ESTIMATION OF SEX−RELATED GENETIC DIVERSITY OF HAGENIA ABYSSINICA (BRUCE) J.F.GMEL USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

KUMILIGN ASMARE

A THESIS PRESENTED TO THE SCHOOL OF GRADUATE STUDIES, ADDISA BABA UNIVERSITY, IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY (APPLIED GENETICS)

JANUARY, 2005

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ABSTRACT

The extent and distribution of sex-related and total genetic variation in Hagenia abyssinica (Bruce) J.F. Gmel collected from four geographical regions of Ethiopia were analyzed using random amplified polymorphic DNA (RAPD) markers for 80 plants representing 8 populations. Three decamer primers generated a total of 19 polymorphic bands, across the 80 plant, with 6-7 bands per primer and an average of 6 bands. The amount of genetic variation within the populations (H_{Pop}=0.32 for female populations; H_{Pop}=0.41 for male population; H_{Pop}=0.45 for the entire population) and within the geographical regions (H_r=0.36 for female population; 0.51 for male population; 0.52 for the entire population) was low to moderate. Similarly, the average genetic distance between populations (0.08 for female population; 0.09 for male population; 0.05 for the entire population) as well as between regions of sampling (0.05 for female population; 0.05 for male population; 0.02 for the entire population) was found to be low. The low genetic variation may be due to the reduced population size of the tree species in Ethiopia because of habitat change and indiscriminate clearing and over-cutting of trees for various purposes. Partitioning of the genetic variation into between and within populations as well as between and within regions of sampling revealed that 65% (for female population), 70% (for male population) and 82% (for the entire population) of the variation was found within the populations whereas 75% (for female population), 88% (for male population) and 93%(for the entire population) of the variation was found within regions. Cluster analysis of genetic distance estimates further confirmed the low level of differentiation of the tree species both on population and regional bases. The genetic diversity of male individuals was found to be greater than that of the female individuals. The relatively lower genetic diversity in the female individuals may be due to the

higher female mortality resulting from the added burden of fruit and seed setting in female plants. Spearman's correlation analysis showed that primer OPA-10 was found significantly correlated with the male sex (locus OPA10-1: r = 0.395 significant at 0.01level) and hence would help to identify the male sex. Discriminant analysis showed that this primer was also found useful to identify which trees belong to which region.

1. GENERAL INTRODUCTION

1.1. Genetic diversity

Genetic diversity refers to the variation among alleles of genes in different members of populations of a species (IPGRI, 1993). The genetic diversity within and between populations is a result of the interplay of the various evolutionary forces (mutation, genetic drift, migration and selection). Generally, forces that increase variation within populations (mutation, migration and balancing selection) prevent the differentiation of populations from each other, whereas the divergence of populations is a result of forces such as disruptive and differential directional selections, and inbreeding or genetic drift that make each population homogenous (Griffith *et. al*., 2000).

Genetic diversity is needed for the survival of a species. It enables plants to better equip themselves to evolve in response to a changing environment. Moreover, with a good genetic variation, plants are less likely to suffer a loss of fitness. For breeders, high genetic diversity offers a great scope for developing varieties with specific desirable traits. Estimation of genetic diversity is therefore highly important especially for endangered species to assess their genetic status and take conservation and/or genetic improvement actions (Hunter, 1996).

In applying the principles of population genetics to the cases of endangered species conservation, biologists are interested primarily in the genetic variation, in particular its distribution and maintenance.

A preliminary analysis of the level and apportionment of genetic diversity contributes to a better understanding of the genetic profile of an endangered species and could then be used to develop strategies for conservation and sustainable utilization (Ang Li *et. al*., 2002).

For most rare species, there is no data on genetic diversity and its patterns. Therefore, the conservation of the species is most often attempted by protecting sufficient habitat to support large enough populations to prevent genetic drift and inbreeding. It is assumed that if sufficient habitat is maintained, loss of genetic diversity is not an immediate concern (Neel and Ellstrand, 2003). However, because genetic diversity contributes to the species persistence, its direct measurement can be an important priority.

Quantifying the organization of genetic variation over populations of an endangered species can help in prioritizing sites and management choices that will capture and maintain that variation (Petit *et. al*., 1998). For example, highly diverse or differentiated populations could be targeted for protection while depauperate populations might be targeted for management actions to restore diversity**.** Petit *et. al*. (1998) demonstrated that a large proportion of populations are required to capture the genetic diversity of a species if populations are chosen without knowledge of genetic diversity patterns, regardless of the how these populations are chosen. Thus, the knowledge of diversity patterns can reduce the number of populations necessary to be conserved and thus reduce the costs and conflicts with competing land uses and other related resources. Information on genetic diversity patterns also provides insight into evolutionary and demographic history of a taxon (Milligan *et. al*., 1994).

Understanding the relative importance of processes that structure diversity within and among populations (specifically inbreeding, gene flow, genetic drift and selection) can provide both a means to assess future risk of erosion of diversity and a means for designing effective conservation strategies for endangered species. For example, if genetic diversity is primarily held within populations, fewer populations would need to be conserved to represent the range of variation within the species. Alternatively, a species with most of its variation partitioned among populations would require protection of a large proportion of existing populations to maintain variation present in the species (Neel and Ellstrand, 2003).

The structure of genetic diversity within and among populations also has an important implication for developing sampling strategies for restoration and reintroductions (Ceska *et. al*., 1997).

Finally, information on genetic diversity can guide future research by focusing attention towards areas of particular potential concern. For example, if genetic diversity is high, and populations do not appear to be at risk of losing that diversity, research and management efforts can be focused on ecological characteristics related to survival of individuals in relation to threats affecting by a species (Neel and Ellstrand, 2003).

Hagenia abyssinica (Bruce) J.F. Gmel, the subject species of the present study, was once abundant in the semi-humid mountain woodlands of Ethiopia with altitudinal range between 2450 and 3250 (Hedberg, 1989). Today, in Ethiopia, the species is endangered and sparsely distributed in parts of Bale, Sidamo, Keffa, Hararghe, Arsi, Gojam, Wello, Gondar, Tigray, Shewa and

Wellega (Legesse, 1995). However, other regions like the highlands of Gamogofa (Chencha) appear to be additional sites.

Despite its endangered state and high call for conservation, no study has been made to assess the genetic diversity status of the species to pinpoint areas and magnitude of diversity in order to take all the necessary actions to rescue this species. The purpose of the present study, therefore, was to estimate the species and sex-related genetic diversity of *Hagenia abyssinica* using RAPD markers for conservation.

1.2. Methods for estimating genetic diversity

The genetic diversity of plants can be estimated using morphological, biochemical, or molecular approaches. Genetic diversity of common morphological traits is difficult to measure in natural populations since the traits are influenced by environmental factors and age of the species to a large degree in addition to interacting genes that contribute to their expression. Assessment of genetic diversity with molecular markers, however, overcomes this problem because these molecular traits have little environmental component and only one or a few genes are involved in their expression (Kean *et al*., 1999).

Most previous investigations of genetic variation within tree species have been based on the use of isozymes (Allnutt *et. al*., 1999). Although isozyme loci are relatively easy to screen, and have particular value for estimating gene frequencies and hetrozygosity, they represent a limited number of coding regions and may therefore not be indicative of the genome as a whole (Schaal *et. al*., 1991).

Moreover, the number of alleles per locus and the percent of polymorphic loci are lower for allozymes than for codominant markers (RFLP, micosatellites) while the percent polymorphic loci is higher in dominant markers (RAPD, AFLP) (Butcher *et. al*., 1998).

Some studies suggest that mutation rates are lower at allozyme loci than at RAPD loci (Peakall *et. al*., 1995). Hence, the amount of genetic diversity revealed by allozymes will be smaller than that obtained with molecular markers such as RAPD.

In recent years, a wide variety of molecular techniques have become available which can be used to assess variation of DNA directly, such as restriction fragment length polymorphisms (RFLP) (Miller and Tanksley, 1990) and methods based on the polymerase chain reaction (PCR), such as microsatellites (Weber and May, 1989), PCR-RFLP (Jarvis *et. al*., 1994) and random amplified polymorphic DNA (RAPD) (Williams *et. al*., 1990). The application of such techniques to tree species has highlighted a higher degree of intra-specific variation than was expected on the basis of previous isozyme analyses.

It seems, however, that both of these techniques and the isozyme analysis are required since the diversity estimate obtained with isozyme studies mainly reflect the degree of natural selection.
The utility of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism (Bardakci, 2001). Currently, the RFLP assay (Botstein *et. al*., 1980) has been the choice for many species to measure genetic diversity and construct a genetic linkage map.

RFLP is the result of the presence or absence of an endonuclease restriction site. A mutation to this site causes absence of this site. However, an RFLP assay which detects DNA polymorphisms through restriction enzyme digestion, coupled with DNA hybridization, is, in general, time consuming and laborious. Hence, over the last decade, PCR technology has become a widely used research technique and has led to the development of several novel genetic assays based on selective amplification of DNA (Erlich, 1989).

The popularity of PCR is primarily due to its apparent simplicity and high probability of success. The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes (Williams *et. al*., 1990). RAPD has proved to be a particularly variable technique, reflecting the wide availability of commercial primers and the lack of any need for DNA sequencing information prior to analysis (Welsh and Mc Celland, 1990).

The simplicity and applicability of the RAPD technique has captivated many scientists' interests. Perhaps, the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amount of DNA without the requirement of cloning, sequencing or any other form of the molecular characterization of the genome of the species in question (Bardakci, 2001).

Most importantly, RAPD technique is useful to analyze rare and endangered species, like *Hagenia abyssinica*, since it is fast and shows a high potential to detect polymorphism even when small amount of genomic DNA is available (Maria *et. al*., 2002).

As indicated by Williams *et. al*. (1990), the principle of the RAPD technique is that it utilizes short synthetic nucleotides (10 bases long) or random sequences as primers to amplify nanogram amounts of genomic DNA under low annealing temperatures by PCR. Amplification products are generally separated on agarose gels stained with ethidium bromide. Nucleotide variation between different sets of template DNAs will result in the presence or absence of bands because of changes in the priming sites.

A major drawback of RAPD markers in population genetic studies is that they are dominant and do not allow to distinguish whether a DNA segment is amplified from a locus that is heterozygous or homozygous (Williams *et. al*., 1990). Thus, gene frequency estimates for such loci are necessarily less accurate than those obtained with codominant markers such as allozymes and RFLPs.

Lynch and Milligan (1994) suggested that 2 to 10 times more individuals need to be sampled for dominant markers to achieve the same degree of statistical power as codominant markers such as allozymes and RFLPs.

Other drawback of RAPD is that the reproducibility problem of the technique. This reproducibility problem is usually the case for bands with lower intensity. Perhaps some primers do not perfectly match the priming sequences, amplification in some cycles may not occur, and therefore bands remain fainter. Generally, the reproducibility problem of the technique can be attributed to the length of a single and arbitrary primer used to amplify anonymous regions of a given genome (Bardakci, 2001).

The most important factor for irreproducibility of the RAPD profile has also been found to be the result of inadequately prepared template DNA (Bardakci, 2001).

The assumption of homology between bands of apparently the same molecular weight from the same primer is potentially another problem for RAPD surveys. This assumption especially may not be true when individuals belong to different species for the chance of comigrating bands being homologous becomes less as populations diverge (Allegrucci *et. al*., 1995).

Together with RAPD markers, such molecular markers as micosatellites or Simple Sequence Repeats (SSR) have also received much recent attention in studies of genetic diversity in natural populations (Lougheed *et. al*., 2000). These markers have high levels of length polymorphism existing in dinucleotide tandem repeat sequences (Weber and May, 1989).

Brown and Litt (1992) have indicated two major drawbacks of SSRs. One impediment to the wide spread use of SSR length polymorphisms by plant geneticists is the time consuming nature of the steps required to identify polymorphic loci. The other impediment may be the perception that the routine detection of PCR products differing only slightly in length is difficult and laborious. In general, many technical questions remain as to the applicability of polymorphic SSR sequences in plant genetic studies. However, these authors added, the informativeness of this type of marker and the potential of tens of thousands of SSR sequences per genome suggest that plant geneticists may wish to consider the use of polymorphic SSR loci as genetic markers. Genetic markers generated via variation in SSR length may provide a useful complement to the RFLP and RAPD markers currently in use.

Generally, from the aforementioned discussion, it is apparent that the RAPD technique is relatively quick, easy, and requires no prior sequence information on the target genome. Hence, the present study used this technique to estimate the genetic diversity of *Hagenia abyssinica*.

2. LITERATURE REVIEW

2.1. Introduction

Ethiopia, being a tropical country, is characterized by the varied climatic and edaphic conditions that endowed her with a very heterogeneous flora (Breitenbach, 1963). The very large area of high land found in the country may be a major reason why so many endemic plants are found in the country (Yalden, 1983)**.** The Ethiopian flora is estimated to contain between 6500 and 7000 species of higher plants, of which 12% are endemic (Tewoldeberhan, 1991).

Extensive forest once covered much of Ethiopia, and the resources of these forests were once considered to be the largest in Africa (Russ, 1974). According to historical estimates, nearly 90% of Ethiopian highlands had forest cover (Eshetu, 1996). It is unfortunate, however, that indiscriminate clearing of the vegetation for cultivation, overgrazing, and exploitations without planting has reduced the forested area from 40% to 16% in the 1950's, 8% in the 1960's, 4% in the 1970's to less than 3% in the 1980's (Lisanework and Mesfin, 1989).

Long-term human occupations of the highlands of Ethiopia, accompanied by sedentary agriculture and extensive cattle herding activities, in combination with population pressure have

resulted in heavy deforestation and subsequent environmental degradation (Eshetu, 1996). Indigenous forests of Ethiopia have also been rapidly destroyed over the last several decades, leaving large tracts of unprotected slopped highland (Legesse, 1995). As of this, important indigenous trees have become endangered. One of those trees is *Hagenia abyssinica*.

Hagenia abyssinica (Bruce) J. F. Gmel is a dioecious mono-specific genus belonging to the family Rosaceae that contains a large number of fruit trees including plums, apples, pears and loquats (Dale and Greenway, 1961). Cytological investigation of the species has shown that *H. abyssinica* has a diploid number of 40 chromosomes (Faris, 1998).

H. abyssinica is usually known in Ethiopia for its medicinal value. As active principles in this species, four phloroglucinol derivatives have been isolated. These are kosotoxin, protokosin, koisdine and kosine (Jansen, 1981). The constituent responsible for its medicinal value is the phenolic compound Kosin which is isolated from the ether extracts of the female flowers of *Kosso.* Kosins exhibits comparable potency as the marketed drugs, Dicholorophin and Nicolsamide. Male flowers are not used traditionally as taeinicides due to their alleged high toxicity (Berahanu and Ermias, 1978)**.**

Recent evidences from Ethiopia as well as other countries indicate that the existence of indigenous medicinal plant based resources is threatened (Fassil and Getachew, 1996). Like for other traditional medicinal plants, the part of the plant collected often poses serious danger to the survival of *Hagenia abyssinica*. Because the part of the plant needed for medicinal role is usually the female flower, collection of this part affects the fruit setting and hence the production of the progeny generation.

So far the greatest danger to the socio-economically important medicinal plants like *Hagenia abyssinica* is due mainly to intensive use of land for agriculture, livestock production, fuel wood and construction material (Fassil and Getachew, 1996).

According to Legesse (1995), causes for the endangered state of *Hagenia abyssinica* are many, but the following two are most important:

1. Great demand for its timber, which is very much needed in the market. For example, in *Merkato* of Addis Ababa, reportedly one of the biggest markets in Africa, it is not uncommon to see low-grade timber on sell. Thus, many trees have to be felled at one time to get the required volume of timber.

Legesse (1995) further emphasized that, worse still, regeneration of the species in its natural habitat has become almost impossible as a result of widespread human interference and, probably, as a result of climatic changes that have usually accompanied the process of deforestation.

2. Forest fire is also responsible, although its vulnerability to fire is less pronounced as compared to other tree species. The causes of fire according to Demel (2000), in Bale-area forests, where *Hagenia abyssinica* is also found, for instance, are:

- Expansion of coffee plantation with emerging markets in the surrounding areas.
- Commercial utilization of timber.

- Careless uses of fire during fumigation of beehives to harvest honey.

Together with the uninterrupted human interference, some life attributes of *Hagenia abyssinica* such as nature of pollinator (wind) and dioecism may have their own contribution to the endangered state of the species. Despite the many possible advantages of dioecy, dioecious flora comprises only 6% of the world's angiosperms (Renner and Ricklefs, 1995). The reason may be a high extinction rate or lower speciation rates that are associated with dioecism.

A monoecious plant is a better colonizer than a dioecious plant as it does not need a mate. If dioecious plants do not accomplish long-range dispersal, it could potentially lead to a lower speciation rate among dioecious clades (Heilbuth, 2000) and this is very likely in habitats of *Hagenia abyssinica* which are fragmented and hence reduce the chances that a wind-borne pollen grain will reach a receptive stigma. Hence, the nature of pollination (anemophillus) contributes to the endangered state of the species when male and female individuals of the tree are widely scattered due to habitat disturbance or fragmentation.

Other reasons for extinction of *Hagenia abyssinica* and other dioecious plants as indicated by Heilbuth (2000) could be**:**

- Only half of the population (female individuals) set seed, and females may not be able to compensate for the loss (inability of male individuals to set seed).

- If they are segregated into small populations in which there are no members of the opposite sex. The likelihood of this problem may increase when difference exists between the sexes in ecological tolerances.

On top of these, wild forest tree species are particularly susceptible to genetic erosion because of the practice of high grading; whereby the best trees are removed each harvest cylcle and the less useful trees become the breeding stock (Chengxin *et al.,* 2003). *Hagenia abyssinica*, being a wild forest tree species, is likely to face the same problem.

In general, the continued exploitation of this species assumes sustainable population growth and sound environmental management, the realization of which largely depends on the will and ceaseless effort of all citizens and particularly of the administrative and political authorities. Without this, we will not only soon be deprived of this resource, but we will also be confronted with a situation which will diminish or even eliminate the prospects of better economic and social benefits (example, modern medical care) resulting from technological advances which surely require a local resource base (Dawit and Ahadu, 1993).

Besides, being a natural resource, the species is a treasure house of biological diversity, which will reap unexpected rewards from the development of drugs and other biotechnological products, which would benefit many people in all corners of the country (Dawit and Ahadu, 1993).

Now-a-days medicinal plants are attracting more attention among contemporary plant researchers because some human diseases have gained worldwide concern, and medicinal plants are hoped

for to cope with this problem as they are sources of genetic diversity for resistance to diseases and pests (Anna *et. al*., 2001). There, is in fact, better chance of discovering new drugs from plants of traditional medicine than other sources, which are screened randomly (Dawit, 1998).

Conservation of *Hagenia abyssinica* is therefore of paramount importance in Ethiopia where various diseases are prevalent, and 80% of the population rely on medicinal plants and have no the access and financial power to buy pharmaceuticals.

In general, being a threatened species, *Hagenia abyssinica* presents scientific, economic, and moral challenges: scientific, because their extinction would remove genetic resources of potential future scientific value; economic, because endangered species or some of their genes might prove economic importance in the future; and moral, because every creature has the right to exist and mankind is to be blamed for having caused, or contributed to their endangered state.

From the aforementioned points, one can see that factual knowledge about the genetic status of the plant is very relevant in Ethiopia to conserve, domesticate or cultivate this tree species for scientific investigations and for the betterment of the health and economy of the country in general and of the rural people in particular.

2.2 Taxonomy

2.2.1. The Family *Rosaceae*

The *Rosaceae* is a large cosmopolitan family with about 2000 species, largely concentrated in temperate regions. The family consists of many herbs, shrubs and trees of economic values, both for food and as ornamentals. These herbs, shrubs and trees can be identified by the alternate stipulate leaves, which are compound in most Ethiopian species (Fichtl and Admasu, 1994).

As described by Hedberg (1989), flowers of this family are very variable; receptacles are well developed, flat, convex or concave, often hollow; calyx are with 5 or 4 sepals, sometimes biserate; ovules are one to two; fruits are of various types, dry or fleshy; and seeds are usually non-endospermic.

2.2.2. The Genus *Hagenia*

As described by (Hedberg, 1989), the genus *Hagenia* is characterized by dioecious (or polygamous) trees with imparipinnate leaves. Flowers are small, anemophillus, in many-flowered panicles. Receptacles are “urn-shaped,” apically closed and carrying two alternating rows of membranous calyx lobes, which provide a parachute for the fruit. Carpels are two, with one pendant ovule in each. This is a monotypic genus confined to Africa.

2.2.3. The species *Hagenia abyssinica* (Bruce) J.F. Gmelin

As described by (Hedberg, 1989), *Hagenia abyssinica* is a tree up to 20m tall with brownish bark which can sometimes be very thick. Young branches are covered with staff hairs which are 3 to 4mm long. Leaves measure up to 40cm in length with 5 to 8 narrowly oblong leaflets on each side; petiole is 5 to 13cm long, winged (andante stipules). Flowers are handsome, multibranched, terminal, and dioecious. The female inflorescences are pinkish red, clearly veined and bulkier than the more feathery orange-buff to white male heads.

2.2.3.1. Species identity

Current name: *Hagenia abyssinica*

Authoriy: Wild

Family: *Rosaceae*

2.2.3.2. Common name

Kosso (Amharic)

Duchia (Oromiffa)

Habbi (Tigrigna)

2.3. Adaptation

2.3.1. Ecology and distribution

Hagenia abyssinica was once abundant in the semi-humid mountain woodlands of Ethiopia with altitudinal rang between 2450 and 3250 (Hedberg, 1989). In forest depressions, it can descend to 2000m (Legesse, 1995). Friis (1992) gives a wider altitudinal range of 1850 to 3700.

The species occurs naturally in undifferentiated afromontane forest (mixed *Podocarpus* forest, *Juniperus-Podocarpus* forest) and dry single dominant afro-montane forest (*Juniperu*s forest or forest dominated by *Hagenia*), especially along the upper limit; often associated with *Schefflera abyssinica, S. volkensii, Galiniera saxifraga, Rapanea melanophloes* and with the mountain bamboo, *Arundinaria alpina*; at lower altitudes often at forest margins. This is a very clear example of an afro-montane endemic (Friis, 1992, Jansen, 1981).

Today, in Ethiopia, the species is endangered and sparsely distributed in parts of Bale, Sidamo, Keffa, Hararghe, Arsi, Gojam, Gondar, Wello, Tigray, Shewa and Wellega (Legesse, 1995). Outside Ethiopia, the species is also reported from the mountainous regions of Kenya, Tanzania, Mozambique, Malawi, Zimbabwe-Rhodesia, Zambia, Zaire, Burundi, Ruanda, Uganda and Sudan (Jansen, 1981).

2.3.2. Husbandry

In Ethiopia, the tree is mainly growing in the wild, but, it is also planted around churches together with *Juniperus*. . In areas where the land is used for agriculture, the tree can be found scattered in the fields. In uncultivated areas, denser stands may occur. *H. abyssinica* is sometimes deliberately left in farmland or derived grassland and may be occasionally planted where it occurs naturally because of its medicinal purposes (Jansen, 1981).

2.4. Reproductive biology

Hagenia abyssinica is either a male or female tree, seldom polygamous. Pollination is animophillus, and since pollination by wind is somewhat inefficient, it is probable that unfertilized flowers of the female inflorescence can occur rather frequently (Legesse, 1995). In Ethiopia, the tree flowers between October and February (usually the dry season). On sunny days, the flowers are much visited by bees for its abundant nectar and pollen. Trees can also be regenerated from seedlings.

2.5. Uses

Hagenia abyssinica is a multipurpose tree species with the following uses:

- Fuel, timber (furniture, poles, flooring, cabinet making)
- Apiculture: - branches are used to keep and stabilize the beehives on them. Besides, both the female and male flowers produce abundant nectar and pollen, respectively for the honeybees (Legesse, 1995).

- Ornamental: - it has attractive appearance with attractive flowers
- Medicine: - roots are cooked with meat and the soup is drunk for general illness and malaria; dried and pounded female inflorescence is used as an anthelminthic to treat tape worm, a very common infestation among Ethiopians due to the age-long practice of eating raw beef (Edemariam *et. al*., 1978). The female inflorescence is also used to cause abortion (Kokwaro, 1993).
- Soil improvement and erosion control: - under favorable conditions, leaf production is very high. Leaf senescence and leaf abscission rates are quite fast. Leaves decompose readily. These three attributed, as well as other important morphological and physiological characteristics of the tree, enable it to produce a great amount of litter per unit time. The litter creates suitable conditions for soil micro-fauna. These organisms are important for improving the fertility of the soil that harbors them. On top of this, the thick mat formed from the fallen leaves of the trees is important for protecting soil erosion by way of reducing the direct impact of the raindrops on to the soil. Moreover, loss of water through surface runoff is very much reduced (Legesse, 1995).

3. OBJECTIVES OF THE STUDY

3.1. General objectives

- To assess the present level of genetic diversity of *Hagenia abyssinica*
- To assess the extinction risk of this tree species

3.2. Specific objectives

- To estimate the total and sex-related genetic diversity between and within populations and regions of sampling of *Hagenia abyssinica*.
- To identify sex-specific gene marker, if any.

4. MATERIALS AND METHODS

4.1. Plant material

A total of 80 *Hagenia* trees were used for this study. The trees were sampled from 8 populations (2 populations from each region) of *Hagenia* forest found in 4 regions of Ethiopia: Bale, Jimma, Sidammo and Arsi (Table 4.1). Ten individual trees (5 males and 5 females) were sampled from each population randomly across a transverse pass through the areas of the sample populations during the period of October to November 2004. Young leaves from these trees were collected in silica gel-containing plastic bags for the analysis.

Table 4.1 List of *Hagenia abyssinica* populations considered in the present study

Population descriptions	Sample size	Administrative zone of collection site	Specific localities	Latitude	Longitude
Bale-1	10	Bale	Dinsho Park, 135km South East of Shashemene town on the way to Robe town.	7^0 06'N	39^0 46'E
Bale-2	10	Bale	109 km South East of Shashemene town on the way to Robe town.	7^0 01'N	39^0 30'E
Jimma-1	10	Jimma	Setama forest, on the ascending slope from Ghibe river bridge on the way from Bedele town.	7^0 43'N	36^0 37'E
Jimma-2	10	Jimma	Tiro-boterbecho forest, 83 km North West of Wolkiete town.	8^0 22'N	37^0 16'E
Sidamo-1	10	Sidamo	15 km South East of Bore town on the way to Kibre Mingist town	6^0 22'N	38^0 43'E
Sidamo-2	10	Sidamo	35km South East of Yirba Muda town on the way to Kibre Mingist town.	6^0 12'N	38^0 42'E
Arsi-1	10	Arsi	Near Mt. Chilalo, East of Asela town.	7^0 56'N	39^0 17'E
Arsi-2	10	Arsi	Kersa, 50 km South of Asela town.	7^0 31'N	38^0 58'E

4.2. DNA extraction

Total DNA was extracted from young silica gel-dried *Hagenia* leaves following the CTAB procedure (Wang *et al.,* 1996) with some modifications. The leaves were frozen in liquid nitrogen and ground to powder. 0.8 to 1.0 gm of powdered leaf was collected in 2ml eppendorf tube, and 750µl of extraction buffer (0.1M Tris pH 7.5, 0.05M EDTA, 0.5M NaCl) and 100%SDS was added. The mixture was incubated for 20 min at 65°C. After incubation, 250µl of 5M KAc was added to the mixture, kept on ice for at least 30 min and centrifuged for 15 min at 14000 rpm. The supernatant was transferred to a new 2ml eppendorf tube, equal volume of cold iso-propanol was added, kept at room temprature for 5 min, and centrifuged for 10 min at 14000 rpm. After air-dried, the pellet was dissolved in 250µl of TAE (10mM Tris HCl, pH 7.6, and 1mM EDTA), 250µl of CTAB buffer (0.2M Tris pH 7.5, 50mM EDTA, 2M NaCl and 2% CTAB), and incubated for 15 min at 65°C. DNA was extracted twice with equal volume of chloroform and subsequent centrifugation at 14000 rpm for 5 min and transfer of the water-phase to a new tube each time.

The final water-phase transferred to a new tube was precipitated with equal volume of chilled iso-propanol and centrifuged at 14000 rpm for 15 min. The DNA pellet was washed twice with 70% ethanol with subsequent centrifugation at 14000 rpm for four min each time. The DNA pellet recovered was air-dried, dissolved in 100µl of TE, and left overnight. Then, 5µl of 1mg/ ml of RNAse was added, incubated at 37°C for 30 min, and kept at –20°C for later use. The DNA quality was checked by electrophoresis in 1% agarose gel and the concentration was estimated by repeated measurements with spectrophotometer at 260 nm optical densities.

4.3. PCR amplification

A total of 3 primers [OPA-07 (5'GAAACGGGTG 3'), OPA-10 (5'GTGATCGCAG 3') and OPA-17 (5'GACCGCTTGT 3')] out of 10 (all from Operon Technologies) were screened for detecting polymorphisms and showing reproducibility, and used for the PCR amplification (Table 5.1). The amplification reaction was performed in a total volume of 25µl reaction mixture, containing 1x reaction buffer (75mM Tris HCl, pH8.8, 20mM $(NH_4)_2SO_4$, and 0.01%(v/v) Tween 20), 2.5 mM $MgCl_2$, 12ng primer, 0.4mM dNTPs (100mM each of dATP, dCTP, dGTP and dTTP), 0.5 units of Taq polymerase and 25ng of genomic DNA.

Amplification was carried out in a Hybaid Omnigene thermocycler for one cycle of initial strand separation at 94°C for 3 min followed by 45 cycles of 1min at 94°C (denaturation), 1min at 37°C (annealing), and 2min at 72°C (chain elongation) using the fastest possible transition temperatures. The last cycle was followed by additional extension at 72°C for 10 min to ensure that the primer extension reaction was completed.

4.4. Agarose gel electrophoresis

After amplification, 5µl of loading buffer (0.12% bromo-phenol blue and 30% glycerol) was added to each tube containing PCR amplification products, and the products were separated on 1.2% agarose gel containing 0.5µg/ ml ethidium bromide, and run in 1xTAE buffer (40mM Tris, pH 8.0, 1mM EDTA, and 19mM HAc) for 2½ hr at 90 volt. The gel was visualized under UV trans-illumination and photographed using Polaroid 667 films. Those bands that were not

reproducible or too difficult to score with certainty were excluded from the data set. A PCR mixture containing all components of the PCR reaction excluding the template DNA was used as a negative control.

4.5. Data scoring and analysis

Each amplified product was treated as an independent character or locus and assigned numbers in order of decreasing molecular weight. The size of each band was estimated using the DNA molecular weight marker (100 bp ladder). A band was scored as present (1) or absent (0). Since RAPD markers are dominant, a locus was considered to be polymorphic if the presence and absence of the bands were observed in various individuals. Though considerable differences in band intensity were observed, such quantitative differences were not considered for data analysis.

The statistical package that was used for the analysis was Pop Gene Software Version 1.32. The magnitude of genetic variation was estimated using the Shannon-Weaver diversity index as described by Lewontin (1972):

$$S = -\Sigma\, pi \log_2 pi$$

Where "pi" is the frequency of the ith amplified polymorphic band among individuals of a population or among populations in a region of sampling.

The pair-wise genetic distances among populations and regions of sampling were estimated as Nei's unbiased genetic distances (Nei, 1978).

Using Nei's unbiased genetic distances as input matrix, relationships among the eight populations and among the four regions of sampling were investigated with a UPGMA (the unweighted pair-group method with arithmetic averages) cluster analysis, and phenetic dendrograms were constructed.

For unbiased estimation of population-genetic parameters, Lynch and Milligan (1994) suggested that the analysis should be restricted to those loci that are not too common as the use of too common loci provide little or no discrimination among relatives. Specifically, they recommended the restriction of the analysis to bands whose observed frequency is less than 1-(3/N) (where, N is the number of individuals in a population; in our case N = 10). Accordingly, loci whose frequency is greater than 0.7 (1-3/10 = 0.7) were excluded from our analysis and then the estimation of the Shannon-Weaver diversity index, the pair-wise genetic distance among populations and regions of sampling, and the construction of the phenetic dendrograms were made again with the consideration of Lynch and Milligan (1994) correction.

Spearman's correlation coefficients were calculated between the loci and the sex and region of the samples considered in the present study.

A discriminant analysis was made to identify those loci that best discriminate regions of sampling of *Hagneia abyssinica*. Predictive new group membership was also built based on the RAPD data of sample trees.

5. RESULT

Ten oligonucleotide primers were first screened using 16 *Hagenia* samples (one female and one male plant sample from each population) of which three primers were selected for generating reproducible polymorphic bands. The remaining seven primers were either monomorphic, produced poor or no amplification products at all. The three primers generated a total of 19 reproducible polymorphic bands across the 80 individuals representing 8 forest *Hagenia* populations (Table 5.1). A band (locus) was considered to be polymorphic if the band differentiates at least any of two of the 80 samples. The number of amplification products varied from 6 to 7, with a mean of 6.33. The size of the amplified fragments ranged from 300 to 2000 base pairs (Table 5.1). The RAPD band profile obtained with each of the three primers (OPA-07, OPA-10 and OPA-17) is shown in Fig. 5.1, Fig. 5.2 and Fig. 5.3.

The number and percentage of polymorphic RAPD bands for the 8 populations and 4 regions of sampling of the female, male and entire (without sex discrimination) individuals is presented in Table 5.2. For female populations, the percentage of polymorphic RAPD bands ranges from 42% for Jimma-1 to 84% for Bale-2 population, with a mean of 56%. For male populations, this value ranges from 53% for Jimma-1 to 84% for Bale-1 population, with a mean of 70%. For the entire population, it ranges from 58% for Jimma-1 to 89% for Bale-1 and Arsi-1 populations, with a mean of 82%.

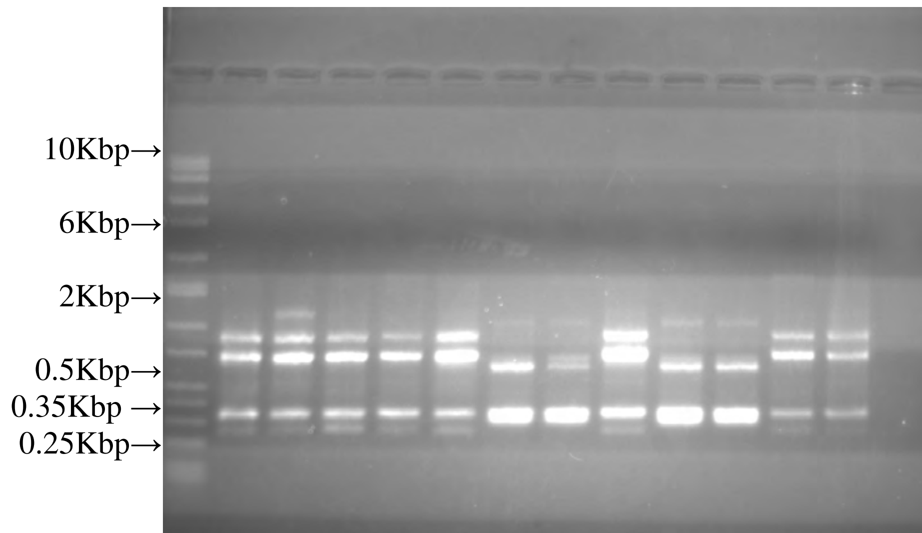

Fig. 5.1 The RAPD fingerprints of genomic DNA of 12 Hagenia *abyssinica* samples using OPA-17 primer.

From left, lane 1 Molecular marker, lanes 2-4, 5-7, 8-10 and 11-13 are Bale, Sidamo, Aris and Jimma populations. Lane 14 is Negative control. Lanes 2-7. Males. Lanes 8-13 Females.

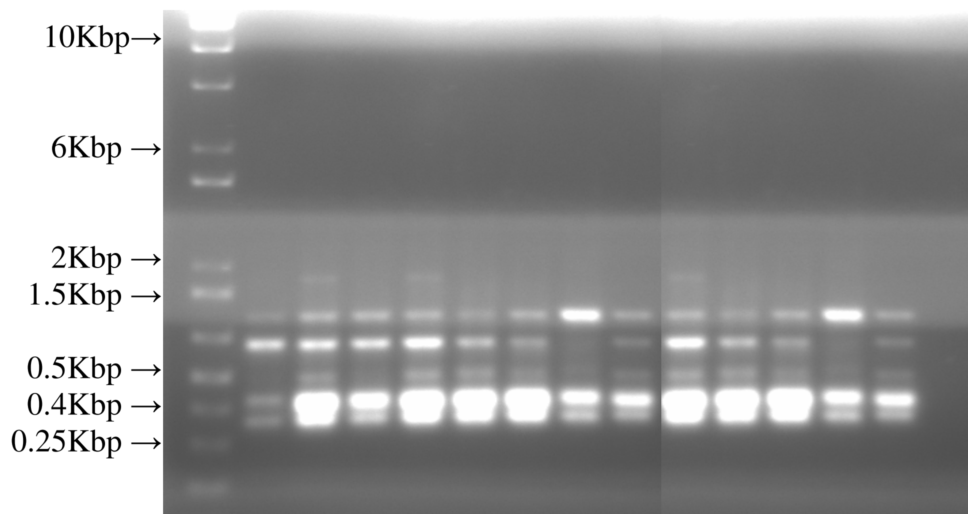

Fig. 5.2 The RAPD fingerprints of genomic DNA of 13 *Hagenia abyssinica* samples using OPA-10 primer.

From left, lane 1 Molecular marker, lanes 2-4, 5-8, 9-11 and 12-14 are Bale, Sidamo, Arsi and Jimma populations. Lane 15 is Negative control. Lanes 2-7 Males. Lanes 8-14 Females.

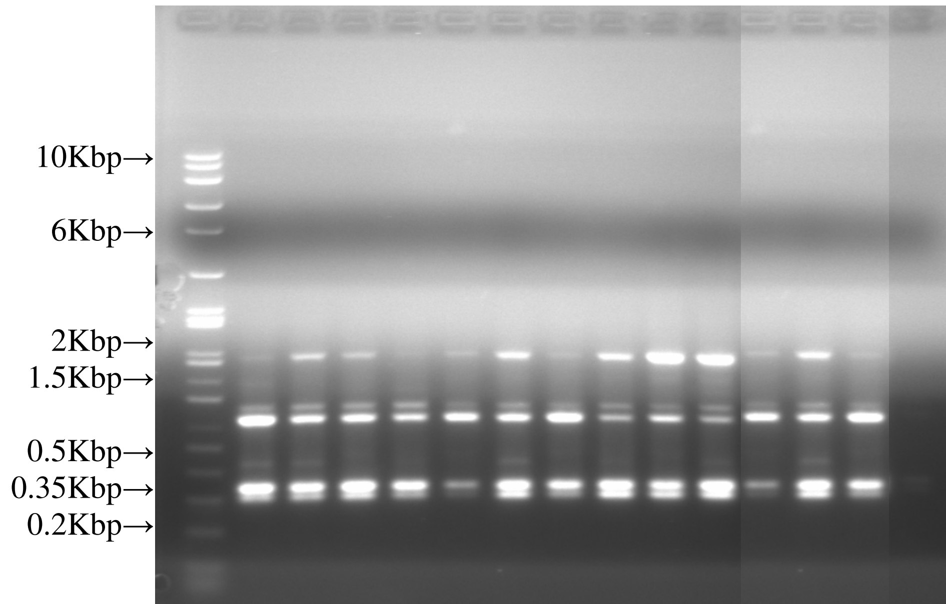

Fig. 5.3 The RAPD fingerprints of genomic DNA of 13 *Hagenia abyssinica* samples using OPA-07 primer.

From left, lane 1 Molecular marker, lanes 2-5, 6-9, 10-12 and 13-14 are Bale, Sidamo, Arsi and Jimma populations. Lane 15 is Negative control. Lanes 2-7 Males. Lane 8-14 Females

Table 5.1 List of selected primers used in the RAPD analysis along with their nucleotide sequences, number of amplified bands, and approximate molecular size range (bp) of amplified bands

Primer	Sequences 5' to 3'	No. amplified bands	Molecular size range
OPA-07	GAAACGGGTG	6	300-2000
OPA-10	GTGATCGCAG	6	300-1750
OPA-17	GACCGCTTGT	7	300-1500
Total		19	
Range		6-7	300-2000
Mean		6.33	

Table 5.2. Number and percentage of polymorphic bands for female, male, and entire populations of *Hagenia abyssinica* and for their four regions of sampling

Category	Female population		Male population		Entire population	
	No. of polymorphic bands	Polymorphic bands (%)	No. of polymorphic bands	Polymorphic bands (%)	No. of polymorphic bands	Polymorphi c bands (%)
Population						
Bale-1	11	57.89	16	84.21	17	89.47
Bale-2	16	84.21	14	73.68	18	94.74
Jimma-1	8	42.11	10	52.63	11	57.89
Jimma-2	9	47.37	12	63.16	15	78.95
Sidamo-1	12	63.16	12	63.16	15	78.95
Sidamo-2	10	52.63	15	78.95	18	94.74
Arsi-1	10	52.63	15	78.95	17	89.47
Arsi-2	9	47.37	12	63.16	13	68.42
Mean	10.5	55.92	13.25	69.74	15.5	81.58
Entire data	18	94.74	19	100.00	19	100.00
Region						
Bale	18	94.74	17	89.47	19	100.00
Jimma	10	57.89	15	78.95	17	89.47
Sidamo	13	68.42	18	94.74	19	100.00
Arsi	10	57.82	18	94.74	18	94.74
Mean	12.75	69.40	17	89.48	18.25	96.05

5.1. Estimation of RAPD variation

Estimation of the Shannon-Weaver diversity index is given for the 8 populations and 4 regions of sampling of females, males and entire individuals in Table 5.3, 5.4, and 5.5, respectively**.** For female populations, pooled over the three primers, the estimated Shannon's diversity varied from 0.20 ± 0.01 for Jimma-1 to 0.45 ± 0.01 for Bale-2 population with an overall mean across populations (H_{Pop}) of 0.32 ± 0.05, indicating a low level of RAPD variation (H_{Pop} values below or above 0.5 are considered to have lower or higher RAPD variation, respectively). For male populations, this value varied from 0.30 ± 0.03 for Jimma-1 to 0.51± 0.02 for Bale-1 with an overall mean of 0.41± 0.01, indicating a low RAPD variation. For the entire population, this value varied from 0.27 ± 0.04 for Jimma-1 to 0.56 ± 0.03 for Sidamo-2 with an overall mean of 0.45 ± 0.02, indicating nearly an intermediate level of RAPD variation. The estimated Shannon diversity for the female, male, and entire population (H_{Tot}) is 0.49 ± 0.02, 0.59 ± 0.02 and 0.55 ± 0.02, respectively.

For female trees, the estimated Shannon diversity for the regions of sampling, varied from 0.27 ± 0.05 for Jimma to 0.54 ± 0.03 for Bale with an overall mean of 0.36 ± 0.02. For male trees, this value varied from 0.40 ± 0.05 for Jimma to 0.55 ± 0.04 for Bale and 0.55 ± 0.03 for Sidamo with an overall mean of 0.51 ± 0.01. For the entire population, the value varied from 0.41 ± 0.02 for Jimma to 0.59 ± 0.01 for Bale with an overall mean of 0.52 ± 0.01.

The estimation of the Shannon-diversity index with Lynch and Milligan (1994) correction is given for the eight populations and four regions of sampling of females, males and entire individuals in Table 5.6, 5.7 and 5.8.

With regard to populations, in all of the cases (female, male and entire populations), the values of the lowest and the highest RAPD variations and the regions that showed those values are nearly the same as the case in the analysis made without Lynch and Milligan (1994) correction except that the values are slightly higher in the results obtained with Lynch and Milligan (1994) correction. Other values of RAPD variation (values of RAPD variation for each population) are also generally higher than the corresponding values obtained in the analysis made without Lynch and Milligan (1994) correction.

With regard to regions of sampling, the situation is the same. That is, the values of the lowest and the highest RAPD variations and the regions that possessed those values are nearly the same as the case in the analysis made without Lynch and Milligan (1994) correction except that the values are slightly higher in the results obtained with Lynch and Milligan (1994) correction.

The values of the overall mean Shannon-Weaver diversity index for the populations (H_{Pop}) and regions of sampling (H_r) still showed no significant change except in the case of female individuals. The values of the total diversity (H_{Tot}) for each case (female, male and entire populations) also showed no significant change.

For the male, female and entire population, the mean estimate of the Shannon-Weaver diversity index for each region is given in Table 5.9. In all of the cases (male, female and entire population), the genetic diversity progressively decreases in the order of Bale, Sidamo, Arsi and Jimma.

The corresponding values obtained with Lynch and Milligan (1994) correction is given in Table 5.9. Here again, the results showed that in all of the cases (male, female and entire population), the genetic diversity progressively decreases in the order of Bale, Sidamo, Arsi and Jimma. Close observation of the diversity values of each region revealed that while some values showed significant change (e.g. regions of male and female individuals) others showed no significant change (e.g. regions of the entire individuals) as compared to the corresponding values obtained in the analysis made without Lynch and Milligan (1994) correction.

Table 5.3 Estimation of Shannon-Weaver diversity index for eight female populations of *Hagenia abyssinica* and for their four regions of sampling

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.40	0.34	0.28	0.34 ± 0.03
	Bale-2	0.45	0.45	0.44	0.45 ± 0.01
	Jimma-1	0.20	0.22	0.17	0.20 ± 0.01
	Jimma-2	0.29	0.34	0.17	0.27 ± 0.05
	Sidamo-1	0.42	0.51	0.17	0.37 ± 0.10
	Sidamo-2	0.29	0.42	0.25	0.32 ± 0.05
	Arsi-1	0.41	0.20	0.30	0.30 ± 0.06
	Arsi-2	0.40	0.20	0.20	0.26 ± 0.07
Mean		0.36	0.34	0.25	0.32 ± 0.05
Entire data		0.47	0.53	0.46	0.49 ± 0.02
Region	Bale	0.49	0.58	0.55	0.54 ± 0.03
	Jimma	0.30	0.33	0.18	0.27 ± 0.05
	Sidamo	0.41	0.37	0.25	0.34 ± 0.05
	Arsi	0.40	0.20	0.30	0.30 ± 0.06
Mean		0.40	0.37	0.32	0.36 ± 0.02

Table 5.4 Estimation of Shannon-Weaver diversity index for eight male populations of *Hagenia abyssinica* and for their four regions of sampling

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.50	0.54	0.49	0.51 ± 0.02
	Bale-2	0.65	0.42	0.32	0.47 ± 0.09
	Jimma-1	0.32	0.34	0.23	0.30 ± 0.03
	Jimma-2	0.29	0.23	0.50	0.34 ± 0.08
	Sidamo-1	0.26	0.43	0.36	0.35 ± 0.05
	Sidamo-2	0.64	0.31	0.51	0.49 ± 0.09
	Arsi-1	0.49	0.52	0.39	0.47 ± 0.04
	Arsi-2	0.28	0.43	0.36	0.36 ± 0.04
Mean		0.43	0.40	0.39	0.41 ± 0.01
Entire data		0.60	0.61	0.55	0.59 ± 0.02
Region	Bale	0.63	0.53	0.50	0.55 ± 0.04
	Jimma	0.39	0.33	0.50	0.40 ± 0.05
	Sidamo	0.59	0.50	0.55	0.55 ± 0.03
	Arsi	0.48	0.63	0.52	0.54 ± 0.04
Mean		0.52	0.50	0.52	0.51 ± 0.01

Table 5.5 Estimation of Shannon-Weaver diversity index for eight populations (without sex discrimination) of *Hagenia abyssinica* and for their four regions of sampling

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.50	0.60	0.47	0.52 ± 0.04
	Bale-2	0.61	0.50	0.55	0.55 ± 0.03
	Jimma-1	0.32	0.29	0.20	0.27 ± 0.04
	Jimma-2	0.35	0.39	0.46	0.40 ± 0.03
	Sidamo-1	0.40	0.59	0.32	0.44 ± 0.08
	Sidamo-2	0.61	0.51	0.56	0.56 ± 0.03
	Arsi-1	0.50	0.58	0.47	0.52 ± 0.03
	Arsi-2	0.40	0.38	0.33	0.37 ± 0.02
Mean		0.46	0.48	0.42	0.45 ± 0.02
Entire data		0.57	0.58	0.51	0.55 ± 0.02
Region	Bale	0.60	0.60	0.57	0.59 ± 0.01
	Jimma	0.41	0.38	0.44	0.41 ± 0.02
	Sidamo	0.58	0.57	0.52	0.56 ± 0.02
	Arsi	0.49	0.56	0.48	0.51 ± 0.03
Mean		0.52	0.53	0.50	0.52 ± 0.01

Table 5.6 Estimation of Shannon-Weaver diversity index for eight female populations of *Hagenia abyssinica* and for their four regions of sampling considering Lynch and Milligan (1994) correction

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.51	0.56	0.28	0.45 ± 0.09
	Bale-2	0.54	0.55	0.42	0.50 ± 0.04
	Jimma-1	0.28	0.29	0.28	0.28 ± 0.00
	Jimma-2	0.29	0.28	0.35	0.31 ± 0.02
	Sidamo-1	0.57	0.46	0.35	0.46 ± 0.06
	Sidamo-2	0.54	0.22	0.31	0.36 ± 0.09
	Arsi-1	0.35	0.28	0.24	0.29 ± 0.03
	Arsi-2	0.39	0.46	0.17	0.34 ± 0.09
Mean		0.47	0.39	0.30	0.39 ± 0.05
Entire data		0.56	0.56	0.50	0.54 ± 0.02
Region	Bale	0.48	0.64	0.44	0.52 ± 0.06
	Jimma	0.38	0.30	0.34	0.34 ± 0.02
	Sidamo	0.53	0.58	0.38	0.50 ± 0.06
	Arsi	0.58	0.40	0.36	0.45 ± 0.07
Mean		0.49	0.48	0.38	0.45 ± 0.03

Table 5.7 Estimation of Shannon-Weaver diversity index for eight male populations of *Hagenia abyssinica* and for their four regions of sampling considering Lynch and Milligan (1994) correction

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.54	0.62	0.51	0.56 ± 0.03
	Bale-2	0.57	0.62	0.47	0.55 ± 0.04
	Jimma-1	0.36	0.42	0.31	0.36 ± 0.04
	Jimma-2	0.54	0.22	0.47	0.41 ± 0.09
	Sidamo-1	0.47	0.22	0.42	0.37 ± 0.08
	Sidamo-2	0.50	0.62	0.35	0.49 ± 0.08
	Arsi-1	0.47	0.22	0.46	0.38 ± 0.08
	Arsi-2	0.53	0.46	0.42	0.47 ± 0.03
Mean		0.49	0.41	0.42	0.44 ± 0.03
Entire data		0.57	0.64	0.51	0.57 ± 0.04
Region	Bale	0.61	0.67	0.56	0.61 ± 0.03
	Jimma	0.56	0.27	0.56	0.46 ± 0.09
	Sidamo	0.54	0.61	0.44	0.53 ± 0.05
	Arsi	0.52	0.51	0.44	0.49 ± 0.03
Mean		0.55	0.52	0.50	0.52 ± 0.02

Table 5.8 Estimation of Shannon-Weaver diversity index for eight populations (without sex discrimination) of *Hagenia abyssinica* and for their four regions of sampling - considering Lynch and Milligan (1994) correction

Category		OPA-07	OPA-10	OPA-17	Mean ± S.E
Population	Bale-1	0.56	0.48	0.45	0.55 ± 0.03
	Bale-2	0.52	0.62	0.41	0.52 ± 0.06
	Jimma-1	0.41	0.40	0.30	0.37 ± 0.04
	Jimma-2	0.46	0.42	0.42	0.43 ± 0.01
	Sidamo-1	0.56	0.50	0.44	0.50 ± 0.03
	Sidamo-2	0.53	0.63	0.51	0.56 ± 0.04
	Arsi-1	0.53	0.58	0.44	0.52 ± 0.04
	Arsi-2	0.56	0.62	0.36	0.51 ± 0.08
Mean		0.50	0.51	0.41	0.47 ± 0.03
Entire data		0.56	0.63	0.52	0.57 ± 0.03
Region	Bale	0.57	0.69	0.53	0.60 ± 0.05
	Jimma	0.47	0.44	0.40	0.44 ± 0.02
	Sidamo	0.59	0.54	0.49	0.54 ± 0.03
	Arsi	0.57	0.63	0.43	0.54 ± 0.06
Mean		0.55	0.58	0.46	0.53 ± 0.04

Table 5.9 Mean estimates of the Shannon-Weaver diversity index across the three primers for four regions of sampling of female, male, and entire *Hagenia abyssinica* populations

		Without considering Lynch and Milligan (1994) correction	Considering Lynch and Milligan (1994) correction
Category	Region	Mean ± S.E	Mean ± S.E
Female population	Bale	0.54 ± 0.03	0.52 ± 0.06
	Jimma	0.27 ± 0.05	0.34 ± 0.02
	Sidamo	0.34 ± 0.05	0.50 ± 0.06
	Arsi	0.30 ± 0.06	0.45 ± 0.07
Male population	Bale	0.55 ± 0.04	0.61 ± 0.03
	Jimma	0.40 ± 0.05	0.46 ± 0.09
	Sidamo	0.55 ± 0.03	0.53 ± 0.05
	Arsi	0.54 ± 0.04	0.49 ± 0.03
Entire population	Bale	0.59 ± 0.01	0.60 ± 0.05
	Jimma	0.41 ± 0.02	0.44 ± 0.02
	Sidamo	0.56 ± 0.02	0.54 ± 0.03
	Arsi	0.51 ± 0.03	0.54 ± 0.06

Table 5.10. Partitioning of genetic variation into within and between populations as well as within and between regions of sampling of *Hagenia abyssinica* populations.

	Population					Region		
	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
Female Population	OPA-07	0.47	0.36	0.77	0.23	0.40	0.85	0.15
	OPA-10	0.53	0.34	0.64	0.36	0.37	0.70	0.30
	OPA-17	0.46	0.25	0.54	0.46	0.32	0.70	0.30
	Mean	0.49 ± 0.02	0.32 ± 0.03	0.65 ± 0.07	0.35 ± 0.07	0.36 ± 0.02	0.75 ± 0.05	0.25 ± 0.05
	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
Male Population	OPA-07	0.60	0.43	0.72	0.28	0.52	0.87	0.13
	OPA-10	0.61	0.40	0.66	0.34	0.50	0.82	0.18
	OPA-17	0.55	0.39	0.71	0.29	0.52	0.95	0.05
	Mean	0.59 ± 0.02	0.41 ± 0.01	0.70 ± 0.02	0.30 ± 0.02	0.51 ± 0.01	0.88 ± 0.04	0.12 ± 0.04
	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
Entire Population	OPA-07	0.57	0.46	0.81	0.19	0.52	0.91	0.09
	OPA-10	0.58	0.48	0.83	0.17	0.53	0.91	0.09
	OPA-17	0.51	0.42	0.82	0.18	0.50	0.98	0.02
	Mean	0.55 ± 0.02	0.45 ± 0.02	0.82 ± 0.01	0.18 ± 0.01	0.52 ± 0.01	0.93 ± 0.02	0.07 ± 0.02

H_{Total} = Mean for total genetic variation computed from the entire data set; H_{Pop} and H_r = Mean genetic variation for the populations and regions, respectively; H_{Pop}/H_{Total} and H_r/H_{Total} = Proportion of genetic variation within the populations and regions, respectively; $(H_{Total} - H_{Pop})/H_{Total}$ and $(H_{Total}-H_r)/H_{Total}$ = Proportion of genetic variation between the populations and regions, respectively

Table 5.11. Partitioning of genetic variation into within and between populations as well as within and between regions of sampling of *Hagenia abyssinica* populations- considering Lynch and Milligan (1994) correction

	Population					Region		
	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
Female population	OPA-O7	0.56	0.47	0.84	0.16	0.49	0.88	0.12
	OPA-10	0.56	0.39	0.70	0.30	0.48	0.86	0.14
	OPA-17	0.50	0.30	0.60	0.40	0.38	0.76	0.24
	Mean	0.54 ± 0.02	0.39 ± 0.05	0.72 ± 0.07	0.28 ± 0.07	0.45 ± 0.03	0.83 ± 0.03	0.17 ± 0.03
Male population	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
	OPA-O7	0.57	0.49	0.86	0.14	0.55	0.96	0.04
	OPA-10	0.64	0.41	0.64	0.36	0.52	0.81	0.19
	OPA-17	0.51	0.42	0.82	0.18	0.50	0.98	0.02
	Mean	0.57 ± 0.04	0.44 ± 0.03	0.77 ± 0.07	0.23 ± 0.07	0.52 ± 0.02	0.91 ± 0.05	0.09 ± 0.05
Entire population	Primer	H_{Total}	H_{Pop}	H_{Pop}/H_{Total}	$(H_{Total} - H_{Pop})/H_{Total}$	H_r	H_r/H_{Total}	$(H_{Total}-H_r)/H_{Total}$
	OPA-O7	0.56	0.50	0.89	0.11	0.55	0.98	0.02
	OPA-10	0.63	0.51	0.81	0.19	0.58	0.92	0.08
	OPA-17	0.52	0.41	0.85	0.15	0.46	0.88	0.12
	Mean	0.57 ± 0.03	0.47 ± 0.03	0.82 ± 0.03	0.18 ± 0.03	0.53 ± 0.04	0.93 ± 0.03	0.07 ± 0.03

H_{Total} = Mean for total genetic variation computed from the entire data set; H_{Pop} and H_r = Mean genetic variation for the populations and regions, respectively; H_{Pop}/H_{Total} and H_r/H_{Total} = Proportion of genetic variation within the populations and regions, respectively; $(H_{Total} - H_{Pop})/H_{Total}$ and $(H_{Total}-H_r)/H_{Total}$ = Proportion of genetic variation between the populations and regions, respectively

5.2. Partitioning of RAPD variation

For female populations, the within population variation accounted for 65% of the total variation and the remaining 35% occurred between populations. Similarly, partitioning of the variation in to within and between regions of sampling revealed that 75% of the total variation was found within and the remaining 25% between the regions of sampling (Table 5.10).

For male populations, the within population variation accounted for 70% of the total variation, and the remaining 30% occurred between populations. Partitioning of the variation into within and between regions of sampling revealed that 88% of the total variation was found within and the remaining 12% between the regions of sampling (Table 5.10). For the entire population, the within population variation accounted for 82% of the total variation, and the remaining 18% occurred between populations. Similarly, partitioning of the variation in to within and between regions of sampling revealed that 93% of the total variation was found within and the remaining 7% between the regions of sampling (Table 5.10), indicating very little differentiation among the regions of sampling in the present sampled regions.

The results of the partitioning of RAPD variation obtained with Lynch and Milligan (1994) correction is given for the eight female, male, and entire populations and their four regions of sampling in Table 5.11. While the entire population showed identical values (to the analysis made without Lynch and Milligan (1994) correction) of proportion of the within population variation (82%) and the within region variation (93%), the male and female populations showed differences. In this analysis, the within population variation accounted for 72% and 77% of the total variation in the female and male populations, respectively. The within region variation

accounted for 83% and 91% of the total variation in the female and male populations, respectively. As is true for the analysis made without Lynch and Milligan (1994) correction, the proportions of the within population and origin variations obtained in this analysis suggested that there is very low genetic differentiation both on population and regional bases.

5.3. Cluster analysis

Cluster analysis of the 8 female populations (Fig 5.4) revealed that while Bale-1 formed a separate group, the remaining 7 populations group to one major cluster which contained one outlier (Bale-2) and one sub-cluster which was further grouped into two sub clusters and an outlier (Sidamo-2). Similarly, clustering of the regions of sampling of female individuals (Fig 5.5) showed that Bale populations appeared to be distinct, while the other three regions were in the same group. A close observation of this second group revealed that Jimma and Sidamo populations were closer to each other than either was to the Arsi population.

Cluster analysis of the 8 male populations (Fig 5.6) revealed three main clusters. Cluster (1) includes Bale-1, Bale-2, and Jimma-1 populations. Cluster (2) includes Jimma-2, Aris-2, and Sidamo-1. Cluster (3) includes Sidmao-2 and Arsi-1 populations**.** Cluster analysis of the entire (both male and female) 8 populations (Fig 5.8) showed that Bale-1 formed a separate group. The remaining 7 populations again formed one major cluster but which was further grouped into two subclusters and an outlier (Jimma-1).

Clustering of the regions of sampling for male (Fig 5.7) and for entire individuals (Fig 5.9) showed that Bale populations appeared to be distinct, while the other three regions were in the same group. Close observation of this revealed that Sidamo and Aris were closer to each other than either was to the Jimma poulation.

Generally, cluster analyses of the male, female and entire populations showed that the populations in all cases failed to completely group on the basis of regions of sampling. A common feature to the clustering of the regions of sampling for the male, female, and entire population is that Bale region formed a separate group in all cases.

The results of the cluster analysis of the populations and regions of sampling of *Hagenia abyssinica,* made after considering the Lynch and Milligan (1994) correction, are given in Fig. 5.10 to 5.15. The overall view of the dendrograms revealed that in most of the cases there are no features that deviate significantly from those obtained with the analysis made without Lynch and Milligan (1994) correction. The only two dendrograms that showed clear differences are the dendrogram constructed for the regions of the entire population where outgrouping of Bale region is not evident [in the analysis made without Lynch and Milligan (1994) correction, Bale formed a separate group] and the dendrogram constructed for the regions of the female populations where Sidamo and Arsi, rather than Jimma and Arsi [the case in the analysis made without Lynch and Milligan (1994) correction], are closer to each other.

As is true for the analysis made without Lynch and Milligan (1994) correction, the cluster analysis obtained in this analysis showed that populations failed to completely group on the bases of regions of sampling.

Pair wise genetic distance among the 8 female populations (Table 5.12) showed that the lowest genetic distance (0.00) was found between Jimma-2 and Sidamo-1, while the highest coefficient of genetic distance (0.25) was found between Jimma-1 and Bale-1. The average genetic distance was 0.08, indicating the existence of a low genetic differentiation among the populations. Likewise, the genetic distance among the regions of sampling (Table 5.14) showed that lowest genetic distance (0.02) was found between Jimma and Sidmao, while the highest (0.07) was between Sidamo and Arsi, with an average of 0.05.

Pair wise genetic distance among the 8 male populations (Table 5.12) showed that the lowest genetic distance (0.03) was found between Arsi-1 and Sidamo-2, while the highest coefficient of genetic distance (0.17) was found between Sidamo-2 and Bale-2. The average genetic distance was 0.09, indicating the existence of a low genetic differentiation among the populations. Likewise, the genetic distance among the regions of sampling (Table 5.14) showed that lowest genetic distance (0.00) was found between Arsi and Sidmao, while the highest (0.08) was between Bale and Arsi, with an average of 0.05.

Pair wise genetic distance among the 8 populations (without sex descrimination) (Table 5.12) showed that the lowest genetic distance (0.00) was found between Arsi-1 and Sidamo-2, while the highest coefficient of genetic distance (0.13) was found between Jimma-1 and Bale-1. The average genetic distance was 0.05, indicating the existence of a low genetic differentiation among the populations. Likewise, the genetic distance among the regions of sampling (Table 5.14) showed that lowest genetic distance (0.01) was found between Arsi and Sidmao, while the highest (0.04) was between Jimma and Bale, with an average of 0.02.

The results of the pair-wise genetic distance among the population and regions of the female, male and entire populations of *Hagenia abyssinica*, made after considering the Lynch and Milligan (1994) correction, are given in Table 5.13 and 5.15.

For the female individuals, the pair-wise genetic distance among the eight populations (Table 5.13) showed that the lowest genetic distance (0.013) was found between Jimma-1 and Bale-2. The values of the average genetic distance (0.09) and the regions (Jimma-1 and Bale-1) that showed the highest genetic distance between them are the same as the case in the analysis made without Lynch and Milligan (1994) correction. The pair-wise genetic distance among the regions of sampling (Table 5.15) showed different value [from that of the analysis made without Lynch and Milligan (1994) correction] of average genetic distance (in this case, 0.03). The lowest genetic distance (0.02) was found between Sidamo and Arsi, while the highest (0.05) was found between Bale and Jimma.

For the male populations (Table 5.13), the values of the average genetic distance(0.08) and the regions (Sidamo-2 and Bale-2) that showed the highest genetic distance between them (but with different values) are the same as the case in the analysis made without Lynch and Milligan (1994) correction. The pair-wise genetic distance among the regions of sampling (Table 5.15) showed different value [from that of the analysis made without Lynch and Milligan (1994) correction] of average genetic distance (in this case, 0.09). Whereas the regions that showed the highest genetic distance (0.18) are Bale and Sidamo, the regions (Arsi and Sidamo) that showed the lowest genetic distance (in this case, 0.03) between them are the same as the case in the analysis made without Lynch and Milligan (1994) correction.

For the entire populations (Table 5.13), the regions (Jimma-1 and Bale-1) that showed the highest genetic distance (in this case, 0.17) between them are the same as those in the analysis made without Lynch and Milligan (1994) correction. The values of the average genetic distance (in this case, 0.03) and the regions that showed the lowest genetic distance (in this case, 0.005) between them (in this case, Arsi-2 and Bale-2) are different, however. The pair-wise genetic distance among the regions of sampling (Table 5.15) revealed that same regions (Arsi and Sidamo) showed the same lowest genetic distance (0.01) between them. The regions (Jimma and Bale) that showed the highest genetic distance (in this case, 0.06) between them are also the same in both cases. What differs [from the analysis made without Lynch and Milligan (1994) correction] here is the average genetic distance which in this case is 0.06.

Fig.5.4. Dendrogram for eight female populations of *Hagenia abyssinica* collected from four geographical regions of Ethiopia.

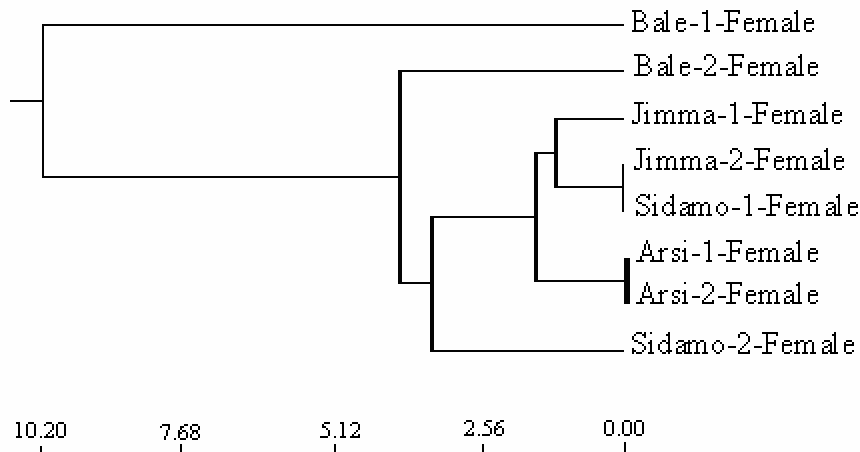

Fig.5.5.Dendrogram for four geographical regions of sampling of female*Hagenia abyssinica*populations in Ethiopia.

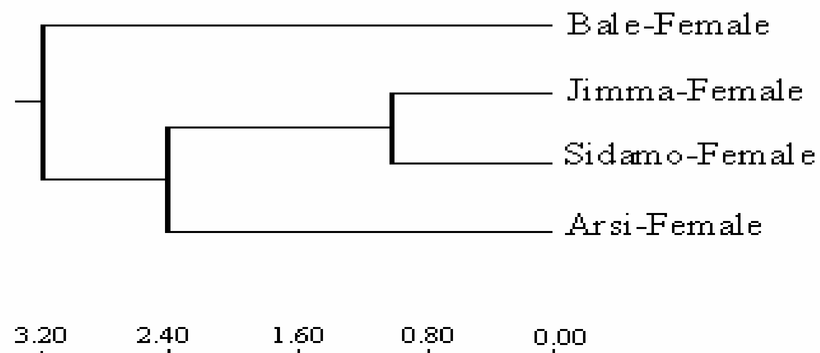

Fig. 5.6. Dendrogram for eight male populations of *Hagenia abyssinica* collected from four geographical regions of Ethiopia

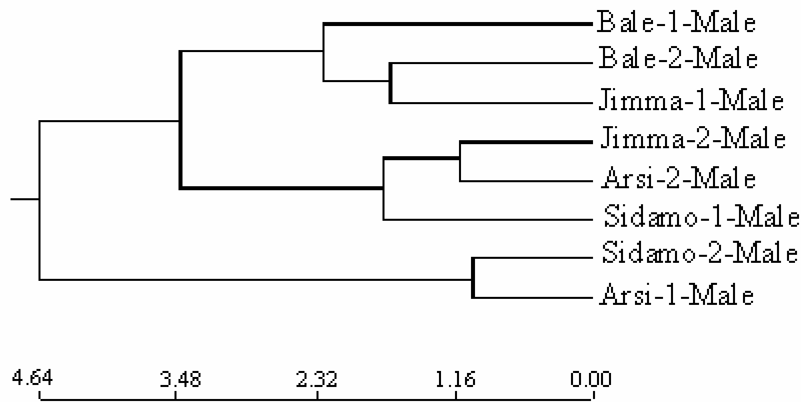

Fig. 5.7. Dendrogram for four geographical regions of sampling of male *Hagenia abyssinica* populations in Ethiopia

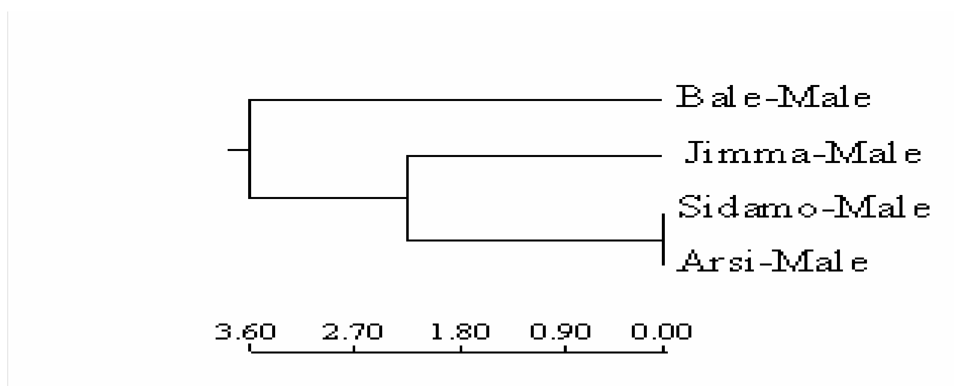

Fig.5.8. Dendrogram for eight populations (without sex discrimination) of *Hagenia abyssinica* collected from four geographical regions of Ethiopia

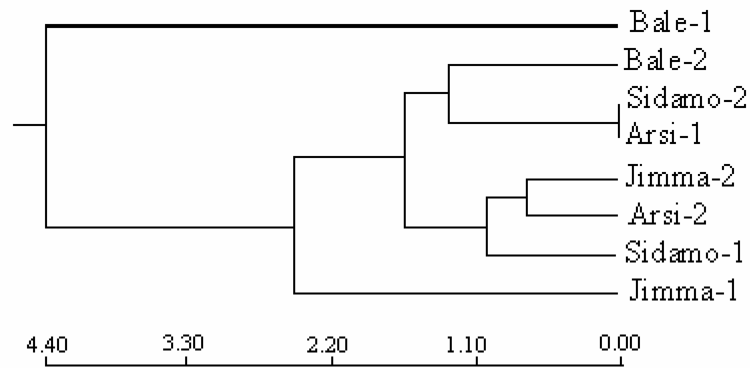

Fig.5.9. Dendrogram for four geographical regions of sampling of *Hagenia abyssinica* populations (without sex discrimination) in Ethiopia.

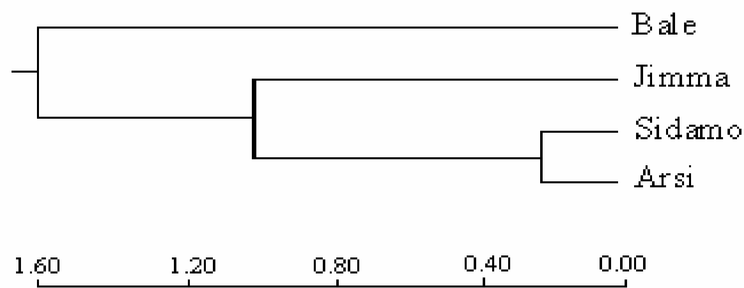

Fig. 5.10. Dendrogram for eight female populations of *Hagenia abyssinica* collected from four geographical regions of Ethiopia - considering Lynch and Milligan (1994) correction

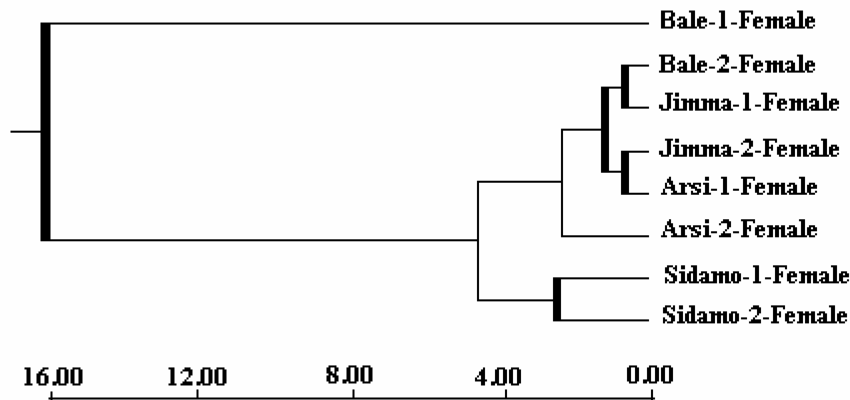

Fig. 5.11. Dendrogram for four geographical regions of sampling of female *Hagenia abyssinica* populations in Ethiopia- considering Lynch and Milligan (1994) correction

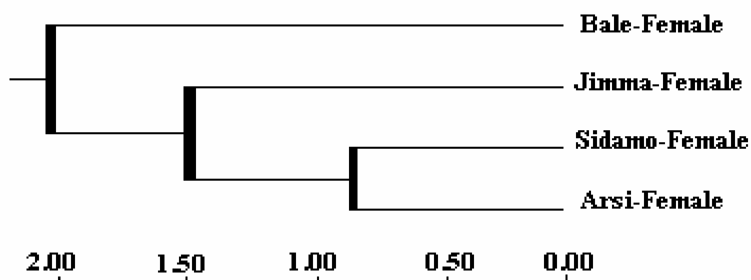

Fig. 5.12. Dendrogram for eight male populations of *Hagenia abyssinica* collected from four geographical regions of Ethiopia- considering Lynch and Milligan (1994) correction

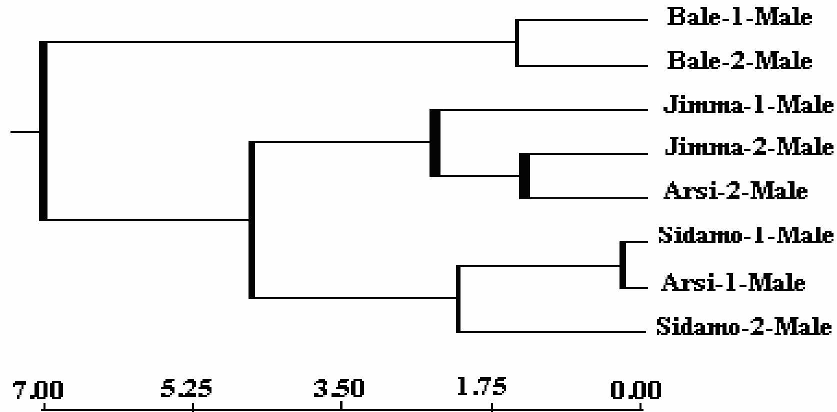

Fig. 5.13. Dendrogram for four geographical regions of sampling of male *Hagenia abyssinica* populations in Ethiopia- considering Lynch and Milligan (1994) correction

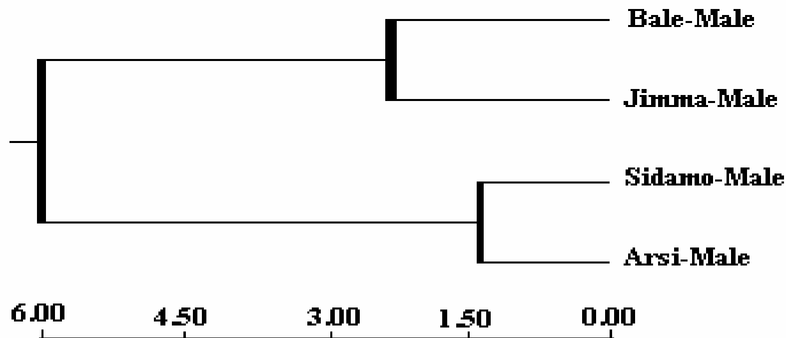

Fig. 5.14. Dendrogram for eight populations (wighout sex discrimination) of *Hagenia abyssinica* collected from four geographical regions of Ethiopia- considering Lynch and Milligan (1994) correction

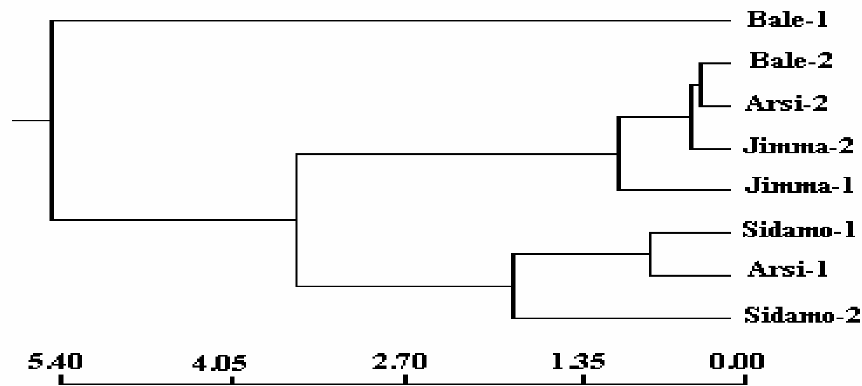

Fig. 5.15. Dendrogram for four geographical regions of sampling of *Hagenia abyssinica* populations (without sex discrimination) in Ethiopia- considering Lynch and Milligan (1994) correction

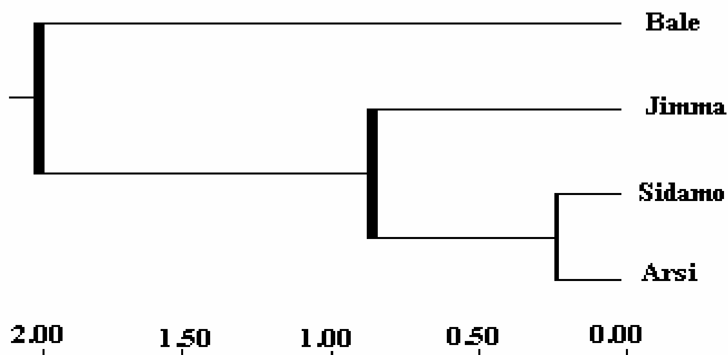

Table 5.12 Nei's genetic distance among eight female, male and entire populations of *Hagenia abyssinica*

Category		1	2	3	4	5	6	7	8
Female population	1.Bale-1-Female	0.0000							
	2.Bale-2-Female	0.2352	0.0000						
	3.Jimma-1-Female	0.2485	0.0692	0.0000					
	4.Jimma-2-Female	0.2064	0.0449	0.0135	0.0000				
	5.Sidamo-1-Female	0.2104	0.0633	0.0358	0.0001	0.0000			
	6.Sidamo-2-Female	0.2303	0.1201	0.0577	0.0457	0.0202	0.0000		
	7.Arsi-1-Female	0.1393	0.0786	0.0313	0.0220	0.0458	0.1120	0.0000	
	8.Arsi-2-Female	0.1602	0.0990	0.0318	0.0234	0.0355	0.0995	-0.0052	0.0000
Male population	1.Bale-1-Male	0.0000							
	2.Bale-2-Male	0.0459	0.0000						
	3.Jimma-1-Male	0.0699	0.0435	0.0000					
	4.Jimma-2-Male	0.1168	0.1356	0.0553	0.0000				
	5.Sidamo-1-Male	0.0834	0.1031	0.0606	0.0487	0.0000			
	6.Sidamo-2-Male	0.1142	0.1673	0.1280	0.0851	0.1306	0.0000		
	7.Arsi-1-Male	0.1423	0.1409	0.1265	0.0868	0.0362	0.0255	0.0000	
	8.Arsi-2-Male	0.0951	0.0967	0.0482	0.0286	0.0406	0.1491	0.1141	0.0000
Entire population	1.Bale-1	0.0000							
	2.Bale-2	0.0555	0.0000						
	3.Jimma-1	0.1265	0.0459	0.0000					
	4.Jimma-2	0.1017	0.0201	0.0318	0.0000				
	5.Sidamo-1	0.0928	0.0414	0.0470	0.0147	0.0000			
	6.Sidamo-2	0.0654	0.0261	0.0707	0.0359	0.0350	0.0000		
	7.Arsi-1	0.0784	0.0271	0.0649	0.0043	0.0096	-0.0014	0.0000	
	8.Arsi-2	0.0988	0.0552	0.0401	0.0145	0.0268	0.0705	0.0277	0.0000

Table 5.13 Nei's genetic distance among eight female, male and entire populations of *Hagenia abyssinica* -considering Lynch and Milligan (1994) correction.

Category		1	2	3	4	5	6	7	8
Female population	1.Bale-1-Female	0.0000							
	2.Bale-2-Female	0.4066	0.0000						
	3.Jimma-1-Female	0.4630	0.0127	0.0000					
	4.Jimma-2-Female	0.3022	0.0196	0.0242	0.0000				
	5.Sidamo-1-Female	0.3369	0.0571	0.0663	0.0851	0.0000			
	6.Sidamo-2-Female	0.2423	0.1320	0.1257	0.1042	0.0508	0.0000		
	7.Arsi-1-Female	0.2590	0.0293	0.0344	0.0138	0.0698	0.0980	0.0000	
	8.Arsi-2-Female	0.2351	0.0450	0.0706	0.0498	0.0758	0.1360	0.0224	0.0000
Male population	1.Bale-1-Male	0.0000							
	2.Bale-2-Male	0.0311	0.0000						
	3.Jimma-1-Male	0.1266	0.1387	0.0000					
	4.Jimma-2-Male	0.1067	0.0850	0.0370	0.0000				
	5.Sidamo-1-Male	0.1446	0.1489	0.1326	0.0603	0.0000			
	6.Sidamo -2-Male	0.1825	0.2418	0.1243	0.1037	0.0597	0.0000		
	7.Arsi-1-Male	0.1525	0.1731	0.1110	0.0594	0.0070	0.0283	0.0000	
	8.Arsi-2-Male	0.0887	0.0913	0.0612	0.0294	0.0777	0.0896	0.0625	0.0000
Entire population	1.Bale-1	0.0000							
	2.Bale-2	0.1143	0.0000						
	3.Jimma-1	0.1650	0.0164	0.0000					
	4.Jimma-2	0.1081	0.0058	0.0112	0.0000				
	5.Sidamo-1	0.0913	0.0353	0.0575	0.0357	0.0000			
	6.Sidamo-2	0.1021	0.1169	0.1084	0.1041	0.0506	0.0000		
	7.Arsi-1	0.0700	0.0577	0.0687	0.0486	0.0122	0.0175	0.0000	
	8.Arsi-2	0.0993	0.0049	0.0241	0.0061	0.0255	0.1097	0.0487	0.0000

Table 5.14 Nei's genetic distance among four regions of sampling of female, male and entire populations of *Hagenia abyssinica*

Category		1	2	3	4
Female population	1.Bale-Female	0.0000			
	2.Jimma-Female	0.0636	0.0000		
	3.Sidamo-Female	0.0675	0.0210	0.0000	
	4.Arsi-Female	0.0664	0.0297	0.0705	0.0000
Male population	1.Bale-Male	0.0000			
	2.Jimma-Male	0.0669	0.0000		
	3.Sidamo-Male	0.0743	0.0462	0.0000	
	4.Arsi-Male	0.0756	0.0422	-0.0034	0.0000
Entire population	1.Bale	0.0000			
	2.Jimma	0.0398	0.0000		
	3.Sidamo	0.0217	0.0236	0.0000	
	4.Arsi	0.0362	0.0176	0.0047	0.0000

Table 5.15 Nei's genetic distance among four regions of sampling of female, male and entire populations of *Hagenia abyssinica* - considering Lynch and Milligan (1994) correction

Category		1	2	3	4
Female population	1.Bale-Female	0.0000			
	2.Jimma-Female	0.0535	0.0000		
	3.Sidamo-Female	0.0450	0.0163	0.0000	
	4.Arsi-Female	0.0173	0.0400	0.0160	0.0000
Male population	1.Bale-Male	0.0000			
	2.Jimma-Male	0.0481	0.0000		
	3.Sidamo-Male	0.1778	0.1227	0.0000	
	4.Arsi-Male	0.1186	0.0661	0.0281	0.0000
Entire population	1.Bale	0.0000			
	2.Jimma	0.0615	0.0000		
	3.Sidamo	0.0378	0.0137	0.0000	
	4.Arsi	0.0194	0.0209	0.0050	0.0000

5.4. Spearman's Correlation

Correlations between sex and loci identified OPA10-1 locus as having a significant (0.01 level of significance) correlation/association with the male sex (Table 5.16). Similarly, correlation between regions of sampling and loci showed that OPA07-3 (0.01 level of significance) and OPA10-2 (0.05 level of significance) are correlated with Bale, Jimma, Sidamo and Arsi in order of increasing association. That is, they are least associated with Bale and most with Arsi region (Table 5.16).

5.5. Discriminant analysis

The discriminant analysis procedure generated three canonical discriminant functions that were used in the analysis to identify those loci that best discriminate the four regions of sampling. Accordingly, OPA10-2, OPA10-4 and OPA10-6 are the only loci that discriminate between Bale and Arsi; between Jimma and Sidmao; and between Jimma and Arsi, respectively (Table 5.17).

The predictive new group membership built based on the RAPD data showed that 83.1% of the original grouped cases are correctly classified (Appendix 5.2).

For further information on the discriminant analysis, see Appendices 5.1, 5.3 and 5.4.

Table 5.16 Spearman's correlation between loci and sex and region of sampling of *Hagenia abyssinica*

Locus	Spearman's rho	
	Sex	Region
OPA07-1	.052	.047
OPA07-2	.076	.034
OPA07-3	-.167	.335**
OPA07-4	.026	.035
OPA07-5	-.197	-.029
OPA07-6	.000	.025
OPA10-1	.395**	-.029
OPA10-2	.031	.260*
OPA10-3	.076	-.068
OPA10-4	.207	-.139
OPA10-5	.142	.114
OPA10-6	.000	-.149
OPA17-1	.206	-.092
OPA17-2	-.040	.088
OPA17-3	-.133	.099
OPA17-4	.077	-.126
OPA17-5	-.115	.000
OPA17-6	.026	-.011
OPA17-7	.088	-.092

Ranking order

Sex
1- Female
2- Male

Region
1-Bale
2-Jimma
3-Sidamo
4-Arsi

**correlation significant at 0.01 level
*correlation significant at 0.05 level (2-tailed)

Table 5.17 Variables (loci) used in the discriminant analysis to discriminate between regions of sampling of *Hagenia abyssinica*

Step		Tolerance	F to Remove	Min. D Squared	Between Groups
1	OPA10-2	1.000	12.600		
2	OPA10-2	.669	18.915	.396	Bale & Arsi
	OPA10-4	.669	8.463	1.225	Jimma & Arsi
3	OPA10-2	.406	28.279	.468	Bale & Arsi
	OPA10-4	.613	8.583	2.887	Jimma & Sidamo
	OPA10-6	.592	4.672	3.546	Jimma & Arsi

6. DISCUSSION

This is the first paper to report on RAPD variation among *Hagenia abyssinica* trees. We have analyzed RAPD variation in 80 *Hagenia abyssinica* trees representing eight populations from four geographical regions of Ethiopia to determine the extent and distribution of genetic variation.

The mean number of bands generated is not as such large may be due to the use of very few primers. In addition to this, the bias in the number of bands may occur in scoring bands of different intensity. The number of amplification products could also be affected by the purity of DNA, sensitivity of gel staining system, constitution of amplification mix and thermal cycling (Williams *et al*, 1993).

In our case, the purity of DNA, in particular, is a worthmentioning factor as the leaves of *Hagenia abyssinica* are fibrous and rich in phenolic compounds (responsible for medicinal value), and such metabolites are known to affect the quality of DNA and hamper the DNA isolation procedures and reactions such as DNA amplification (Khanuja *et. al*., 1999). However, the procedure that was followed to optimize this extraction condition seems to minimize such a problem.

6.1. Genetic variation

All the four regions of sampling generally showed a low to moderate level of RAPD variation whose value decreases in the order of Bale, Sidamo, Arsi and Jimma. This is in agreement with the present population size which also becomes relatively smaller and smaller in the same order of regions (observation during collecting trip). There is in fact a positive correlation between population size and neutral genetic variation within populations for many tree species (Hilfiker *et. al*., 2004). Moreover, of the characteristics affecting the level of genetic variation, the size and distributions of populations received the most attention, particularly in the context of species' responses to anthropogenic disturbances (Ellstrand and Elam, 1993).

During collecting trip, we observed that Bale and Sidamo regions have relatively better population size. In Arsi and Jimma, the populations are so small that we were sometimes forced to cover a large area to get few sample trees. The smaller population size and hence the lower RAPD variation in Arsi and Jimma regions could be attributed to the large human population size who collect the tree for different purposes.

Investigations of the stomatal physiology of the leaflets have revealed that *Hagenia abyssinica* is one of those tree species that do not conserve water rather well. The stomata are relatively wide open, allowing for the escape of much water vapor per unit leaf area and per unit time (Legese, 1995). Hence, it is apparent that the plant will be more endangered if dry conditions prevail in the sampling regions.

In contrast, Bale and Sidamo regions are relatively moist and hence favour the survival of the plant. In Bale areas where we have sampled, though ineffective, some of the natural forests are protected against the impact of man to some extent. Hence, this could be another reason why there is relatively better population size and hence better genetic diversity of *Hagenia abyssinica* in Bale than in the other three regions. As mentioned above, although Bale and Sidamo have relatively better diversity, the RAPD in all regions was generally found to be low to moderate.

In conclusion, the male, female and entire population showed a lower level of RAPD variation suggesting the endangered state of this species. This genetic erosion is mainly due to habitat fragmentation, expansion of land for crop cultivation, timber cutting and over collection of the different parts of the tree. Over collection of female inflorescences for their medicinal value, for instance, has a direct impact on fruit setting and could endanger the establishment of the next generation of trees. This practice definitely contributes to the rarity and endangered state of the species.

All the above-mentioned practices ultimately lead to the reduction in the population size of the tree. This reduction of population could in turn alter the mating structure within populations by increasing the mating among relatives, thereby increasing inbreeding depression (Husband and Schemske, 1996).

Increased inbreeding in any plant population does not only result from increased self fertilization of individuals within a population, but also from mating of close relatives, i.e. sib-mating. Inbreeding depression may thus occur in small populations of almost completely outbreed species

such as *Hagenia abyssinica* as well, because many crossing events are likely to be among relatives (Young *et. al*., 1996).

Fragmentation of previously connected populations could have deleterious effects by disrupting gene flow among populations and could exacerbate effects of loss of diversity through genetic drift and further increases inbreeding in the remaining fragments (Templeton *et. al*., 1990).

The most significant point to note here is that any subdivision of the species population would lead to an increase in the proportion of homozygotes in the entire population as compared to the proportion present if mating were random. This phenomenon called-the Wahlund effect-arises when the subdivided population are subjected to different evolutionary factors and their different alleles are get fixed in their own subpopulations, thereby increasing the overall amount of homozygosity. This effect is highly pronounced when the allelic frequencies in the subpopulations deviate greatly from that found in the entire population.

The subsequent effects of genetic drift in small isolated population would lead to a loss of genetic diversity, leaving plants less able to adapt to changes in their environment and ultimately increasing the risk of extinction (Keller and Waller, 2002).

Habitat fragmentation as a reflection of Wahlund effect could also lead to founder effect in which case a consequence of recent habitat fragmentation reduces population sizes and forces some colonies to reestablish from a limited number of surviving sources. Hence, both genetic drift and founder effect that are the ultimate results of habitat fragmentation could be the reason for the low level of RAPD variation found in the present study.

Since the results of RAPD variation obtained with Lynch and Milligan (1994) corrections are nearly the same (i.e. a low to moderate level of RAPD variation whose values decreases in the order of Bale, Sidamo, Arsi and Jimma), all the above discussions of this section also hold true for the results obtained with Lynch and Milligan (1994) corrections. The only difference in the results is that the values obtained in the analysis with Lynch and Milligan (1994) correction are higher, but still showed low to moderate level of RAPD variation. The increment in these values is attributed to the removal of some loci that are exceedingly common to most individuals and hence could otherwise have contributed to a lower RAPD variation that is comparable to the result obtained with the analysis made without Lynch and Milligan (1994) correction.

6.2. Partitioning of genetic variability and cluster analysis

In all of the three cases (male population, female population, and entire population), more RAPD variation is found within regions and populations than among populations and regions. Being long-lived, predominantly out-crossed, and often wind-pollinated and dispersed, dioecious tree species have usually a large proportion of their total neutral genetic variation within populations (Hilfiker *et. al*., 2004). Hence, the genetic variation distribution in *Hagenia abyssinica* may be explained by its mating system (obligate cross fertilization) and other life history traits such as wind pollination, seed dispersal, and survival and germination rate of seedlings which in turn are affected by the environment and the intra- and inter-specific competitions prevailing among them.

The fact that a large proportion of the genetic variation was found within populations as well as within regions of sampling indicates that *kosso* populations are poorly differentiated either on

population or regional bases. This was further confirmed by the cluster analysis which revealed lack of distinctiveness among the populations. This was true for the case of male, female, and entire population.

One may assume gene flow to be the cause for the low level of genetic differentiation among the populations. This is, however, very unlikely as any of two populations of different regions are not closer enough to allow the reach of the wind-borne pollen from one population to the other. Wind usually promotes little pollen and seed dispersal at long distances (Loveless and Hamrick, 1984).

Hence, the weak geographical differentiations of the populations may be the reflection of the pronounced local (within population) differentiation or variation. It is not, therefore, surprising to find genetic differences between close populations of same region than between populations that belong to different regions and are farther apart. Such independence of genetic distance on geographical distance has been observed in crop species (Endashaw, 1983).

Although it is likely that gene flow occurs between two populations of the same region, the dendrogram still showed that there are no two populations of same region that group together. Genetic differentiation for such close populations could be due to inefficient pollen and seed dispersal resulting from undesired direction of wind blow. Hence, the direction of wind and the spatial location of the male and female trees is another important factor that might govern the genetic variation distribution in wind-pollinated dioecious plant species.

Legesse (personal communication), while collecting fruits of female kosso trees, observed that some female trees did not set fruits although their location, with respect to the location of the

male trees, is in such a way that precipitation of the light wind-borne pollen from the male flower down to the female tree is best favored. This may suggest that the direction of the wind blow may be unpredictable and could sometimes fail to favor pollination and fruit setting. This has got a bearing in conservation strategy where in this particular case the female trees need to be circled by the male trees for appropriate seed setting.

Since the key features of the results of the partitioning of genetic variation and the cluster analysis [i.e. in all of the cases (female, male and entire populations) more RAPD variation was found within populations and regions than among populations and regions, and all dendrograms showed a weak geographical differentiation of populations] obtained with Lynch and Milligan (1994) correction are the same, all the above discussions of this section can hold true for results obtained with Lynch and Milligan (1994) correction.

6.3. Comparison of the male and female genetic diversity

In the present study, in all of the cases (male, female and entire populations), the male genetic diversity was found to be greater than that of the female. There are two main approaches to an understanding of the causes of such sex-biased genetic diversity. The first approach views this biased genetic diversity as a consequence of the genetic mechanisms of sex determination. The second approach views it as a consequence of differential allocation by the different sexes to reproductive vs. vegetative reproduction (Elle, 1999; Alstrom *et. al*., 1997).

To explain the male-biased genetic diversity in *Hagenia abyssinica* based on the first approach is difficult. This is because the sex determination mechanism of this species like in many other

dioecious species is yet unknown. In dioecious species, there are many differences in the mechanism of sex determination, and only a few dioecious plants have a well-defined sex-chromosome system (Shibata *et. al*., 2000).

When we come to the second approach, the male-biased genetic diversity is explained in terms of the variation between the two sexes in the relative allocation to reproductive vs. vegetative functions (Elle, 1999).

Male and females both use resources for flowering. After flowering, female plants continue to allocate resources to seed and fruit production, where as males revert to vegetative growth. The added burden of fruit and seed production is thought to result in high female mortality especially when resources and environmental conditions are not good (Elle, 1999; Lloyd and Webb, 1977; Ortiz *et. al*., 2002; Allen and Antos, 1988; Antos and Allen, 1994).

Several authors have indicated that males allocate a higher proportion of resources to vegetative growth and suggested that this would give them higher tolerance to environmental stresses (Grant and Mitton, 1979; Hoffmann and Alliende, 1984; Allen and Antos, 1988; Antos and Allen, 1994).

Leigh and Nicorta (2003) estimated the reproductive allocation for each sex by calculating the relative biomass allocated to flowers and fruits per gram of leaf tissue, based on one branch per sample plant. They found that reproductive allocation was nine times greater in females than in males.

Corriea and Barradas (2000) found that, under stress conditions, photosynthetic capacity of females decline more than that of males due to much water loss. They suggested that female's adaptive strategies to limit water loss during periods of drought result in a restriction of photosynthetic CO_2 uptake and could give rise to the problem of effective dissipation of excessive radiation (Corriea and Barradas, 2000).

The reason why stomatal conductance is much lower in females could be due to their higher internal resistance to water flow (Corriea and Barradas, 2000).

Generally, since in Ethiopia there has been an occurrence of periodic drought and since *Hagenia abyssinica* is one of those trees that loss much water (Legesse, 1995), females of this tree are more likely to be affected than their male counterparts for the reasons mentioned above. Hence,

relative allocation by the two sexes to reproductive vs. vegetative functions could be another possible explanation for the male-biased genetic diversity found in the present study.

Here again, all the above discussions of this section holds true for the results obtained with Lynch and Milligan (1994) correction. This is because, as can be seen from Table 5.9, in all of the cases (female, male and entire populations) and in all of the regions, the male genetic diversity was found to be greater than that of the female. Once again, the values of the male and female genetic diversity in this analysis are higher than those obtained in the analysis made without Lynch and Milligan (1994) correction for the same reason given at the end of section 6.1.

6.4. Spearman's correlation

The Spearman's correlation showed that OPA-10 primer is significantly associated with the male sex. Hence, it will be useful to differentiate the male sex. Similarly its progressive association in the order of Bale, Jimma, Sidamo and Arsi (i.e. most associated with Arsi and least associated with Bale) might help to identify which plants belong to which regions. Although this primer showed significant correlation with the male sex or with the regions in the stated order, the values of spearman's correlation ($r = 0.395$ for sex; $r = 0.335$ for region) are not still larger enough to make this primer or locus as a best sex-determining marker. Moreover, no primer or locus was found to identify the female sex. The failure to get loci that are significantly associated with the female sex and that can best discriminate sex and region (with better and higher "r" value) may be attributed to the use of only a few primers.

To find a good sex-determining marker, it seems crucial to get a large amount of bands. Levels of polymorphism from few numbers of primers may not always be a good indicator for the chance of obtaining useful sex markers (Persson and Nybom, 1998). For instance, Jiang and Sink (1997) and Hormaza *et. al.* (1994) were forced to use as many as 760 and 700 primers, respectively, to identify a sex-specific locus. It might also be possible that combinations of bands used as markers.

6.5. Discriminant analysis

The loci OPA10-2, OPA10-4 and OPA10-6 were the only loci that discriminate between Bale and Arsi; between Jimma and Sidmao; and between Jimma and Arsi, respectively. This is in agreement with the Spearman's correlation analysis where OPA-10 was showed useful to discriminate regions.

Apart from its uses mentioned above, the primer OPA-10 may be especially useful for diversity analysis at the species level as DNA amplification with this primer resulted in the highest value (H_{Tot} = 0.58) of the Shannon-Weaver diversity index estimated for the entire population. This primer was also the most efficient for detecting variation within sites (H_{Pop} = 0.48). For differentiation among sites, primer OPA-07 [$(H_{Total} – H_{Pop})/ H_{Total}$ = 0.19] appear to be the most efficient.

6.6. Implications for conservation

In the aforementioned discussion, it is mentioned that the RAPD variation of *Hagenia abyssinica* is generally low. It is, therefore, apparent that conservation measures need to be taken for this species to survive. Conservation efforts should focus on maintaining large populations as well as connections among those populations. In addition, restoration of degraded sites to increase sizes of population that have been impacted by human activities could also be useful, but should be a lower priority than protecting existing sites.

Selecting populations that are divers for several measures of genetic diversity appears to be an efficient way to capture genetic diversity and to decrease apparent level of inbreeding (Neel and Ellstrand, 2003). Hence, conserving only the highly genetically diverse populations may not be enough as the populations may not reflect the ecological and geographical range of the species nor would it serve to maintain gene flow among the populations. Capturing the ecological and geographical range of a species is thought to be the most effective way to capture adaptive variation and facilitating gene flow by maintaining connections among populations increase the probability of maintaining diversity overtime. Thus, while conserving the most diverse populations is a good start, it is not a sufficient conservation on its own.

With regard to maintaining connections among the populations of *Hagenia abyssinica*, transplanting seedlings to the natural population may maximize population size and improve genetic exchanges among populations. Moreover, the persistence of small populations that are suffering the effects of increased frequencies of deleterious alleles may be enhanced by gene flow from neighboring populations, which would tend to counteract the potentially negative effects of

genetic drift (Ellstrand and Elam, 1993). Strong interpopulational connectedness is therefore good for promoting the maintenance of overall genetic diversity of *Hagenia abyssinica*. Moreover, rare alleles are less likely to disappear in a larger population of this species.

In the present study, since much of the RAPD variation was found within populations and regions of sampling, it is misleading to suggest that a germplasm sampling strategy in which many trees from a few populations may capture a large proportion of the genetic variation. However, as discussed earlier, sampling from as many regions and populations as possible, with a wide range of ecological conditions, is still recommended as this strategy is useful to capture genes for adaptive traits and rare alleles.

Identification of rich centers of genetic diversity is necessary for undertaking conservation of plant genetic resources. The overview of our results revealed that Bale and Sidamo regions are relatively rich in genetic diversity of *Hagenia abyssinica*, and hence could serve as regions of focus for conservation.

The best means of conserving *Hagenia abyssinica* is to ensure that this species continues to grow and evolve in its natural habitat, i.e. *in situ* conservation. This is because *in situ* conservation, unlike conservation in the gene bank (*ex situ* conservation), generates the establishment of new variations that provide different patterns of gene survival which in turn affects the potential for future evolution and adaptability under changing environment. That is, unlike *ex situ* conservation, *in situ* gene conservation does not freeze evolutionary processes (Endashaw, 1986).

The *in situ* conservation has, therefore, the potential for long-term preservation of this species together with the surrounding plant populations and communities, under conditions of continuing adaptation. Indeed for any plant species to survive in nature unaided by humans, it must do so within a community of interacting organisms (Frankel *et. al*., 1995). Therefore, an approach of conservation that gives this species a separate fitness value is an inappropriate approach towards the understanding of a long-term conservation strategy.

For an endangered species, like *Hagenia abyssinica*, it is usually a difficult and expensive task to reverse its present endangered situation. We would thus suggest that the *in situ* conservation of this species will require a higher level of human intervention.

Although the existing ever-increasing socio-economic demands of people do not seem to allow protection of this species in its natural habitat, some incentive mechanisms should be put in place together with education of the local people of the wise use of the tree to promote its *in situ* conservation. In this regard, the IBCR, which is mandated for germplasm collection, and other sister institutions should be strengthened for the *in situ* and *ex situ* conservation of *Hagenia abyssinica.*

Priority being given to the *in situ* conservation, due to the alarming rate of deforestation and loss of this species, the potential role of *ex situ* conservation of this species, as a complement to the *in situ* strategy, should be further considered in parallel. The *ex situ* strategy could form a back-up in case of a disaster that requires the need for restoration of the primary *in situ* population. Hence, the *ex situ* conservation to a large degree is subsidiary and complementary to the *in situ* conservation.

Most important of all, *ex situ* conservation should not be used as a reason for failing to safeguard representative samples of this species and its habitat.

7. CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

The following major conclusions are made from the present study. The conclusions hold true for both analyses [with and without Lynch and Milligan (1994) correction].

1. The level of RAPD variation is generally low to moderate when estimated both on the population and regional bases.
2. More of the genetic variation was found within population and regions of sampling than among populations and regions of sampling. This is true for the male, female and entire population.
3. The genetic diversity of male individuals is greater than that of female individuals.
4. The genetic variation of female individuals is lower than that of male individuals probably due to the added burden of fruit setting on the female that may cause mortality in females, especially in adverse environmental conditions.
5. The level of genetic differentiation of *Hagenia abyssinica* trees on regional bases seems to be low.

6. The level of RAPD variation is low probably due to reduction in population sizes resulting from various human activites.
7. OPA-10 primer would help to identify sex of the species and its various loci would help to identify which groups of trees belong to which regions.

7.2. Recommendations

The following approaches should be used in varying mixes to conserve the genetic diversity of *Hagenia abyssinica*:

1. Achieve in situ conservation by setting aside natural reserves and national parks and by ensuring that as many trees as possible can continue to survive in managed habitats, such as farms and plantation forests.
2. Establish botanic gardens that are also important centers for research and have a vital educational role as the shop windows of botany to the outside world.
3. The government should control trade in this medicinal plant and its product
4. The government should regulate the collection of this tree from the wild. The regulation should also respect ethical, legal and social interests of all those concerned, in particular those living in the area where the natural population of this species are found. Regulations can be boosted by public information campaigns, to convince traders and users of the need to conserve wild stocks.
5. Using traditional medicine is usually regarded as a sign of illiteracy among health professionals. This may impede the effort made to conserve the species as such people will give little or no attention to the plant. Hence, attempt should be made to draw the

attention of health workers towards the contribution of medicinal plants like *Hagenia abyssinica* to the modern medicine. In this regard the MOH should be responsible for approving the potential uses of such medicinal species

6. Build public awareness to enable people appreciate the value of genetic resources and their conservation through formal education and informal community participation
7. Train local healers on the appropriate strategy of medicinal plant genetic resource conservation.
8. Promote and encourage community participation and support in conservation of medicinal plants.
9. Establish *in situ* conservation sites in localities with rich genetic diversity.
10. Establish *ex situ* facilities such as cold storage, botanic gardens and field gene bank.

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Appendix 5.1. Pooled within-group correlations between discriminating variables and standardized canonical discriminant functions

Structure Matrix

		Function	
	1	2	3
TOPA17-5 [a]	.514*	-.371	.314
TOPA17-7 [a]	.494*	-.334	.025
TOPA10-1 [a]	.456*	.113	.321
TOPA07-1 [a]	-.368*	.264	.014
TOPA17-3 [a]	.329*	-.035	.026
TOPA07-2 [a]	.314*	-.118	.200
TOPA07-3 [a]	-.275*	-.262	-.085
TOPA10-4	.219	.803*	.554
TOPA17-2 [a]	.474	-.587*	-.038
TOPA10-5 [a]	-.187	.537*	.239
TOPA17-1 [a]	.118	.525*	.413
TOPA10-3 [a]	-.092	.503*	.304
TOPA17-6 [a]	.072	.456*	-.360
TOPA17-4 [a]	.131	.375*	.161
TOPA07-6 [a]	.314	.354*	-.147
TOPA07-5 [a]	-.058	.293*	.192
TOPA10-6	.032	-.440	.897*
TOPA10-2	-.496	.285	.820*
TOPA07-4 [a]	-.073	.231	.240*

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions
Variables ordered by absolute size of correlation within function.

*. Largest absolute correlation between each variable and any discriminant function

a. This variable not used in the analysis.

Appendix 5.2. Predicted new group membership of regions of *Hagenia abyssinica* populations generated from discriminant analysis

Classification Results(a)

			Predicted Group Membership				Total
		Region	Bale	Jimma	Sidamo	Arsi	
Original	Count	Bale	4	0	0	0	4
		Jimma	0	2	1	1	4
		Sidamo	0	0	4	0	4
		Arsi	0	0	1	3	4
	%	Bale	100.0	.0	.0	.0	100.0
		Jimma	.0	50.0	25.0	25.0	100.0
		Sidamo	.0	.0	100.0	.0	100.0
		Arsi	.0	.0	25.0	75.0	100.0

a 81.3% of original grouped cases correctly classified.

Appendix 5.3. Eigen values of the first three canonical discriminant functions that were used in the discriminant analysis

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	11.308[a]	89.7	89.7	.959
2	.858[a]	6.8	96.5	.679
3	.442[a]	3.5	100.0	.554

a. First 3 canonical discriminant functions were used in the analysis.

Appendix 5.4. Variables entered / removed in the discriminant analysis

Variables Entered/Removed[a,b,c,d]

Step	Entered	Min. D Squared					
		Statistic	Between Groups	Exact F			
				Statistic	df1	df2	Sig.
1	TOPA10-2	1.225	Jimma and Arsi	2.449	1	12.000	.144
2	TOPA10-4	3.546	Jimma and Arsi	3.251	2	11.000	.078
3	TOPA10-6	5.178	Jimma and Arsi	2.877	3	10.000	.089

At each step, the variable that maximizes the Mahalanobis distance between the two closest groups is entered.

a. Maximum number of steps is 38.

b. Minimum partial F to enter is 3.84.

c. Maximum partial F to remove is 2.71.

d. F level, tolerance, or VIN insufficient for further computation.