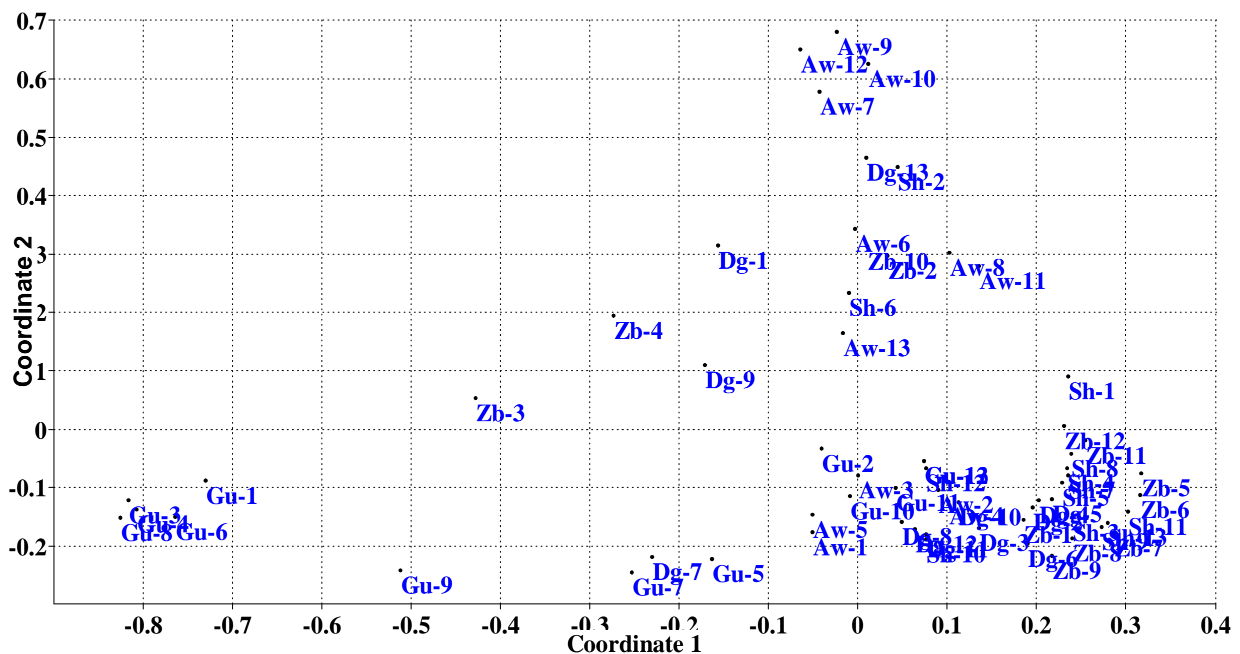


Figure 5 Neighbor-joining analysis of 64 individuals based on 39 PCR bands amplified by five primers. The neighbor joining algorithm is based on Jaccard's coefficients obtained after pair wise comparison of the presence-absence fingerprint.

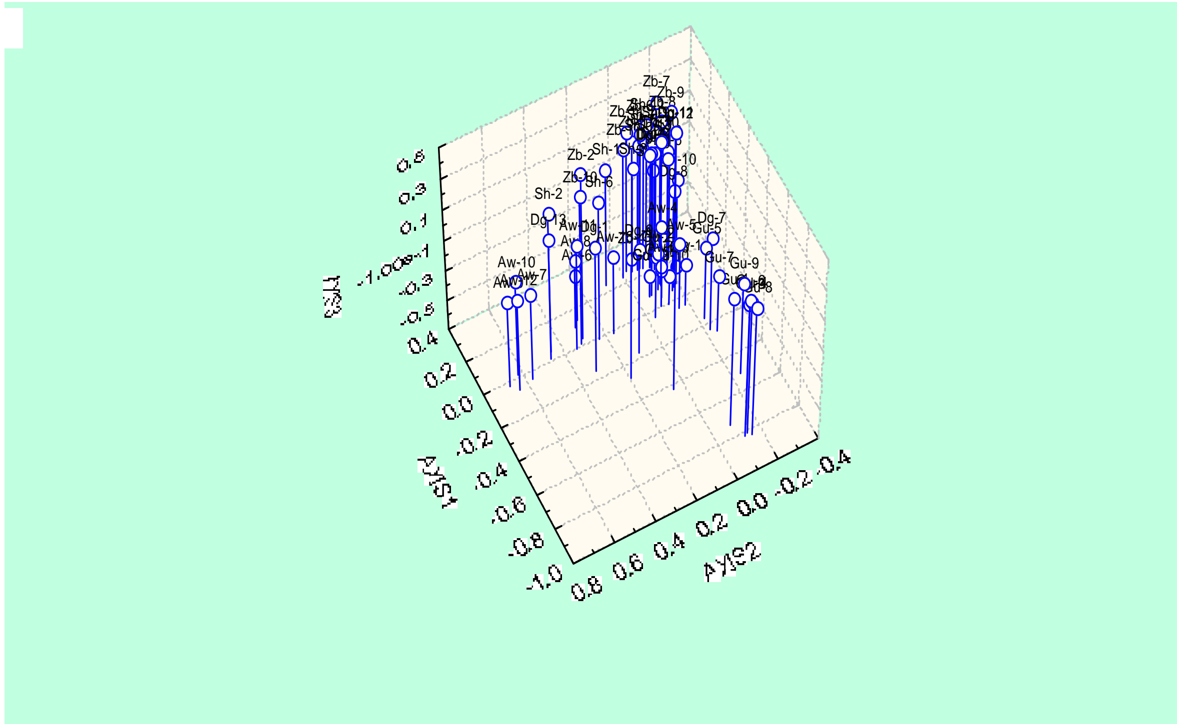
5.6 PCO analysis

All the data obtained using the five ISSR primers were used in PCO plotting. The analysis was carried out using Past and Statistica softwares by employing Jaccard's coefficients of similarity. The first three coordinates of the PCO having eigenvalues of 5.24, 3.69 and 3.59 with variance of 12.6%, 8.9% and 8.6%, respectively used to reveal the grouping of individuals using two and three coordinates (Figure 6 and Figure 7). In 3D most of Agam wuha and Gubay individuals tended to form their own grouping. Individuals from Zebach Bahir, Das Gundo and Shinfa were inter-mixed with each other and made separate group from the other populations. Using two coordinates (Figure 6) almost similar result was observed like that of three coordinates.



Key- Zb-1-12-Zebach Bahir, Sh-1-13-Shinfa, Gu-1-13Gubay, Aw-1-13-Agam Wuha and Dg-1-13-Das Gunda

Figure 6. Two dimensional representation of principal coordinate analysis of genetic relationships among 64 individuals of five Metema populations.



Key- Zb-1-12-Zebach Bahir, Sh-1-13-Shinfa, Gu-1-13Gubay, Aw-1-13-Agam Wuha and Dg-1-13-Das Gunda

Figure 7. Three dimensional representation of principal coordinate analysis of genetic relationships among 64 individuals of five Metema populations.

5.7 Phenology

Diameter at breast height (DBH) and height of the ten trees are characterized in table 6.

Leaf fall starts at the end of September and continue to the mid of November. After the leaf fall was completed, it was observed that the trees produced new leaves which are green and small in size than the primary leaves. The trees remain leafless for 2–3 weeks prior to flowering and during the entire period of flowering and fruiting.

Table 6. DBH and height of the trees

Tree	Height (m)	DBH (cm)
1	5.5	80
2	7.1	90
3	5.75	80
4	5.4	78
5	6	80
6	6.4	93
7	7.4	109
8	6.4	73
9	6	76
10	6.4	96

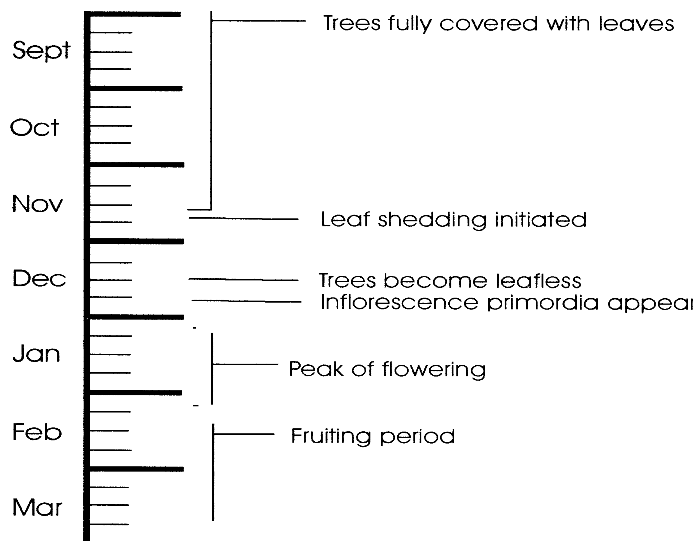


Figure 8. Various phenoevents in *Boswellia papyrifera*

Flower initiation begins after 1 week to 3 weeks of leaf fall is completed and the trees remain leafless throughout the remaining period (period of flowering and fruiting). Flower initiation started at the end of November and continued up to January. During flower initiation the tip of the branch became green and produced flower buds in cluster. However, the flower initiation and flowering was not uniform throughout the population. During observation, flowering was highly variable, there was about one to two weeks gaps of flowering period between trees and within trees (between branches).

During phenological observations, *B. papyrifera* carried 8-15 flowers per inflorescence. However, in some cases, flower buds could increase up to 60 flower buds under optimum environmental conditions. Moreover, during maximum flowering period, on average, 14.5 inflorescences per branch was recorded. After the female flower received pollen, the flower become closed and start fruiting. Fruiting starts late January and continues to end of March.

5.8 Pollinator activity

In this study, it was observed that the flowers of *B. papyrifera* are frequently visited by honeybees and rarely by wasps.

Figure 9 Figure showing flowers of B. papyrifera being visited by honey bees. Photo by Abush Zinaw, 2011.

It was found that a flower is visited by an average number of 6.35 insect visitors every 15 minutes. Maximum number of visitors recorded was nine and minimum number was 4 per 15 minutes, respectively. On average, each insect visitor spent 5.8 seconds on a flower.

6. DISCUSSION

6.1. Molecular diversity

Genetic diversity is essential to the long-term survival of species; without it, species cannot adapt to environmental changes and are more susceptible to extinction. The amount of genetic variation available within species also determines the potential for improving species through breeding and selection programs. Knowledge of pattern of genetic variability is useful for defining heterotic patterns in hybrid breeding and for relating the observed pattern with presence of certain economically important traits. Such information can be used to design effective germplasm conservation and for setting germplasm collection mission as well as to estimate or predict the risk of genetic erosion in certain area. Pattern of genetic variability can be studied by morphological, isosyme or molecular markers. Among the molecular markers ISSR markers are important to study genetic variations in plant species, as they are effective in detecting very low levels of genetic variation (Zietkiewicz et al., 1994).

The present study provides the first estimates of genetic diversity of the frankincense tree population of *B. papyrifera* of Ethiopia. Five populations from Metema area of Amhara region, northern Ethiopia were analysed by five ISSR primers yielding 39 scorable and reproducible bands. Under the assumption that each fragment represents a genetic locus, it was possible to estimate allelic frequency and therefore to proceed further into estimating classical population parameters.

ISSR primers have high resolution power in fingerprinting and diversity analysis of *Boswellia* (Coppi *et al.,* 2010). The present study also confirms that ISSR markers are efficient in detecting polymorphism within and among populations of *Boswellia*. All the 39 scored fragments

generated from five primers were polymorphc. This result is in agreement with previous studies, (Coppi, *et al.* 2010), whom reported 97.9% polymorphism using ISSR marker. The genetic diversity parameters: percent polymorphic loci, gene diversity (GD), and Shannon's diversity index (I) indicated that the genetic diversity in the *Boswellia* populations of Zebach Bahir and Das Gundo is indeed high. Shannon diversity index result showed that populations of *Boswellia* from Zebach Bahir were more diverse (0.60) than populations from Das Gundo area (0.48). Similarly, gene diversty result indicates that *Boswellia* population from Zebach Bahir (0.42) has higher genetic diversity than from that of Das Gundo (0.33). The overall total Nei's gene diversity and Shanon's diversity index were high, 0.42 and 0.60, respectively. This result is in agreement with other woody species from tropical, subtropical or warm temperate regions that have been recently studied with ISSR markers, such as *Hagenia. abyssinica* (GD=0.40, Tileye *et al.,* 2007), *Eucalyptus grandis* (GD=0.338, Okun *et al.,* 2008) and *Asparagus acutifolius* L. (GD=0.262, Sica *et al.,* 2005).

The success of a tree-improvement programme largely depends on the availability and knowledge of the genetic resources in a germplasm collection. In the present study, all the diversity parameters confirm that there is medium to high gene diversity in Metema populations of *B. papyrifera*. Even though the small area representing the Ethiopian *Boswellia* population thinking one of the largest populated areas is the Metema area this fact would give us some hint on the diversity of Ethiopian populations. Since areas of high genetic diversity contribute more accessions than those with a low diversity for further and future collection, breeding and conservation activities high priority should be given to areas with high genetic diversity. The result of this study show among the five areas higher genetic diversity is shown by the individuals coming from the Zebach bahir area.

Analysis of molecular variance (AMOVA) using four primers for *Boswellia* populations of Oman showed inter-population differentiation and within-population variation, 38.2% and 61.9%, respectively, which is relatively close to the result of present study 26% and 74%, respectively (Coppi *et al.,* 2010). The slight difference may be due to the fact that the present result is based on small geographical area and sample size.

A number of factors contribute to the high levels of within population genetic diversity typically found in forest tree populations: (1) Large population size; (2) Longevity; (3) High levels of outcrossing; (4) Strong migration between populations; and (5) Balancing selection (Ledig, 1986, 1998; Hamrick *et al.,* 1992). Generally, in outbreeding species with more or less continuously distributed populations, the proportion of internal variation is often over 70%, while inter-population differentiation is often lower than 30% (Hamrick and Godt 1989; Nybom and Bartish 2000; Ge *et al.,* 2005b).

A cluster analysis (UPGMA and Neighbor joining) was used to construct a dendrogram in order to see relationships among all individuals. Both dendrograms indicate a strong separation between the five *Boswellia* populations except few individuals which are distributed through out the dendogram. The separation and formation of a cluster by the five populations may suggest that these populations may have been isolated from each other for a longer period in time and limited long distance geneflow. However, PCO analysis based on two and three coordinates show populations from Das Gundo, Zebach Bahir and Shimfa inter-mixed each other and tend to form separate group. Overall, the structure observed in all the clustering analysis showed that there is moderate to low geneflow among populations of *Boswellia* in Metema region. In the intermixed populations only Das Gondo and Shinfa share bounderies.

6.2 Phenology and pollinator activity

Phenological patterns in tropical plants are key factors in understanding their reproductive strategies, and therefore, fluctuations in resource availability that directly affect demographic dynamics and reproductive cycles in herbivores (Van Schaik *et al.,* 1993). The present study is the first study on the reproductive biology of *B. papyrifera* of Ethiopia. The trees remain leafless during the whole of the flowering and fruiting period. The reproductive phases in the phenology of *B. papyrifera* are constrained to the dry season in the study site, a trait shared by many other tree species from the tropical forest (Sunnichan *et al.,* 2005). The absence of leaves on the tree during the entire period of flowering and fruiting might be the trees have to utilize limited water available and resources stored in the stem for fruit and seed loading (Baker *et al.,* 1983).

Variation in the abundance and availability of pollinators influences the reproductive success of plants. For example, under continuous pollen limitation because of low pollinator activity or availability, autogamous selfing may be advantageous by providing reproductive assurance (Kalisz and Vogler, 2003; Moeller, 2006). In contrast, preferential fertilization with outcross-pollen in a mixed-mating system and obligate outcrossing due to self-sterility are common for plants with frequent pollinator visits. However, the effectiveness of these mating systems should vary if the activity and availability of pollinators fluctuate spatially and temporally. Even under high pollinator activity, geitonogamous pollination may result in the abundant production of inbred seeds and a decrease in siring success due to pollen discounting in mixedmating plants (de Jong *et al.,* 1993). Therefore, pollinator activity may cause large variations in reproductive success among populations, depending on the mating system of individual plants. In this study the flowers of *Boswellia* are largely visited by honey bees only. This result is in agreement with Sunnichan *et al.* (2005), whom worked on *B. serata* of India. Authors like Wilson (1977) and

Kindeya et al., (2002) reported lack of regeneration in *Boswellia* which contribute to the dwindling of *Boswellia* populations, may be due to only few pollinaters are available for pollination.

7. CONCLUSION

The present study was conducted with the main objective of assessing the extent of genetic diversity among Metema population of *B. papyrifera* using Inter Simple Sequence Repeat marker.

This study shows that ISSR markers are important for genetic diversity study in boswella. The present analysis of genetic variation could provide a basis for its term conservation and sustainable use of *B. papyrifera*. In general, all the five populations showed high to moderate genetic diversity, which will be valuable for the future collection, conservation and improvement strategies. Zebach Bahir population shows relatively high level of genetic diversity whereas Agam Wuha population shows lower genetic diversity. Hence, conservation effort should start from Zebach Bahir and then consider the most threatened populations.

Clustering and PCO analysis for the Metema populations suggest that there was a strong association between individuals and their geographic origin. The most likely important factor for this strong association is; either these populations were separate for long time, or lack of seed and pollen flow.

Analysis of molecular variance for the populations studied showed that the highest proportion of genetic variation was attributed to within population than among population. This is also further confirmed with the result of the clustering analysis.

Phenological observation study showed that there is strong association between phonological pattern and seasonal variation. Flowering and fruiting of *Boswellia* trees lack leaves and takes

place during dry season. In this study it was observed honey bee is the major pollinator agent for *Boswellia* trees.

8. RCOMMENDATIONS

- This study is the first attempt to address the genetic diversity of *Boswellia* population along with its phenology. However, the current study has a limitation that only populations from Metema were studied. Therefore, boswellia genetic diversity and phylogeography of Ethiopian populations from different agro-ecology should be studied in detail.
- Analysis of genetic diversity in forest tree species using more than one methods helps to better understand the levels of genetic variation, the genetic structure of populations and determine migration root; when compared to the results obtained using only one method. Analysis with co-dominant markers system, like microsatellites, needs to be conducted to better understand and estimate the gene flow, and determine the size of a population and levels of inbreeding.
- Similar to genetic diversity study, reproductive biology study is also important for breeding programs and conservation. Thus, detail reproductive biology study should be done to understand floral structures, stigma receptivity, pollen flow, seed dispersal, etc.
- *B. papyrifera* has a great potential both from economic and ecological perspectives. But continued pressure on the dry forests through unregulated use has affected the population status of the species. Therefore, researchers, policy makers and other stakeholders need to come-up with sustainable use and conservation strategies.

9. REFERENCES

Abeje Eshete (2002). Regeneration Status, Soil Seed Banks and Socio-economic Importance of *B. papyrifera* in Two Woredas of North Gonder Zone, Northern Ethiopia. MSc thesis, Swedish University of Agricultural Sciences, Skinnskatteberg, Sweden.

Abeje Eshete, Demel Teketay and Hulten, K. (2005). The socio-economic importance and status of populations of *B. papyrifera* (Del.) Hochst. in northern Ethiopia: The case of North Gondar Zone. *Forest, Trees and Livelihoods.* **15:**55-74.

Abercrombie, T. (1985). Arabia's frankincense trail. *Nat. Geog.* **168:**474513.

Adams, W. T., White, T. L. and Neale, D. B. (2007). *Forest Genetics.* CABI, London, UK.

Aman Dekebo., Medihn Zewdu. and Ermias Dagne (1999). Volatile oils of Frankincense from *Boswellia papyrifera*. *Bulletin of Chemical Society of Ethiopia.* **13** (1): 93-96.

Aregawi Berhe (1997). Preliminary Survey on Forest Products Utilization and Marketing in Tigray. Tigray Bureau of Agricultural Development and Natural Resources, Mekelle, Ethiopia. 67 pp. Unpub.

Baker, H. G., Bawa, K. S. and Opler, P. A. (1983). Reproductive biology of plants in tropical forests. **I**n: *Ecosystems of the World: Tropical Rain Forest Ecosystems: Structure and Function.* (Golley, F. B. ed.). Elsevier, New York.

Bennett, S. J. and Maxted, N. (1997). An ecogeographic analysis of the *Vicia narbonensis complex. Genet. Res. Crop Evol.* **44:** 411-428.

Bornet, B. and Branchard, M. (2001). Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant. Mol. Biol. Rep.* **19:**209–215

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

Bucco, G. (1998). Joint Relief Herbs for Health Magazine, Nov/Dec. 1998.

Cabrita, L., Elisiário, P., Leitão, J. and Guerreiro, A. (2001). Assessment of the genetic relationships among citrus species and varieties by Isozyme and RAPD Markers. *Acta. Hort.* **546:**177-181.

Caetano-Anollés, G., Bassam, B. J. and Gresshoff, P. M. (1992). DNA fingerprinting: Mapping out a RAPD redefinition? *Biotechnology* **10** (9):937.

Coppen, J. (1995). *Flavours and Fragrances of Plant Origin*. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. 63 pp.

Coppi, A., Raffaelli, M., Cecchi, L. and Selvi, F. (2010). The Frankincense tree (*Boswellia sacra*, Burseraceae) from Oman: ITS and ISSR analyses of genetic diversity and implications for conservation. *Genet Resour. Crop. Evol.* **57:**1041–1052.

de Jong, Waser, T. J. and Klinkhamer, P. G. L. (1993). Geitonogamy: the neglected side of selfing. *Tren. Eco. Evol.* **8:**321–325.

De Vicente, M. C. and Fulton, T. (2003). Using molecular marker technology in studies on plant genetic diversity. Illus. Nelly Giraldo. IPGRI, Rome, Italy.

De Vienne, D., Santoni, S. and Falque, M. (2003). Principal sources of molecular markers. **I**n: *Molecular Markers in Plant Genetics and Biotechnology* (De Vienne, D., *ed.*). Springler, England.

Dean, R. E., Dahlberg, J. A., Hopkins, M. S., Mitchell, S. E. and Kresovich, S. (1999). Genetic redundancy and diversity among 'Orange' accessions in the U.S. national sorghum collection as assessed with Simple Sequence Repeat (SSR) markers. *Crop Sci.* **39:**1215-1221.

Excoffier, L. (2006). An Integrated Soft Ware Package for Population Genetics Data Analysis. Computational and Molecular population Genetics Lab.(MPG), Institute of Zoology, University of Bern, Switzerland.

Fichtl, R. and Admasu Adi (1994). *Honeybeef Flora of Ethiopia.* Margraf Verlag, Weikersheim.

Finkeldey, R. and Hattemer, H. H. (2007). Genetics of tropical forests. **I**n *Tropical Forest Genetics* (Finkeldey, R. and Hattemer, H. H. eds.). Springer, Berlin, Heidelberg.

Frankel, O. H., Brown, A. H., Burdon, J. J. (1995). *The Conservation of Plant Biodiversity*. Cambridge University Press, Cambridge.

Ge, X. J., Yu, Y., Yuan, Y., Huang, H. and Yan, C. (2005a). Genetic diversity and geographic differentiation in endangered Ammopiptanthus (Leguminosae) populations in desert regions of northwest China as revealed by ISSR analysis. *Ann. Bot.* **95:**843–851

Ge, X. J., Zhang, L. B., Yuan, Y. M., Hao, G. and Chiang, T. Y. (2005b). Strong genetic differentiation of the East-Himalayan Megacodon stylophorus (Gentianaceae) detected by inter-simple sequence repeats (ISSR). *Biodiver. Conserv.* **14:**849–861.

Gebhardt, C., Ritter, E., Barone, A., Debener, T., Walkemeier, B., Schachtschabel, U., Kaufmann, H., Thompson, R. D., Bonierbale, M. W., Ganal, M. W., Tanksley, S. D. and Salamini, F. (1991). RFLP maps of potato and their alignment with the homeologous tomato genome. Theor. *Appl. Genet*. **83:**49–57.

Goettsch, E. (1986). Traditional aromatic and perfume plants in Central Ethiopia (a botanical and ethno-historical survey). *J. Ethiopian Studies* **19:** 81-90.

Groom, N. (1981). *Frankincense and Myrrh: A Study of The Arabian Incense Trade*. Longman, London and New York.

Hammer, O., Harper, D. and Ryan, P. (2001) PAST: Paleontological statistics software for education and data analysis. *Paleontología Electrónica* **4:** 1-9.

Hamrick, J. L. and Godt, M. J. W. (1989). Allozyme diversity in plant species. **In**: *Plant Population Genetics, Breeding and Genetic Resources* (Brown, A. H. D., Clegg, M. T., Kahler A. L. and Weir B. S. eds). Sinauer, Sunderland.

Hamrick, J. L., Godt, M. J. W. and Sherman-Broyles, S. L. (1992). Factors influencing levels of genetic diversity in woody plant species. *New Forests* **6:**95-124.

Hatcher, P. E., Wilkinson, M. J., Albani, M. C. and Hebbern, C. A. (2004). Conserving marginal populations of the food plant (*Impatiens noli-tangere*) of an endangered moth (*Eustroma reticulatum*) in a changing climate. *Biol. Conserv.* **116:**305–317

Hearne, C. M., Ghosh, S. and Todd, J. A. (1992). Microsatellites for linkage analysis of genetic traits. *Tren. Genet.* **8:**288–294.

Hedenäs, L. and Kooijman, A. M. (1996). Phylogeny and habitat adaptations within a monophyletic group of wetland moss genera Amblystegiaceae. *Plant Syste. Evol.* **199:** 33–52.

Hepper, F. N. (1969). Arabian and African frankincense trees. *Egy. Ecol.* **18:**166-171

Holsinger, K. E., and Gottlieb, L. K. (1991). Conservation of rare and endangered plants: Principle and Prospect. **In**: *Genetics and Conservation of Rare Plants* (Falk, D. A. and Holsinger, K. E., *eds.*). Oxford University Press, New York.

Hurni, H. (1988). Degradation and conservation of the resources in the Ethiopian highlands. *Mount. Res. Develop.* **8**(2/3):123-130.

Jaccard, (1908). Nouvelles researchers Sur la distribution florale. *Bull. Soc. Vaud. Sci. Nat*. **44:**223 – 270.

Kalisz, S. and Vogler, D. W. (2003). Benefits of autonomous selfing under unpredictable pollinator environments. *Ecol.* **84:**2928–2942.

Karp, A., Seberg, O. and Buiatti, M. (1996). Molecular techniques in the assessment of botanical diversity. *Ann. Bot* **78:**143–149.

Karp, A. and Edwards, K. J. (1997). Molecular techniques in the analysis of the extent and distribution of genetic diversity. **I**n: *Molecular Genetic Techniques for Plant Genetic Resources* (Ayad, W. G., Hodgkin, T., Jaradat, A. and Rao, V. R., eds.). IPGRI, Rome, pp. 11–22

Karp, A., Edwards, K. J, Bruford, M., Funk, S., Vosman, B., Morgantem, M., Seberg, O., Kremer, A., Boursot, P., Arctander, P., Tautz, D. and Hewitt, G. M. (1997a). Molecular technologies for biodiversity evaluation: opportunities and challenges. *Nat. Biotec.* **15:**625–628.

Karp, A., Kresovich, S., Bhat, K. V., Ayad, W. G. and Hodgkin, T. (1997b). Molecular tools in plant genetic resources conservation: a guide to the technologies. IPGRI Technical Bulletin No. 2. IPGRI, Rome, Italy.

Kermali, I. R. (1994). Morphometric *Diversity in Short-season Maize (Zea mays L.) germplasm.* Ph.D thesis, University of Guelph.

Kindeya Gebrehiwot, Muys, B., Miteku Haile and Mitloehner, R. (2002). *B. papyrifera* (Del.) Hochst: a tropical key species in northern Ethiopia. Conference on International Agricultural Research for Development October 9-11. Deutscher Tropentag, Witzenhausen.

Kindeya Gebrehiwot, Muys, B., Mitiku Haile and Mitloehner, R. (2003). Introducing *B. papyrifera* (Del.) Hochst and its non-timber forest product, frankincense. *Intern. Fores. Rev.* **5:**348–353.

Kudo, G. (2006). Flowering phenologies of animal-pollinated plants: reproductive strategies and agents of selection, p. 139-158. **I**n: *Ecology and Evolution of Flowers* (Harder, L. D and Barrett, S. C. eds). Oxford University Press, Oxford, England.

Lawless, J. (1995). *The Illustrated Encyclopaedia of Essential Oils: The Complete Guide to the Use of Oils in Aromatherapy and Herbalism.* Rockport, MA. Element Books.

Ledig, F. T. (1986). Heterozygosity, heterosis, and fitness in outbreeding plants. **I**n: *Conservation Biology* (Soule, M. E. ed.). Sinauer Associates, Sunderland.

Ledig, F. T. (1998). Genetic variation in Pinus. **I**n: *Ecology and Biogeography of Pinus* (Richardson, D. M. ed.). Cambridge University Press, Cambridge, UK.

Lee, A., Suh, J. K., Roh, M. S. and Slovin, J. P. (2001). Analysis of Genetic Relationships of Ardisia spp. Using RAPD Markers. Tektran, United State Department of Agriculture, Agricultural Research Service

Li, J. and Jin, Z. (2007). Genetic variation and differentiation in *Torreya jackii* Chun, an endangered plant endemic to China. *Plant. Sci.* **172:**1048–1053

Luo, X., Zhuang, X.and Yang, Y. (2007). Genetic diversity of *Camellia changii* Ye (Theaceae) using ISSR markers. *J. Trop. Subtrop. Bot*. 15(2):93–100

Marshal, N. (1998). Searching for a cure: Conservation of medicinal wildlife resources in East and Southern Africa. A Species Endangered Report, Nairobi, Kenya.

Maxted, N., Van Slageren, M. W. and Rihan, J. R. (1995). Ecogeographic surveys. **I**n: *Collecting Plant Genetic Diversity* (Guarino, L., Ramanatha, V. and Reid, R., eds.). CABI.

Metema Pilot Learning site Diagnosis and program (2005). Improving Productivity and Market Succes (IPMS) of Ethiopia Farmer, team report. Canadian International Development Agency (CIDA).

Moeller, D. A. (2006). Geographic structure of pollinator communities, reproductive assurance, and the evolution of self-pollination. *Ecol.* **87:**1510–1522.

Morgante, M., Hanafey, H. and Powell, W. (2002). Microsatellites are preferentially associated with nonrepetitive DNA in plant genome. *Nat. Genet.* **30:**194–200.

Mueller, U. G. and Wolfenbarger, L. L. (1999). AFLP genotyping and fingerprinting. *Tren. Ecol. Evol.* **14:**389–394.

Mulugeta Lemenih and Demel Teketay (2003). Frankincense and myrrh resources of Ethiopia: II. Medicinal and industrial uses. *SINET: Ethiopian J. Sci.* **26**(2):161-172

Mulugeta Lemenih, Tarekegn Abebe and Olsson, M. (2003). Gum-resins from some Acacia, *Boswellia* and *Commiphora* species and their economic contributions in Liban zone, Ethiopia. *J. Ari. Envir.* **55:**465–482.

Nybom, H. and Bartish, I. (2000). Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. *Perspect. Plant Ecol. Evol. Syst.* **3**(2):93–114.

Okun, D. O., Kenya, E. U., Oballa, P. O., Odee, D. W. and Muluvi, G. M. (2008). Analysis of genetic diversity in *Eucalyptus grandis* (Hill ex Maiden) seed sources using inter simple sequence repeats (ISSR) molecular markers. *Afr. J. Biotec.* **7**(13):2119–2123.

Oqbazghi, W. (2001). *The Distribution and Regeneration of Boswellia papyrifera (Del.) Hochst. in Eritrea*. PhD thesis, Wageningen University and Research Centre, Wageningen.

Parasharami, V. A. and Thengane, S. R. (2012). Inter population genetic diversity analysis using ISSR markers in *Pinus roxburghii* (Sarg.) from Indian provenances. *Int. J. Biodiv. Cons.*4(**5**):219-227.

Parker, P. G., Snow, A. A., Schug, M. D., Booton, G. C. and Fuerst, P. A. (1998). What molecules can tell us about populations: choosing and using a molecular marker. *Ecol.* **79:**361–382.

Pavlicek, A., Hrda, S and Flegr, J (1999). Free-tree-freeware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of genus *Frenkelia*. *Folia Biol. (Praha)* **45:** 97-99.

Peeters, J. P. and Martinelli, J. A. (1989). Hierarchical cluster analysis as a tool to manage variation in germplasm collections. *Theore. Appl. Genet.* **78:**42–48.

Perez-Collazos, E. and Catalan, P. (2007). Genetic diversity analysis and conservation implications for the Iberian threatened populations of the irano turanian relict Krascheninnikovia ceratoides (Chenopodiaceae). *Biol. Linn. Soc.* **92:**419–429.

Rakoczy-Trojanowska, M. and Bolibok, H. (2004). Characteristics and comparison of three classes of microsatellite-based markers and their application in plants. *Cell Mol. Biol. Lett.* **9:**221–238

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

Rijkers, T., Ogbazghi, W., Wessel, M. and Bongers, F. (2006). The effect of tapping for frankincense on sexual reproduction in *Boswellia papyrifera. J. Appl. Ecol.***43:**1188–119

Robinson, A. J., Love, C. G., Batley, J., Barker, G. and Edwards, D. (2004). Simple sequence repeat marker loci discovery using SSR primer. *Bioinformatics.* **20**(9):1475–1476

Rohlf, F. J. (2000). NTSYS-pc ver 2.11T. Exter Software, Setauket, New York.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406-425.

Schlötterer, C. (2004). The evolution of molecular markers—just a matter of fashion?. *Nat. Rev. Genet*. **5:**63–69.

Sharma, K., Degan, B., Wuehlisch, G. V.and Singh, N. B. (2002). Allozyme variation in eight natural population of *Pinus roxburghii Sarg.* in India. *Silvae. Genet.***51:**246-253.

Sica, M., Gamba, G., Montieri, S., Gaudio, L. and Aceto, S. (2005). ISSR markers show differentiation among Italian populations of *Asparagus acutifolius* L. *Genet.* **6:**17.

Sinclair, W. T., Morman, J. D. and Ennos, R. A. (1999). The postglacial history of Scots pine (*Pinus sylvestris* L.) in western Europe: evidence from mitochondrial DNA variation. *Mol. Ecol.* **8:**83-88.

Siregar, I. Z. (2000). *Genetic Aspects of the Reproduction System of Pinus merkusii Jungh. et de Vriese in Indonesia.* Ph.D thesis, George-August University of Gottingen. Germany

Sneath, P. H. A. and Sokal, R. R. (1973). *Numerical Taxonomy*. Freeman, Sanfrancisco.

Spooner, D., van Treuren, R. and de Vicente, M. C. (2005). Molecular markers for genebank management. IPGRI Technical Bulletin No. 10. IPGRI, Rome, Italy.

Stat soft. Inc. (2001). Statistica data analysis system, Statistica software.

Studier, J. A. and Keppler, K. L. (1988). A note on the neighbor-joining algorithm of Saitou and Nei. *Mol. Biol. Evol.* **5:**729-731.

Sunnichan, V. G., Mohan, Ram, H. Y. and Shivanna, K. R. (2005). Reproductive biology of *Boswellia serrata*, the source of salai guggul, an important gum-resin. *Bot. J. Linn. Soc.* **147:**73–82.

Tefera Mengistu (2011). *Physiological Ecology of the Frankincesce Tree.* MSc Thesis, Wageningen University.

Thulin, M. (2001). Two new species of frankincense trees (*Boswellia, Burseraceae*) from Socotra. Kew Bulletin **56** (4): 983-988.

Tilahun G. (1997). *Boswellia papyrifera (Del.) Hochst. from Western Tigray: Opportunities, Constraints and Seed Germination Responses.* MSc thesis. Swedish University of Agricultural Sciences. Faculty of Forestry, Sweden.

Tileye Feyissa, Nybom, H., Bartish, I. V. and Welander, M. (2007). Analysis of genetic diversity in the endangered tropical tree species *Hagenia abyssinica* using ISSR markers. *Genet. Resour. Crop. Evol.* **54:**947–958.

Tohme, J., Jones, P., Beebe, S. and Iwanaga, M. (1995). The combined use of agroecological and characterization data to establish the CIAT Phaseolus vulgaris core collection. **I**n: *Core Collections Genetic Resources* (Hodgkin, T., Brown, A. H. D., van Hintum, Th. J. L. and Morales, E. A. V. eds.). John Wiley and Sons, Chichester, UK.

Tucker, A. (1986). Frankincense and myrrh. *Econ. Bot.* **40** (4): 425-433.

Van Schaik, C. P., Terborgh, J. W. and Wright, S. J. (1993). The phenology of tropical forests: adaptive significance and consequences for primary consumers. *Ann. Rev. Ecol. Syst.* **24:** 353-377.

Van Beek ,G. (1960). Frankincense and Myrrh. *Bibli. Archaeol.* **23** (3): 7095.

Verghese, J. (1988). Olibanum in focus. *Perfumer and Flavorist* 13 (1): 1-12.

Vollesen, K. (1989). Burseraceae. **I**n: *Flora of Ethiopia*, Volume 3 (Hedberg, I. and Edwards, S., eds.). National Herbarium, Addis Abeba University, Addis Abeba and Uppsala University, Uppsala. pp. 442-478.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van De Lee, T., Hornes, M., Frijters, A., Pot, J, Peleman, J., Kuiper, M. and Zabeau, M. (1995). AFLP: a new technique for DNA Fingerprinting. *Nucl. Aci. Res.* **23:** 4407-4414.

Weising, K., Nybom, H., Wolff, K. and Kahl, G. (2005). *DNA Fingerprinting in Plants, Principles, Methods, and Applications.* Taylor & Francis Group, USA.

White, F. (1983). The vegetation of Africa: A descriptive memoir to accompany the UNESCO/AETFAT/UNSO, Vegetation map of Africa. United Nations, UNESCO, Switzerland.

Whitkus, R., Doebley, J. and Wendel, J. F. (1994). Nuclear DNA markers in systematic and evolution. **I**n: *DNA-based Markers in Plants* (Phillips, R. L. and Vasil, I. K., eds.). Kluwer Academic Publishers, Dordrecht, The Netherlands.

Williams, J. G. K., Kubelik, A. Livak, R. K., J. Rafalski, A. J., and Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Aci. Res.* **18:**6531–6535.

Wilson, R. (1977). The vegetation of central Tigre, Ethiopia in relation to its land use. *Webbia* **32:** 233-270.

Wilson, R. (1995). *Aromatherapy for Vibrant Health and Beauty*. Avery Books, Penguin Puntam Inc. India.

Winfield, M. O., Arnold, G. M., Cooper, F., Le Ray, M., White, J., Karp, A. and Edwards, K. J. (1998). A study of genetic diversity in *Populus nigra* subsp. betulifolia in The Upper Severn Area of The UK using AFLP markers. *Mol. Ecol.* **7:** 3-10.

Wubalem Tadesse, Demel Teketay, Mulugeta Limenih and Girmay, F. (2002). Country report for Ethiopia. **I**n: *Review and Synthesis on The State of Knowledge of Boswellia Species and Commercialization of Frankincense in the Dry land of Eastern Africa* (Chokma,i B. ed). FAO/EU/, FORENESSA Publication, FAO, Rome.

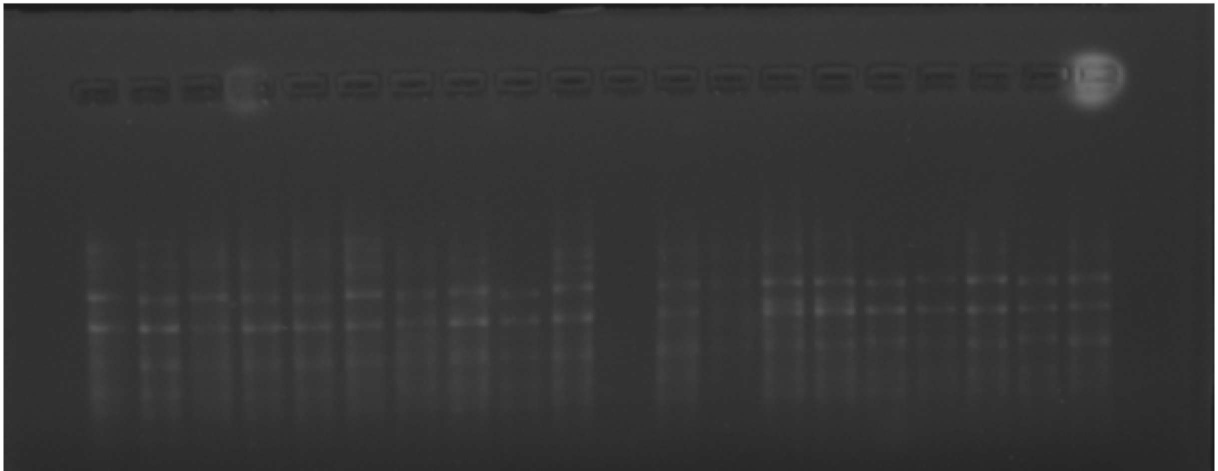
Yeteh, F. C., Yang, R. C., Boyle, T. J. B., Ye, Z. and Mao, J. X. (1999). *POPGENE, the user Friendly Shareware for Population Genetics Analysis, Version 1.3.1.* Molecular Biotechnology center, University of Aleberta, Canada.

Zietkiewicz, E., Rafalski, A. and Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* **20:**176-183.

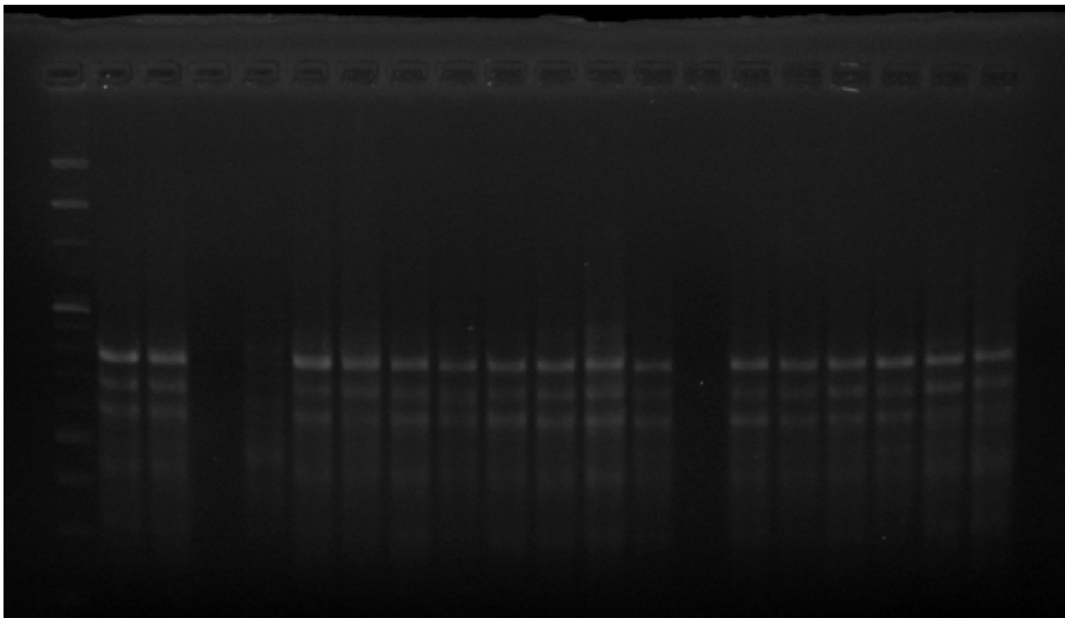
10. APPENDICES

Appendix 1. ISSR fingerprints of *B. papyrifera* by different primers

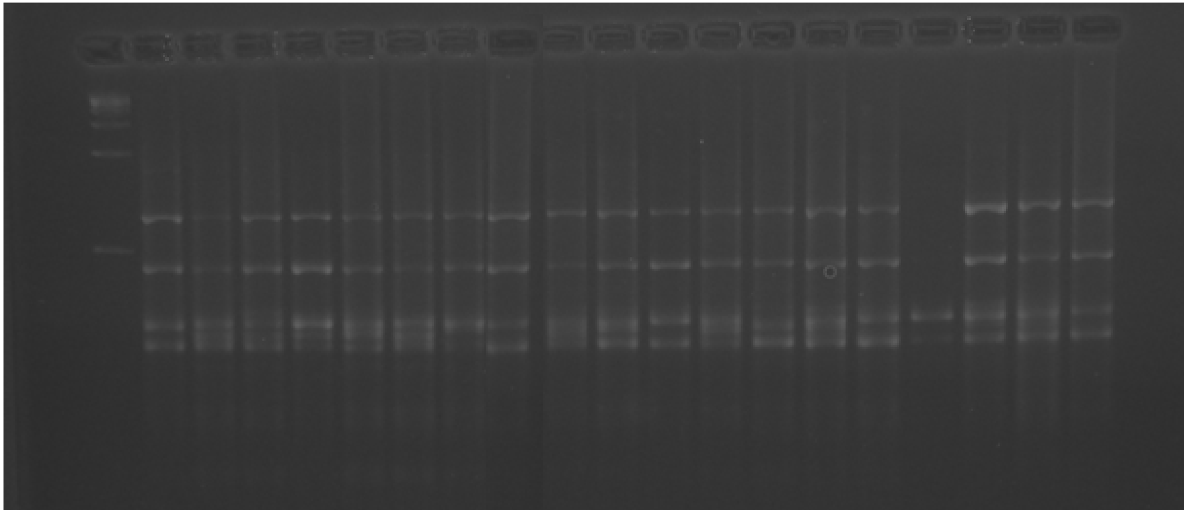
a) Primer 810



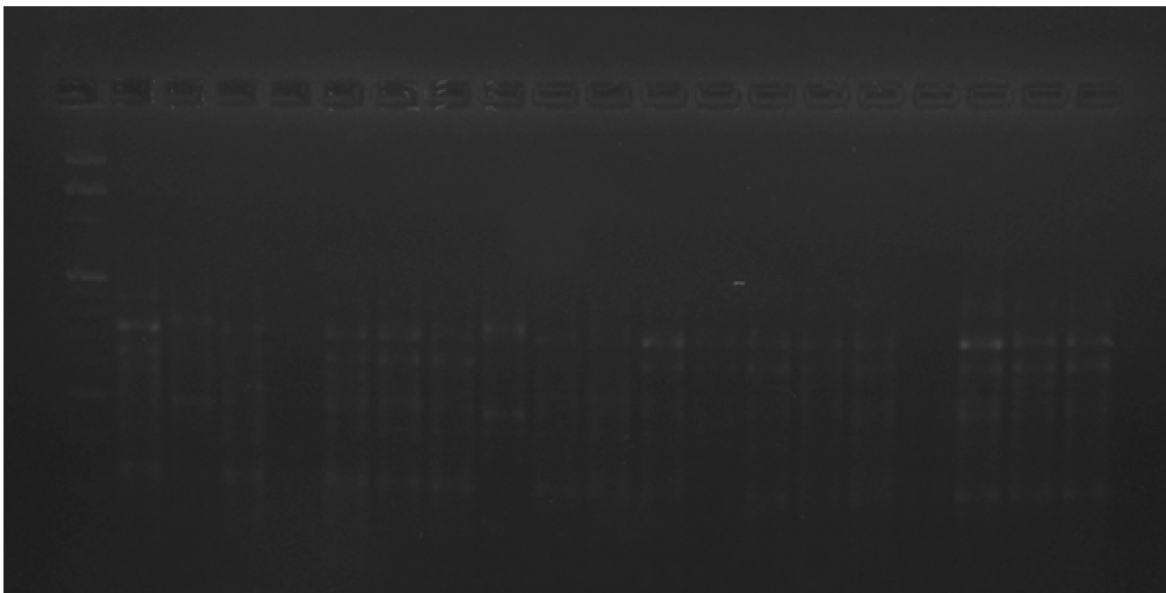
b) Primer 812



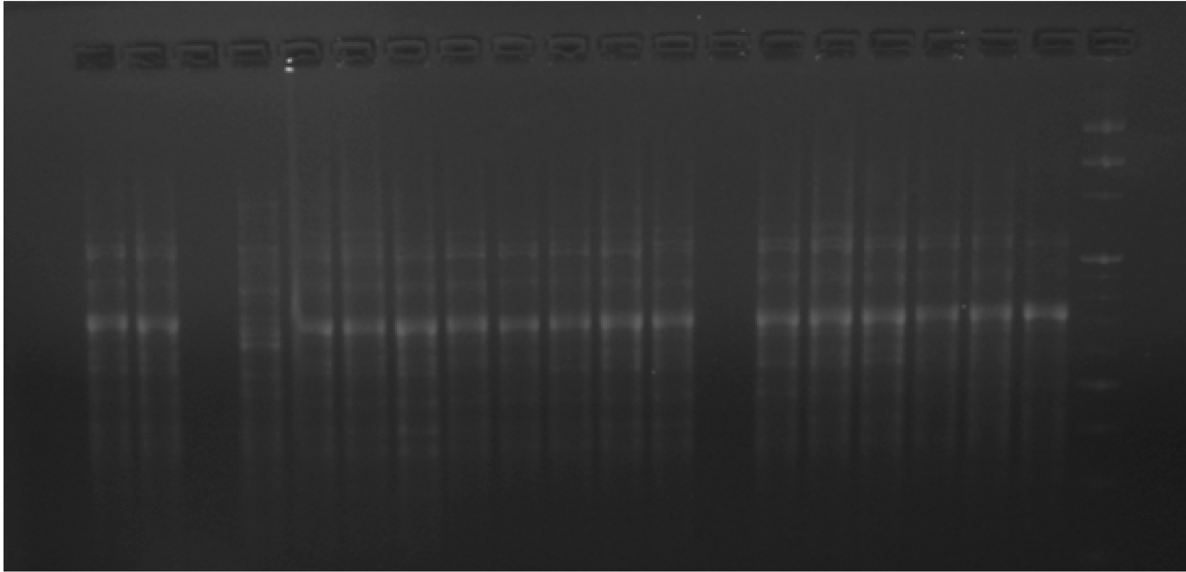
c) Primer 818



d) Primer 834



e) Primer 880



Appendix 2. Boswellia tree at different stages

a. Before leaf fall

b. After leaf fall

c. Branches showing flower buds

d. Branches with different flowering stages

e. Trees at maximum flowering stage

f. branches showing closed flower

Apendix 3. Boswellia fruit

Declaration

I, the undersigned declared that this Thesis is my original work and that all sources of material used for the thesis have been correctly acknowledge.

Name: Abush Zinaw

Signature__________

Date of submission____________

Advisors: Dr. Kassahun Tesfaye

Signature___________

Date ___________

Dr. Kifle Dagne

Signature___________

Date___________