Genetic Variation in Some Natural Populations of *Prunus africana* (Hook.F) Kalkman (Rosaceae) from Ethiopia as Revealed by Random Amplified Polymorphic DNA (RAPD)

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Abstract

Prunus africana (Hook.f.) Kalkman (Rosaceae) is a multipurpose Afromontane tree species which is threatened by over-exploitation and habitat destruction in a number of African countries, including Ethiopia. As a result, there is a growing concern of conservation and sustainable utilization of the species. Understanding of the patterns of variation within and among populations of the species is a prerequisite for devising optimum management strategies for its conservation and sustainable utilization. Here, random amplified polymorphic DNA (RAPD) analysis was used to assess the extent of genetic variation and the partition of within and among six populations of *P. africana* sampled from six different geographical regions of Ethiopia. From the total number of amplified loci, 82.3% revealed to be polymorphic for the whole data set. The within population diversity estimated by Nei's gene diversity estimates ranged from 0.307 for Tepi to 0.150 to Bulki, with a mean of 0.234. Genetic differentiation between populations was estimated with Nei's G_{ST} (0.262) and AMOVA based F_{ST} (0.257; $P<0.00001$), which appears to be slightly higher than the values obtained from various RAPD based studies on outcrossing and long-lived species. Both G_{ST} and AMOVA derived F_{ST} revealed higher proportion of variation within populations (74%) relative to the amount of variation among populations (26%). Nei's genetic distance estimated between pair of populations ranged from 0.061 (between Agere Mariam and Lepisi) to 0.213 (between Chilimo and Agere Mariam), with the mean of 0.147 among the six populations. Genetic relationships among the populations examined by UPGMA cluster analysis based on Nei's genetic distance separated the six populations into two primary clusters which some what reflects their geographical locations: one composed of Agere Mariam, Lepisi, Bulki and Tepi, and the other composed of Bedele and Chilimo. The Bulki and Tepi populations were further separated from the Agere Mariam and Lepisi populations within the first cluster. The results of the present study suggest that the Tepi, Lepisi and Chilimo populations have higher genetic variability and need to be given priority for both in situ and ex situ conservation. Those of Agere Mariam, Bedele and Bulki populations should also be conserved as they may have some unique genes.

Key words: *Prunus africana,* RAPD, population, conservation, Ethiopia.

Abbreviations and units

AMOVA	analysis of molecular variance
G_{ST}	between populations genetic diversity relative to the overall species genetic diversity
F_{ST}	AMOVA derived between population variation relative to the total variation
H_S	overall within population genetic diversity
H_T	overall species genetic diversity
H_W	mean within population genetic diversity
m.a.s.l.	meter above sea level
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
UPGMA	unweighted pair group methods with arithmetic average

1. Introduction

Prunus africana (Hook. F.) Kalkman (Rosaceae) (syn. *Pygeum africanum*), is a montane forest tree species that naturally occurs in most of tropical African countries; from Ethiopia in the North, down to South Africa, as far West as Nigeria, as well as on outlying islands as Madagascar (Cunningham and Mbenkum, 1993). It is a multipurpose, ever green hardwood tree species that may grow to a height of more than 40 meters and a diameter of above 1 meter, with straight trunk.

The species is commercially important for its bark, which is in high demand in the treatment of benign prostatic hyperplasia, and for its wood, which is a good source of timber (Bombardelli and Morazzoni, 1997). This has led to serious destruction of the species throughout Africa, leading to concerns on the long term sustainability of harvesting and conservation of this and associated Afromontane species (Cunningham *et al*., 1998). As a result, *P. africana* is listed as endangered under appendix II of the Convention on International Trade in Endangered Species of wild fauna and flora (CITES) (Simpoha, 1986). In addition, the FAO Panel of Experts on Forest Gene Resources lists *P. africana* as one of 18 top priority species for action in Africa (FAO, 1997).

In Ethiopia, the natural populations of this species are threatened by habitat destruction, excessive logging and illegal tree felling that has led to a widespread deforestation (Gure, *et al.,* 2005). *P. africana* is now found as a remnant of isolated and scattered populations (Negash, 2002), which are still under sever pressure of human impacts. Clearly, the development of

management strategies for the conservation of the remaining wild populations of this valuable species is urgently needed.

At the forefront of management decisions concerning endangered species is the necessity to understand the biology of the species and the factors acting against its natural survival. Population genetics provides critical guidance for interpreting the present status and future prognosis of threatened species as it allows to study relevant parameters such as extent and distribution of genetic diversity available within the species (Ellstrand and Elam, 1993).

Investigation of the level and apportionment of genetic diversity of endangered species has been identified as one of the main priorities for conservation biology (Holsinger and Gottlieb, 1991; Ellstrand and Elam, 1993) and the integration of these data with those from other disciplines has permitted the taking of the better informed decisions for the appropriate management of endangered species (Avise, 1994; Avise and Hamrick, 1996).

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. For instance, highly diverse or differentiated populations could be targeted for protection while depauperate populations might be targeted for management actions to restore diversity.

Moreover, understanding the relative distribution of the total genetic variation of the species within and among populations is vital for determining the number of populations necessary to be conserved for capturing the genetic diversity of a species (Haig, 1998). For example, if the

higher portion of genetic diversity is maintained within populations, fewer populations would to be conserved to represent the range of variation within the species. On the other hand, a species with most of its variation partitioned among populations would require protection of a large proportion of the existing populations to maintain variation present in the species.

However, despite its imperil state and high call for conservation, to date no genetic diversity study has been made for Ethiopian *P. africana.* Thus, the need of undertaking genetic study aiming at assessment of available intra and interpopulations genetic variation of this endangered, valuable tree species, is unquestionable to pinpoint its genetic status that may facilitate reasoned scientific decisions on its management and conservation..

Random Amplified Polymorphic DNAs (RAPDs), generated by polymerase chain reactions (PCR) using arbitrary primers, is a method of choice for studying genetic variation for plant species (Huff *et al*., 1993; Peakall *et al*., 1995). RAPDs marker was found much better at revealing within and between species genetic variability than allozymes or RFLPs in *Populus* (*Aspens*), which showed very low level of allozymes variability (Liu and Furnier, 1993). RAPDs have been successful in discerning genetic variation within and among populations of a diverse array of tree species, for example, *Cederela odorata* L. (Gillis *et al*., 1997), *Banksia cuneata* (Maguire *et al*,. 1997), *Eucalyptus globules* (Nesbitt *et al*., 1995), *Prunus africana* (Dawson and Powell, 1999; Muchuga *et al*., 2005), and *Hagenia abyssinica* (Kumilign, 2005). On the basis of these background, this project was designed to asses the level and patterns of genetic variation within and among populations of *Prunus africana* in Ethiopia using RAPD marker.

2. Literature Review

2.1 Prunus africana

2.1.1. Description and geographical distribution

Prunus africana (Hook. F.) Kalkaman (Rosaceae), commonly known as African cherry, bitter almond, and red stinkwood, is a geographically wide spread forest tree species whose natural distribution is restricted to Afromontane forest islands of main land Africa (Angola, Cameroon, Ethiopia, Kenya, Malawi, Nigeria, Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zaire, and Zimbabwe) and in the outlying islands (Fernando Po, Grand Comore, Madagascar, Sao Tome) (Kalkman, 1965; Cunningham and Mbenkum, 1993). It is a long lived, ever green hardwood tree species that may grow to a height of more than 40 meters and a diameter of above 1 meter, with straight trunk.

Its leaves are alternate and simple, 5-15 cm long, shiny green and with finely toothed margins; the leaves have a faint smell of almonds when crushed. Flowers are bisexual, small, white and fragrant, solitary or in 3-7 cm long inflorescences. Its bark is dark brown to black with a rough blocky texture; the freshly broken bark smells of bitter almonds (Schippmann, 2001).

The species grows more commonly at altitudes between 900 to 3000 m.a.s.l. in areas with mean annual rainfall of about 2000 mm (Danida, 2003). But, Hall *et al*. (2000) reported that the mean annual rainfall at which the species occurs is ranging from 500 to 3000 mm. In Ethiopia, *P. africana* is restricted to Afromontane forests and occurred at altitudes between 1500-2300 m.a.s.l.

(Azene, 1993), although the species occurs in some areas such as Tepi and Agere Selam with an elevation of 1290 and 2700m.a.s.l., respectively (Aalbeak, 1993). The species has a high light requirement and grows best in forest gaps. It tolerates mild frost and water logging and is little drought-resistant (Danida, 2003).

Taxonomically, *P. africana* belongs to the genus *Prunus*, which includes more than 200 species that have originated mainly in the Northern hemisphere and widely represented in Europe (Hall *et al*., 2000). Botanical classification of species within this genus is sometimes controversial, partly because of the easiness of interspecific hybridization (Casas *et al.*, 1999), which creates numerous intermediate types, and fades the limits between species. *P. africana* is the only species in the genus *Prunus* that is native to Africa (Hedberg, 1989) and thus deserves a particular attention.

2.1.2 Reproductive biology

Few studies on the reproductive biology of *P. africana* have been conducted. Generally, in common with other *Prunus* species, *Prunus africana* is assumed to be predominantly outcrossing and pollinated by insects or birds (Baird *et al*., 1996; Granger, 1997; Munjuga *et al*. 2000). Flowering and fruiting in a given population may be spread over a relatively long period of time, with short period of stigma receptivity of individual flowers (Munjuga *et al*., 2000).

Seed of *P. africana* is intermediate in nature in terms of retaining its viability for long term storage, which limits *ex situ* seed storage (Sunderland and Nkefor, 1997). The best conditions for

seed storage were obtained when seed from mature (purple) fruit was harvested directly from trees and depulped immediately after collection, followed by storage, without drying, at 5° Celsius. However, even under these conditions, germination was only 35% after 12 months of storage (Dawson *et al.,* 2000). Long term seed storage of *P. africana* as a means of *ex situ* conservation is not yet properly worked out and therefore this practice is not advisable, although short-term storage across planting seasons is possible.

2.1.3 Uses of *Prunus africana*

Prunus africana is an important multiple-use species throughout its range. The species provide diverse types of uses including its use in apiculture, for timber, erosion control, shade or shelter, soil fertility improvement, and as a beautiful, dark ornamental plant (Demel, 1993; Cunningham and Mbenkum, 1993). Its timber is excellent; the wood is heavy, hard, durable, straight grained, strong, red brown, planes well, takes a high polish, but splits and twists. It is used for heavy construction work, furniture, flooring, poles and mortars and household utensils (Marshall and Jenkins, 1994).

The bark of *P. africana* is also highly valued for its purgative and medicinal property. Herbal preparations made from the bark of *Prunus africana* historically have been employed by Africans to treat chest pain, malaria, inflammation, fever and kidney disease, as well as for producing a cattle purgative (Cunningham and Mbenkum 1993). It is traded on the international market for the manufacture of products used to treat prostate gland hypertrophy (enlarged prostate gland) and the closely related but more serious condition of benign prostatic hyperplasia (BPH) (Bombardelli and Morazzoni, 1997), diseases that commonly affect older men over 50

years old (Page, 2003). The extract from the pulverised bark is incorporated into capsules and sold under various trade names, including Pygenil, produced in Italy, and Tadenan, produced in France (Schippmann, 2001).

The bark of *Prunus* and its extracts are traded on a larger scale than those of any other wild-collected African tree. The bark of a mature wild tree is worth on average $200 to $2000 to a collector (Simons *et al*., 1998). By comparison, this bark is worth $6500 to $65000 to industry. In Cameroon, bark is bought for about 60 or 70 US cents a kilo (Cunningham and Mbenkum 1993). Current international export demand for *Prunus* bark is in the order of 3000-5000 tonnes per year, that is estimated to be worth between US$150 and US$220 million (Alternative Medicine Review, 2002), and the main sources are in Cameroon, Madagascar, Equatorial Guinea, Kenya, Uganda and Tanzania. Given the increasing interests in 'natural' remedies and also that men are likely to live longer, the demand for *Prunus* can be expected to rise in the future.

2.1.4 Population Status

High demand for *P. africana* has led to over-exploitation of this species for its medicinal properties and its timber (Dawson and Rabevohitra, 1996). In addition to commercial exploitation, habitat loss for mainly agricultural expansion has resulted to a rapid decline in *P. africana* populations; threatening conservation of its genetic diversity (Cunningham and Mbenkum, 1993). As a result, the species is becoming scarce throughout Africa to a level that makes it to be listed as vulnerable in the world list of threatened trees, owing to its rapid population declines (Schippmann, 2001).

In Ethiopia, although the species has not been commercially exploited for its bark as of those regions of the species natural distribution, natural populations of this species have also been threatened seriously. *P. africana* is now found as a remnant of isolated and scattered populations (Legesse, 2002). This is primarily due to habitat loss (forest clearance for agricultural expansion and settlement) and selective- illegal cutting of the species for its timber and for the production of construction and house hold equipment (Ahmed *et al.,* 2005).

Apart from large scale deforestation in Ethiopia, which has resulted in depletion of *P. africana* and other many valuable indigenous tree species, selective removal of large, reproductively matured, *Prunus* trees in a population would reduce seed dispersal and gene flow between already isolated montane "islands", further increasing their isolation (Cunningham and Mbenkum, 1993). Selective felling of the largest trees may also results in lower production for the next generation (Connell *et al.,* 1984).

Clearly, urgent conservation efforts for maintaining the remaining wild populations of this valuable species are needed. The conservation of this species in the face of their perceived decline represents a major challenge in the halting of the current decrease of the country's biological diversity. Moreover, with existing high international demands of the species for a natural remedy of prostate disorders, the biodiversity of *Prunus africana* have promising potential for tapping attractive income for the country if sustainable harvesting coupled with establishment of commercial plantations is in place.

2.1.5 Genetic variation

The species is widely distributed in montane forest across Africa, but its populations are isolated from each other, and genetic variation can be expected to have diverged accordingly. Despite no results of genetic studies found for Ethiopian *P. africana*, it is already known that there are genetic differences between the populations in some of its main regions of occurrence. Analysis by Dawson and Powell (1999) using molecular markers (random amplified polymorphic DNA RAPD) indicated this to be indeed the case at the gene level. Analysis of 10 populations sampled from Cameroon, Ethiopia, Kenya, Madagascar and Uganda revealed that most genetic variation is among countries (66%, $P < 0.001$), indicating the importance of regional approaches for conservation. Variation among populations sampled within Cameroon and Madagascar was also highly significant.

The RAPD analysis of five populations sampled from South Africa, Uganda and Cammeroon by Barker *et al*. (1994) also indicated that these populations are genetically distinct. More importantly, the study of Muchugi *et al*. (2004) on 9 populations from Cameroon and 8 from Kenya using RAPD marker revealed significantly more variation among Kenyan populations than among Cameroonian populations. Their result indicates that different populations of the species found in different geographical localities within a country could genetically be unique, and thus need to receive different attention in the context of management. Further, the results of Muchugi *et al*. (2004) indicates that the extent of genetic exchange and hence the patterns of genetic variation that would be maintained within and among populations of the species may vary from one geographical area to another.

2.2 Genetic diversity and its relevance in forest tree populations

2.2.1 Patterns and Levels of genetic variation in forest tree populations

The most important application of genetic markers in population genetics concerns the study of genetic structure and genetic diversity. Analysis of the current genetic structure of forest tree species gives insights into an ongoing process of genetic differentiation among populations. However, the level of genetic variation and its pattern in plant populations at any one given time is the product of interacting natural and historical factors. Selective forces, size and interplay of populations in their glacial refugia, and post glacial migration routes are important natural factors that have shaped and still modify the genetic structure of species in their distribution range (Geburek, 1997; Haig, 1998; Newton *et* al., 1999). Shifting selection, genetic drift and mutations constantly destabilize potential genetic equilibria. Furthermore, life-history traits, such as generation length, geographical distribution, pollination mechanism, mating pattern and seed and pollen dispersal are other important determinant factors affecting the amount and distribution of genetic variation within and among populations of forest tree species (Loveless and Hamarick, 1984; Ranker, 1992; Carthew, 1993; Hamarick *et al.,* 1992; Philipp *et al.,* 1992). Besides these natural factors, a direct human impact has partly altered natural genetic differentiation in certain forest tree species (Ledig, 1993).

The studies accumulated during the 1980s and 1990s have contributed a great deal to our understanding of various population processes. In general, the long-lived, outcrossing wind

pollinated forest trees were found to harbor much more genetic variation than annual, selfing and insect pollinated plants. Partitioning of the genetic variability often reveals that more than 90% of the total genetic variation resides within populations and less than 10% is due to differentiation among tree populations (Hamarick *et al.,* 1992; Wang and Shimidt, 2001). However, this data is obtained and mostly refer for temperate forest tree species.

Although much of the woody plant diversity resides within the tropics (Gentry, 1990), relatively little is known concerning the genetics of tropical species (Ward *et al.,* 2005). To date, genetic diversity in tropical forest trees has only been investigated for a limited number of tree species (e.g. Loveless, 1992, 2002; Dawson and Powell, 1999; Lowe *et al.*, 2002; Caron *et al.*, 2004; Ward *et al.,* 2005). Obviously, previous population genetics and evolutionary studies of forest tree species have a distinct north temperate bias.

It seems that some of the species in the tropics are currently being considered. However, this still needs improvement. Because, due to differences in density, dispersion or the breeding systems of temperate and tropical tree populations, it may not be possible to use our knowledge of temperate forest tree species to predict the levels and distribution of genetic variation in tropical trees. Of special importance is the very different population structure of most tropical trees.

Tropical forest trees typically occur in assemblages of very high density, with the result that the great majority of species occur at low density (Ward *et al.,* 2005). Of critical interest in this regard is that the maintenance of genetic diversity over time and the genetic differentiation of populations in space. Few allozyme studies have indicated that tropical tree species have high

levels of outcrossing and genetic differentiation increases among individuals and populations with increasing geographical distances (Hall *et al*., 1994; Murawski *et al*., 1994). In addition, gene flow among neighborhoods has been found to be important in the maintenance of genetic variation within metapopulations in the neotropical tree, *Cordia alliodora* (Chase *et al*., 1995; Boshier *et al*., 1995b).

Sexual systems have been demonstrated to have an effect on spatial heterogeneity and partitioning of genetic variation among populations of tropical tree species (Murawski *et al*., 1994). Of all tropical trees, it has been estimated that about 23% are dioecious and most species are pollinated by insect pollinators and there is a high degree of self-incompatibility in many species (Bawa *et al*., 1985).

The very few allozyme marker based researches available indicate that the average genetic diversity found in tropical tree populations (0.125) is somewhat higher than the average found in short-lived plant species (0.113) (Hamrick, 1992). Tropical tree species, however, have somewhat less the average genetic diversity than temperate tree species (0.158). On the other hand, tropical tree species tend to have more genetic diversity among their populations than their temperate counterparts (Fst = 0.119 and 0.079, respectively). This is probably due to the high number of wind-pollinated conifers in the temperate data. Wind-pollinated species probably experience high levels of gene flow that would reduce genetic differentiation among populations and increases gene diversity within populations (Loveless and Hamrick, 1984; Hamrick and Godt, 1989).

In the most detailed genetic study of tropical forest tree species, Hamrick and Murawski (1991) studied 29 species of forest trees, among which 16 were classified as common species (densities >4 individuals per hectare) and 13 species as uncommon (densities 0.5 individuals per hectare), that occur on Barro Colorado Island, Republic of Panama. Their result indicates that the common species had significantly higher levels of genetic diversity than the uncommon species. The authors concluded as the low effective population sizes of uncommon species allow the loss of genetic diversity due to genetic drift.

2.2.2. Relevance of Genetic Diversity

Genetic diversity refers to the variation of genes within species, i.e., the heritable variation within and between populations of organisms (Brown, 1983). It is the foundation for survival, adaptation and evolution of any species, because the higher genetic diversity, the higher evolutionary potential exists for the population or species if the environment changes (Barett and Kohn, 1991; Ellstrand and Elam, 1993). Genetic diversity helps to overcome stresses imposed by pests, parasites, pathogens and environment on crop varieties that range from a reduced productivity to extinction in evolutionary perspective (Endashaw, 1985). Booth and Grime (2003) demonstrated in experimental communities with different mixture of cloned genotypes how species diversity declined with declining genetic variation, although they were not able to conclude which mechanisms were operating behind the decline. Low genetic variation can cause a reduction in fitness (Reed and Frankham, 1994), for example by reproductive output (Schmid and Jensen, 2000), but also by increasing susceptibility to pathogens (Altizer *et al*., 2003).

Ultimately, the genetic diversity of trees forms the basis of forestry and related forest industries. Diverse gene pools are the foundation of an effective tree improvement program, since it does not only provide the necessary building blocks for further tree improvement, but also are essential if high levels of productivity are to be sustained (Mauna, 1990). For example, most breeding programs that are aimed at improving wood quality and growth traits of *Eucalyptus globules* and established both in Australia and overseas are currently at the stage of exploiting collections made from wild populations in Australia (Jordan *et al*., 1993). The existence of diverse gene pools can also support future efforts of reforestation program by providing genetically diverse planting material that may have better potential of adapting and surviving their planting environment.

Genetic diversity is an important parameter in conservation effort as it is essential for the long term survival of particularly endangered species (Frankel *et al*., 1995). Long-term sustainability is affected by the ability of the populations to adapt to changing environments. Ellstrand and Elam (1993) recapitulated the theoretical relationship between genetic diversity, adaptation, demography, and population extinction. Under normal circumstances, a temporary decrease in population fitness due to a change in the environment is corrected via the effect of selection acting on genetic diversity. Each population is able to track the environment as it changes. How ever, under the circumstances of low genetic diversity where insufficient alleles are available for selection, adaptation will be slowed and is insufficient. Instead, the population size will be reduced due to poor fitness, which reduces genetic diversity and, in turn, reduce fitness. The separate process of fitness loss, population loss, and demographic effects interact with each other until the population finally crashes.

2.3 Genetic erosion

Currently there is a great concern over the loss of genetic diversity in plant species (Harlan, 1992). Loss of genetic diversity in species reduces the potential for adaptation to new environmental conditions and the potential for selection and breeding for new objectives. Such a loss can be due to a variety of causes and might take place unnoticed even in common species that appear to be in no danger (Ledig, 1993).

Forest tree species becomes extinct or endangered for a number of reasons, but the primary cause is the destruction of habitat by human activities (Ledig, 1986). Habitat fragmentation, the isolation and division of habitats into smaller areas, has caused plant and animal species in the remaining islands of habitat to lose contact with other populations of their own kind. This reduces their genetic diversity and makes them less adaptable to environmental or climatic change. These small populations therefore become highly vulnerable to extinction (Ledig, 1986; Koski, 1996).

This concern is particularly valid in tropical countries, where more than 15 million hectares of tropical forest are lost each year (Rao, 2004). Same to this, in Ethiopia, the natural high forests that were estimated to have once covered nearly 90% of the country's highlands (Eshetu, 1996) has disappeared at alarming rate, and, according to Lisanework and Mesfin (1989), it is only about 2.8 million hectares of forest (excluding open Acacia and the woodland Savannah) that is extremely fragmented remain. However, there seems because of different classification used by different authors, there is no consistency in the available information on forest resources. For

example, according to the estimate made by the State Forest Conservation and Development Department (SFCDD) in 1990, the natural high forest covers 3.5 million ha in Ethiopia.

Deforestation, developmental activities such as hydroelectric projects, road construction, urbanization and changes in agricultural practices, and finally modern agriculture and introduction of new and uniform varieties are the principal reasons that led to the loss of forest trees and other forms of plant genetic resources (Plant Genetic Resources Center, 1996; Rao, 2004). Over-grazing and changes in land-use pattern are also taking heavy toll on diversity available in the wild species.

P. africana is one of those species which is becoming threatened by extinction due to long-term human occupations of the highlands of Ethiopia, accompanied by sedentary agriculture and cattle herding activities, and excessive logging and illegal tree felling on the montane rain forests of Ethiopia (Plant Genetic Resources Center, 1996; Gure *et al.,* 2005). The species is now found as a remnant of isolated and scattered populations (Negash, 2004). The tendency once a forest has been destroyed is to give up on the remaining fragments. This is, however, a mistake, for these fragments, if maintained and preserved, can serve as reservoirs of genetic diversity.

Fragmentation of once continuous forest, will not in itself lead to the overall loss of genetic diversity. Since outcrossing trees have most of their genetic variation within populations, moderate sized fragments containing 15-20 individuals would be expected to contain most of the genetic diversity of the species (Hamrick, 1992; Wang and Shimidt, 2001). However, fragments of this size would also be expected to experience genetic drift and the genetic diversity in each

fragment would gradually decrease. Individual fragments become increasingly monomorphic, the almost inevitable destruction of fragments would lead to the overall loss of genetic diversity of the species. Until necessary research is done one will not know whether fragments represent small isolated stands that are slowly loosing the genetic diversity necessary for the long-term survival of the species, or a fragmented forest represents a net work of genetically interconnected patches in which genetic diversity can be easily maintained (Hamrick, 1992).

2.4 Genetic Conservation

The purpose of conserving forest genetic resources is to maintain hereditary variation in species and local populations far into the future so that their viability and adaptability would be sufficient to cope with changing environmental conditions (Mari *et al.,* 2004). Thus, maintaining adequate levels of genetic diversity within and among populations of forest tree species is one critical aspect to consider, yet in some cases it may be futile to be concerned with long-term goals when habitat is being destroyed at a rate such that even short-tern population survival cannot be assured (Haig, 1998).

The principal aim is to make sure that forest stands have ample genetic diversity and that this diversity is transferred to the next generation, thereby representing the basis for evolution. The two main components of the strategy for conserving forest genetic resources are conservation of these genetic resources in the original environment (*in situ*) and conservation in special collections established outside the original environment (*ex situ*). *In situ* conservation normally requires that a representative area of natural forest or a naturally regenerated commercial forest is

set aside as a gene reserve forest. Whereas, *ex situ* conservation is implemented by establishing collections of individual trees in an orchard or by storing seeds, pollen or tissue (FAO *et al*., 2004).

Outcrossing forest tree species with wide and continuous distribution area usually contain abundant genetic diversity within each stand. Their mode of pollen and seed dispersal allow transfer of genes from one stand to another over long distances, and therefore single stands contain a large part of the entire genetic diversity of the species. For these particular types of forest tree species, establishing gene-reserve forests (*in-situ* conservation) is the principal method of genetic conservation (Mari *et al.,* 2004).

In-situ conservation involves maintaining genetic resources in the natural habitats where they occur, allowing evolution to continue and increasing genetic diversity to be conserved (Endashaw, 1986). In terms of adaptation, each gene-reserve forest represents natural evolution of the site. Gene-reserve forests are naturally regenerated and managed following best forest management practices. It is essential for conservation that as many trees as possible produce seed, resulting in a new generation that is genetically diverse and able to adapt to changes in the environment (FAO *et al*., 2004).

Ex situ conservation is appropriate when the tree species is rare and grows only as small patches, when the site is threatened or when regeneration is uncertain. With these species, gene migration from one stand to another as seed or pollen is limited. The genetic diversity of these tree species is conserved by collecting material from several stands into specific conservation collections,

which are intensively managed. These collections do not contain selected types representing particular characteristics, but they represent random samples of the genetic diversity of the species in its distribution area. It is expected that this artificially established stand in the long run produce genetically more diverse seed than would be produced in the small natural stands (Mari *et al.,* 2004).

Ex situ conservation of forest tree genetic resources is mainly concerned with sampling and maintaining as much of the genetic variation as possible that resides within and among populations of selected target species. *Ex situ* conservation requires substantial levels of human intervention, in the form either of simple seed collections, storage and field plantings or of more intensive plant breeding and improvement approaches. Unlike breeders of agricultural crops, forest tree breeders cannot rapidly produce new varieties, nor can they quickly breed for new variations among populations. Therefore, the existing genetic diversity among populations is important and fundamental to the conservation of forest genetic resources, particularly as it may relate to maintaining genetic diversity in viable populations in the long term. This also suggests that special attention must be given to conserving intraspecific genetic variation in peripheral or isolated populations, as they could possess higher levels of characteristics such as drought resistance, tolerance to various soil conditions (Sterne and Roche, 1974), or features that will help to protect them from future climate change (Muller-Starck and Schubert, 2001).

In Ethiopia, the Forest Genetic Resources Conservation and Utilization Research Program of the Forestry Research Center (FRC) once launched an effort to establish *in-situ* conservation stands of *P. africana* in South Western parts of the country. Although this program received about 20

hectare of natural forest in which the species found at Dega Woreda of Illuababor zone, from the local administration, the program failed to undertake further activities, even to guard the site from local encroachment and grazing. Furthermore, no prior evaluation was done for choosing among the available stands for *in-situ* conservation of the species.

Choice of sites for *in-situ* conservation may be based on two types of data, (1) high diversity estimates based on markers, or (2) knowledge of adaptive traits linked to certain ecological conditions. Under appropriate ecological and environmental condition, *in situ* population of the species may not only maintain a large level of variation but a high frequency of desirable genes (Demissie, 1996).

2.5 Genetic Markers

The past 20 years have seen rapid advances in the technologies available for assessing genetic diversity and differentiation in natural, managed and breeding populations. Traditionally, the genetic variation of forest trees is studied following the approach of progeny tests and provenance trials (Wang and Schimidt, 2001). These studies are established in different environments and focus on quantitative and/or physiological traits of economic values and biological importance such as survival, volume growth, tolerance to environmental stress, wood characteristics and resistance to pests and pathogens.

Most of these traits are polygenic in nature and their expression is influenced by the environment. Traditional studies relies naturally on quantitative genetic methods for assessing the amount of variation and its apportionment among the various classes of effects on phenotypic, genetic,

environment, and genotype x environment interaction (Mitchell-Olds and Rutledge, 1986). These methods are expensive, laborious and time-consuming, but necessary for obtaining information needed for breeding and testing. However, it is not certain that they can provide accurate assessment of genetic variation and its apportionment among and within populations (Wendel and Weeden, 1989).

The other, relatively recent, approach involves the use of proteins, such as allozymes, electrophoresis techniques. This approach is among the most cost effective methods of investigating genetic variability in natural populations at molecular level (Hillis *et al*., 1999). Protein electrophoresis technique has been used for population genetic study of some tree species (e.g. Dulloo *et al*., 1997; Harris *et al*., 1997). However, this techniques has several limitations, including the weakness of the method to randomly sample the genome, limits to the number of loci resolved, alleles per locus, and individuals required for population studies (Parker *et al*., 1998)

The most recent and preferred approach is, however, the use of DNA based markers. Highly variable regions of DNA sometimes provide a unique "fingerprint" for each individual, and access to such fine scale genetic variation is one of the most compelling reasons for choosing to work with DNA markers (Parker *et al*., 1998). Various types of DNA based markers, such as Restriction Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Inter Simple Sequence Repeats (ISSRs), Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), have been developed to date. Depending on the level of genetic variation in the population these markers

can provide enough polymorphic loci to investigate genetic questions within and among populations. Table 2.1. shows comparison of some of the commonly used molecular markers in plant population genetics studies.

Table 2. 1. Comparison of commonly used molecular markers: Source: Young *et al*., 2000

features	**Isozyme**	**RFLP**	**SSR**	**RAPD**	**AFLP**
Theoretical no. of loci	30-50	Unlimited	10 000	Unlimited	Unlimited
Degree of Polymorphism	30-50	100s	10s	1000s	1000s
Dominance	Codominant	Codominant	Codominant	Dominant	Codominant
Null alleles	Rare	Extremely rare	Occasional	Presence/absence	Presence/absence
Reliability (Reproducibility)	Easy	Very high	High	Low to moderate	Medium to high
Required amounts of samples (per assay)	Milligrams of tissue	2-10 mg DNA per lane	25-50 ngDNA	5-10 ng DNA	25ng DNA
Ease of assay	Easy	Difficult	Easy to moderately difficult	Easy	moderately difficult
Multiplexing (loci per assay)	Gel slices (6)	1-3	1-9	5-20	20-100
Cost of development	Cheap	Expensive	Very Expensive	Moderate	Moderate
Cost of assay	Cheap	Expensive	Moderate to Expensive	Moderate	Moderate

2.5.1 Random Amplified Polymorphic DNA (RAPD)

The RAPD marker technique is a modification of PCR in which a single, short primer of arbitrary sequences (usually 10 bases long) is used to amplify at random anonymous genomic sequences (Williams *et al*., 1990). The amplification of anonymous polymorphic DNA fragments is based on PCR methodology, with the distinction that usually one primer is used in RAPD, and to allow binding of this short primer, a constant low annealing temperature (usually 34-37^{o}C) is used in RAPD reactions. However, the simultaneous use of two different primers is also possible and can yield additional information (Reddy and Soliman, 1997). Since the nucleotide orders within the 10mer RAPD primers is arbitrary, by chance, sequences matching a given RAPD primer will occur in many places throughout a large, eukaryotic genome. Occasionally, two such matching sequences will occur within a PCR amplifiable distance (less than 3000bp) and in the

proper orientation (i.e. on opposite strands of the DNA) to allow PCR amplification. In large genomes, several loci are usually amplified with a given RAPD primer, resulting in 3 to 20 detectable bands (Young *et al*., 2000). The amplification products are typically separated on an agarose gel and detected by staining with ethidium bromide.

The RAPD marker method detects DNA polymorphisms that results in the loss or gain of amplification at a locus. Amplification can be lost owing to substitution within a primer binding site, deletion of a primer binding site or a large insertion between two primer binding sites. Conversely, amplification can be gained from substitution or insertions that create new binding sites, or deletions between two existing priming sites (bringing them within PCR amplifiable range of each other). Hence, there are typically only two possible alleles at a RAPD marker locus-that lead to amplification of a band (the 'presence' allele) and that from which a band is not produced (the 'absence' or 'null' allele) (Parker *et al*., 1998). Alternative presence alleles of different size are seldom observed. Different sized bands produced from a single RAPD primer are generally assumed to be derived from different RAPD loci.

RAPD fragments often vary in band intensity. In addition, methods of DNA detection that are more sensitive than ethidium bromide have regularly demonstrated that many bands exist that are not observed in standard protocols (Williams *et al*., 1993). Band intensity differences could result from primer mismatch or relative sequence abundance (Devos and Gale, 1992; Williams *et al*., 1993). However, Thormann *et al*. (1994) found no correlation between copy number and band intensity. The fact that fainter bands are generally less robust in RAPD experiments (Ellesworth *et al.,* 1993; Heun and Helentjaris, 1993) suggests that varying degrees of primer mismatch may account for many band intensity differences.

The RAPD process typically reveals several polymorphic genetic segments per primer within populations; other segments may appear as monomorphic bands within or across populations (Hadrys *et* al., 1992). While RAPD analysis detects size variation among the fragments but is insensitive to sequence differences among them, the degree of variability observed for many primers suggests that the technique will be useful for the evaluation of genetic diversity and relatedness within a species (Parker *et al.,* 1998).

The technical ease of RAPD markers and their application to any species has led to their use in many forest trees population studies (e.g. Isabel *et al*., 1995; Vicario *et al*., 1995; DeVerno and Mosseler, 1997; Furman *et al*., 1997; Gillis *et al*., 1997; Maguire *et al*,. 1997; Legionnet *et al*., 1997; Nesbitt *et al*., 1997; Jordano and Godon, 2000). The rapid pace of obtaining RAPD data is an important factor in their popularity (Nybom, 1994), making the acronym particularly appropriate. The broad use of RAPD markers can be attributed to several factors: (1) no prior sequence information is needed; (2) small amounts of DNA can be used; (3) the method requires a minimal amount of laboratory supplies and equipment; (4) detection is non radioactive; and (5) a large number of potential markers can be generated using readily available primers. All of these factors contribute to efficiency and speed of data gathering (Wolf and Szmidt, 2001).

For many applications of RAPD markers, the dominant behavior of RAPD fragments is a disadvantage. RAPD markers are rarely inherited as codominant alleles (Roy *et al*., 1992; Dawson *et al*., 1993). Loss of a priming site results in complete absence of the enclosed amplified fragment, not simply a shift in mobility on the gel. In heterozygotes, therefore, difference may appear only as difference in band intensity, which is not usually a reliable

phenotype for PCR analysis. As a consequence, information on a parental origin of alleles may be inaccessible for RAPD markers, as compared to codominant markers such as RFLPs (Lewis and Snow, 1992). Furthermore, because of their short length, RAPD markers may produce some artifactual amplification products, and careful control of DNA quality and amplification conditions is necessary to insure reproducible banding patterns (Reidy *et al.,* 1992; Scott *et al.,* 1993).

3. Objectives of the Study

3.1 General Objective

- To asses the present level of genetic diversity of some populations of *Prunus africana* in Ethiopia and to identify natural populations of hot spots for *in-situ* conservation of the species and utilization of its genetic diversity.

3.2 Specific Objectives

- To investigate the extent of genetic variation within populations of the species

- To characterize population differentiation by quantifying the genetic variability within and between populations of *P. africana* and to infer the relationship among these populations

- To generate information for targeting *P. africana* populations for *in situ* conservation and for *ex situ* conservation collection.

4 Materials and methods

4.1 Plant material

Six populations of *Prunus africana,* with eight individuals each, collected from six different localities of Ethiopia were used for this study (Table 4.1). Selections of the populations were made to represent geographic regions where natural populations of *P. africana* are found within the country. All samples were collected directly from the field in these localities from the end of January to the mid of February 2006.

In each population, fresh leaf samples were collected from young, actively growing branches of eight individual standing trees and placed in tubes containing silica gel to dry and preserve samples. To decrease the probability of collecting samples from closely related individuals, sample trees within each population were chosen with about 100 m distance from each other in either direction.

Table 4.2 indicates geographical distances between sampled populations based on their map distances from Atlas of Ethiopia (National Atlas of Ethiopia, 1988).

Table 4- 1. List of *P. africana* populations with their respective climatic and geographical information

Locality	Sample size	Administrative region	Altitude (m.a.s.l.)	Annual Rainfall (mm)	Latitude, Longitude	Remark
Tepi	8	South Nations	1290	1555	$6^0$59'N, $35^0$14'E	Protected natural Forest
Bedele	8	Oromia	2005	1966	$8^0$27'N, $36^0$20'E	Remnant trees in patchy, coffee planted forest and farm lands
Arsi (Lepsie)	8	Oromia	2300	1400	$7^0$20'N, $38^0$45'E	Protected Natural Forest
Chilimo	8	Oromia	2200	1300		Protected Natural Forest
Bulki	8	South Nations	2420	1887	$6^0$20'N, $36^0$50'E	Remenant trees in farm and grazing lands and home yards
Agere Mariam	8	South Nations	2000	847	$5^0$36'N, $38^0$20'E	Few trees in patchy natural forests, and in agricultural and grazing lands.

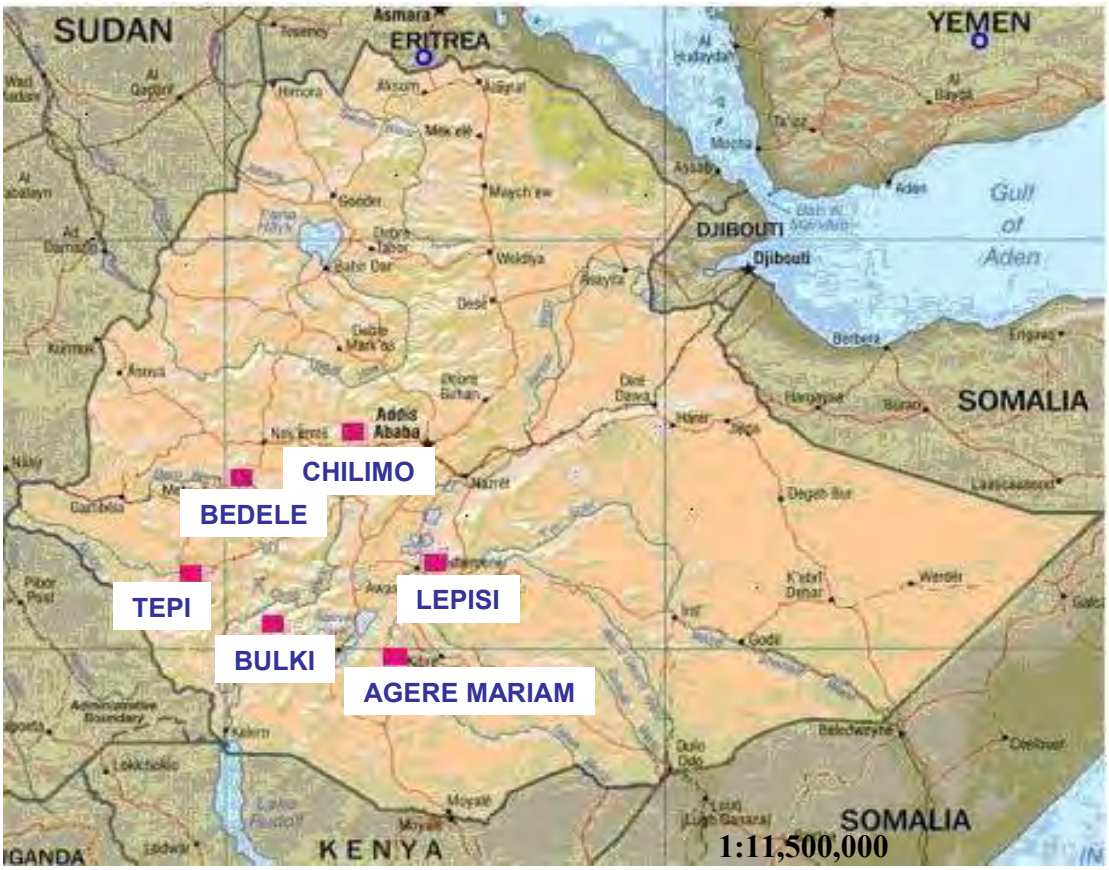

Figure 4- 1. *Map of Ethiopia showing the 6 regions from where the Prunus africana populations were sampled (Shaded red).*

Table 4- 2. Matrix of geographic distances (in km) between sampled populations obtained from their map distances in Ethiopian Atlas. Source: National Atlas of Ethiopia, (1988)-Transportation Map

Populations	Agere Mariam	Bedele	Chilimo	Lepisi	Tepi	Bulki
Agere Mariam	0.00					
Bedele	390	0.00				
Chilimo	370	180	0.00			
Lepisi	200	320	250	0.00		
Tepi	380	190	330	400	0.00	
Bulki	220	260	360	270	180	0.00

4.2 DNA Extraction

Silica-gel dried young leaves of *P. africana* were used for total genomic DNA extraction. DNA was extracted by using a modified version of CTAB method as described by Borsch *et al*., (2003). The isolation procedure consists of triple CTAB extractions to yield optimal quantities of high quality of DNA from tissues with considerable amounts of secondary compounds.

For each sample, about 100 mg of dried leaf samples were first grinded in sand (quartz) using pestle and mortar manually, and added into 1.5 µl Eppendorf tube. Then 700 µl extraction buffer (2% cetyltrimethylethylamonium bromide, 1% polyvenylpyrolidone, 100 mM Tris (pH 8), 20 mM EDTA, 1.4 mM NaCl, 0.2% β-mercaptoethanol) was added into Eppendorf tube containing the powdered leaves and incubated at 65^{o}C for 30 minutes. After centrifuging and transferring the supernatant into new Eppendorf tube, the same tissue was re-incubated twice with CTAB solution. All the three preparations were kept separate. The CTAB solutions were then extracted with addition of 600 µl chloroform twice, and the DNA was subsequently precipitated with addition of cooled isopropanol (-20^{o}C) about 2/3 of the solution volume and allowed to freeze overnight at -20^{o}C. After separately re-suspending the pellets from all extraction steps in 100 µl TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), two cleaning steps were carried out: first 50 µl of cooled 7.5 M ammonium acetate (4^{o}C) and precipitating with 300 µl of 100% ethanol (4^{o}C), and second by adding 50 µl of 3 M sodium acetate (4^{o}C) and precipitating with 300 µl of 100% ethanol (4^{o}C). The pellet from all extraction steps were then air dried, dissolved in 100 µl TE and stored at -20^{o}C.

Only one of the three extractions was used for RAPD-PCR analyses. Selection was made based on the quality (band clarity and intensity) of genomic DNA by agarose gel (NEEO, Germany) electrophoresis at concentration of 0.8%. In most cases, the third extraction was relatively pure and selected.

4.3 RAPD PCR Amplification and Electrophoresis

4.3.1 Primer Screening and RAPD PCR Amplification

A number of protocols that have been used for plant DNA amplification were tested in a T3 PCR machine (Biometra, Personal) and the one with best amplification profiles was chosen. After choosing the PCR protocol, 7 RAPD primers (series OPB (OPB15, OPB 14, OPB, 11, OPB 8) and OPC (OPC16, OPC 10 and OPC 4), Operon Technologies, Inc.) were evaluated using DNA of two randomly chosen samples with the objective of screening primers that can detect polymorphism, show clearly resolvable banding patterns and amplify larger number of loci per sample (Table 4.3). Accordingly, 3 primers (OPB15, OPC 10 and OPC 16) that fulfill the above criteria were used.

Table 4.3. List of RAPD primers that were screened along with their nucleotide sequences

Primer	Sequences 5’ to 3’
OPB-07	5’-GGTGACGCAG-3’
OPB-11	5’-GTAGACCCGT-3’
OPB-14	5’-TCCGCTCTGG-3’
OPB-15	5’-GGAGGGTGTT-3’
OPC-04	5’-CCGCATCTAC-3’
OPC-16	5’-CACACTCCAG-3’
OPC-10	5’-TGTCTGGGTG-3’

For the RAPD reaction, 2.5 µl sample DNA were used as a template in a total volume of 20µl containing 10x reaction buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% NP-40 and 0.01% TritonX 100), 3.5 mM $MgCl_2$, 10 ng/ µl primer, 0.1 mM of each dNTPs, and 0.12 µl of 1 units of *Taq* DNA polymerase from PeqLab. The DNA amplifications were performed with the following temperature profiles: 1 cycle of 3 minutes at 94^0C for initial denaturing, 45 cycles of 1 minute at 94^0C, 1 minute at 37^0C, and 2 minutes at 72^0C for denaturing, primer annealing and primer extension, respectively. The last cycle was followed by a final incubation for 10 minutes at 72^0C. The amplified product was stored at 4^0C until electrophoresis.

4.3.2 Gel Electrophoresis and Visualization

For separation of fragments, 9 µl DNA amplification products pre-mixed with 2 µl 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol) were loaded on 1.5% (w/v) agarose gels and electrophoresed in 1x TBE buffer (containing 10.8 g of Tris, 5.5 g of boric acid and 0.74 g Na-EDTA) for 1.45 hours at constant voltage of 90V. A 100-basepair ladder (*ROTH*) was loaded on marginal lanes to estimate the size of the fragments. After electrophresis, the gel was stained with ethidium bromide (10 µg/ml) on a shaker containing 400 ml of distilled water for 30 minutes and then destained in distilled water for 20 minutes. The stained DNA was visualized under UV transluminator (BioDoc Analyser^{TM}) and photographed by digital camera, connected to PC BioDoc Analyse program and saved for later data scoring.

4.4 Data Scoring and Analysis

Each RAPD band was considered as a two-allele system, with only one of the alleles being amplifiable by the PCR. Data were scored as 1 for the presence and 0 for the absence of a DNA

band for each locus across the 48 genotypes. The locus was considered as polymorphic when the frequency of present allele or null allele is less than 95% across the whole genotypes investigated.

Genetic diversity was calculated based on Nei's unbiased gene diversity (Nei, 1978), $H_j(i) = 2q(1-q)$, where q is the frequency of null allele at a locus, with modification provided by Lynch and Milligan (1994) using polymorphic loci only. Gene diversity for each population was calculated for every locus according to Lynch and Milligan (1994) as:

$H_j(i) = 2q_j(i)[1 - q_j(i)] + 2Var[q_j(i)]$, where q is the frequency of null allele at a locus for a given population and was calculated from x, which is the frequency of individuals within the population that lack the RAPD band, as $q = x^{1/2}[1 - var(x)/8x^2]^{-1}$. $Var(q)$ and $var(x)$ were calculated as $(1 - x)/4N$ and $x(1 - x)/N$, respectively, where N is number of individual per population. The mean observed gene diversity within each population was then calculated as $H_w = 1/L \sum_{i=1}^{L} Hj\,(i)$, where L is the number of polymorphic loci.

Estimates of Among population diversity (D_{ST}) and coefficient of genetic differentiation between populations (G_{ST}) was calculated as $D_{ST} = H_T - H_S$ and $G_{ST} = (H_T - H_S)/H_T$, respectively, where H_S is the mean gene diversity per population averaged across all population for each polymorphic locus and H_T is the total gene diversity calculated from the overall frequency of the amplifiable and null allele for each polymorphic locus. F_{ST} was also calculated from analysis of molecular variance (AMOVA) (Excoffier *et al.,* 1992).

POPGENE version 1.31 (Yeh and Boyel, 1997) was used for analysis of percentage of polymorphic loci for each population. The extent of population differentiation (F_{ST}) was

determined from analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) using Arlequin version 2 (Schnider *et al.,* 2000). For the estimation of genetic distances between populations, an unbiased genetic distance matrix (Nei, 1978) was generated by PopGene version 1.31. The Nei's unbiased genetic distance matrix was subjected to UPGMA (unweighted pair group methods with arithmetic average) cluster analysis using SAHN (sequential agglomerative hierarchical nested cluster analysis) with NTSYSpc program (Rohlf, 2000).

5. Results

From an initial analysis of 7 Operon technologies (OPB and OPC series) RAPD primers using two randomly selected individuals from two populations, 3 primers were found detecting multiple bands per sample. The remaining 4 primers were either produced poor or no amplification products at all. These 3 primers of arbitrary nucleotide sequence were then used in this study to amplify DNA segments from the genomic DNA of 48 *P. africana* genotypes. All the 3 primers produced multiple and clear bands that had a degree of heterogeneity across the 48 individuals representing 6 natural *P. africana* populations. In some instances, certain individuals in a population failed to amplify. Where this was the case, the absence of bands was coded as 'unknown'. The characteristics of the three primers used in this study, the total number of bands and polymorphic bands generated per primer, and the level of genetic variation and extent of population differentiation revealed by each primer are summarized in Table 5.1. Figures 5.1, 5.2 and 5.3 show the amplification products of each primer which were resolved by agarose-gel electrophoresis and visualized by staining with ethidium bromide.

The 3 primers generated a total of 34 different bands across the 48 individuals. The size of the amplified fragments ranged from ≈ 300 to 1500 base pairs (bps) across the three primers (Table 5.1). Twenty eight of the 34 bands (82.35%) were polymorphic and used subsequently to evaluate genetic diversity in the populations. Six bands, three from primer OPC 16 (~800 and 675bps), two from OPC 10 (~700 and 350bps), and 1 from OPB 15 (~750bps), were found to present in all specimens. When averaged across primers, the mean number of amplified loci and percentage of polymorphic loci per primer were 11.3 (ranging from 9 to 15), and 9.3 (ranging from 6 to 14), respectively.

A high level of marker polymorphism was found for the six Ethiopian *P. africana* populations. Only 17.7% of the bands were monomorphic across the six populations. The percentage of polymorphic loci, considering those loci with the frequency of the most common allele is ≤ 0.95, was considerably wide across populations, for a single population it ranged from 50% for a population from Bulki to 70.5% from Tepi, with a mean of 62.26% across the three primers. The number and percentage of polymorphic RAPD bands for the 6 populations is presented in Table 5.2.

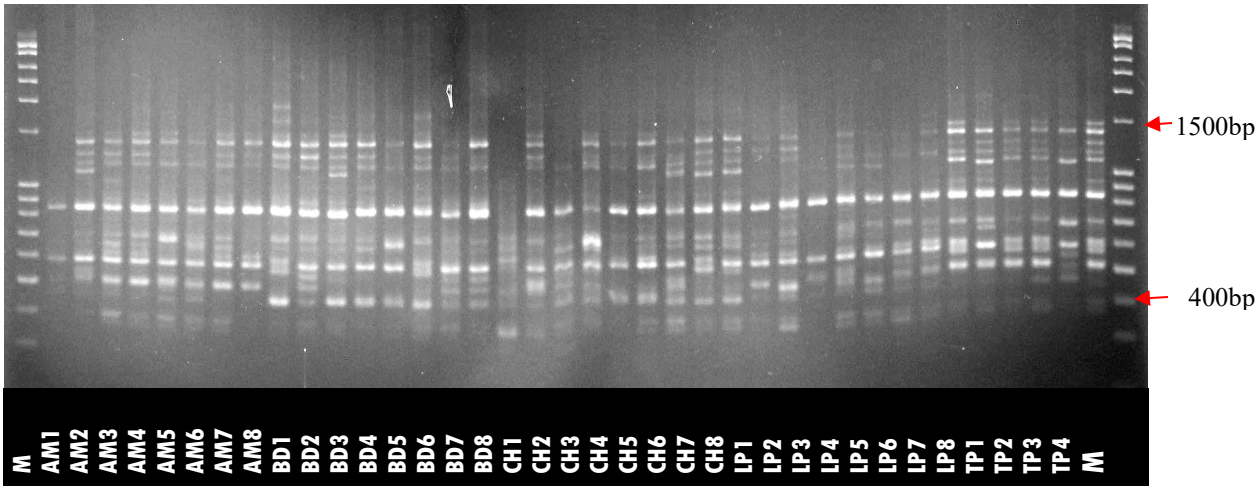

Figure 5- 1. Ethidium bromide stained gel of RAPD fragments using primer OPB 15 DNA samples from Agere Mariam (AM), Bedele (BD), Chilimo (CH), Lepisi (LP), and Tepi (TP). Outside lanes (M) show an extended 100-bp ladder.

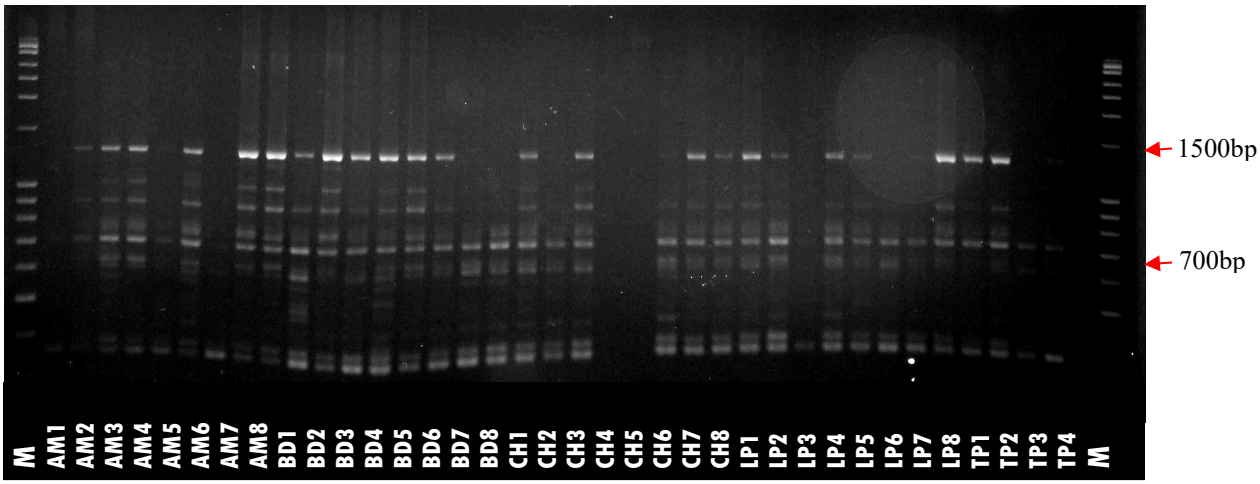

Figure 5- 2. Ethidium bromide stained gel of RAPD fragments using primer OPC 10. DNA samples from Agere Mariam (AM), Bedele (BD), Chilimo (CH), Lepisi (LP), and Tepi (TP). Outside lanes (M) show an extended 100-bp ladder.

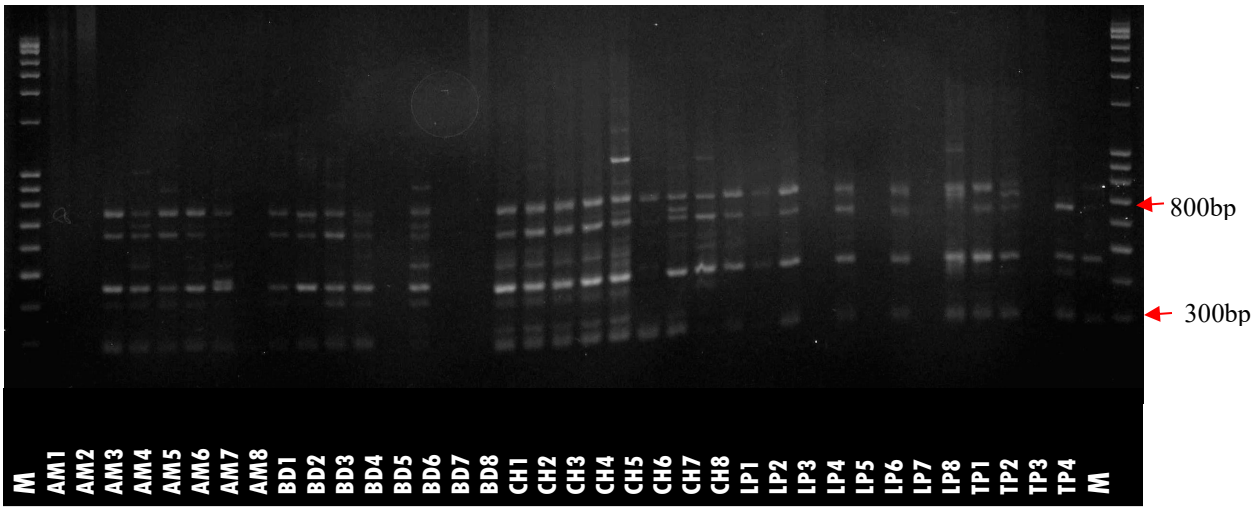

Figure 5- 3.Ethidium bromide stained gel of RAPD fragments using primer OPC 16. DNA samples from Agere Mariam (AM), Bedele (BD), Chilimo (CH), Lepisi (LP), and Tepi (TP). Outside lanes (M) show an extended 100-bp ladder.

Table 5- 1. RAPD primers used for the study, their nucleotide sequences, total number of amplified fragments and polymorphic fragments, and approximate size of fragment amplified by each primer

Primer	Base sequence	NAL[a]	NPL[b]	SRF[c]
OPC-16	5'-CACACTCCAG-3'	9	6	300-800
OPC-10	5'-TGTCTGGGTG-3'	10	8	375-1400
OPB-15	5'-GGAGGGTGTT-3'	15	14	400-1500
Total		**34**	**28**	
Range		**9-15**	**6-14**	**300-1500**
Mean		**11.3**	**9.3**	

[a]Number of amplified loci, [b]Number of polymorphic loci and [c]Size range of amplified fragments. [*]Mean obtained by averaging per locus values across all loci.

Table 5- 2. Number and percentage of polymorphic bands obtained for the six populations of *P. africana*

Population	Number of polymorphic bands	***Percentage of polymorphic bands (%)***
Agere Mariam	20	58.8
Bedele	19	55.8
Chilimo	24	70.59
Lepisi	23	67.65
Tepi	24	70.59
Bulki	17	50.0
Mean	**21.17**	**62.26**

Estimation of the Nei gene diversity that was calculated for each populations by using Nei unbiased (Nei, 1978) gene diversity with modification of Lynch and Milligan (1994) as H_W, which is the average value across the whole loci, is given in Table 5.3. Pooled over the three primers, the diversity within each population ranged from 0.150 ± 0.044 for Bulki to 0.307 ± 0.037 for Tepi, with greatest genetic diversity estimates in the larger populations (Chilimo, Lepisi and Tepi) compared to the smaller populations (Agere Mariam, Bedele and Bulki). The average genetic diversity across the six population was found to be 0.234, indicating medium RAPD variation. Similarly, assuming Hardy-Weinberg equilibrium, the overall species genetic diversity calculated across the three primers using Nei unbiased (Nei, 1978) gene diversity with modification of Lynch and Milligan (1994) as $\overline{H}_T$ was found to be 0.320 (± 0.05). The overall within population genetic diversity estimated by as $\overline{H}_S$, which is the mean value obtained by averaging per locus values across all loci, was found to be 0.236 (±0.04) (Table 5.4).

Table 5-3. Mean genetic diversity (H_W) of the six populations of *P. africana* calculated from RAPD data using Nei unbiased gene diversity (Nei, 1978) with modification of Lynch and Milligan (1994).

Population	Primers			***Mean H_W* ± S.E.**
	OPC 10	OPC 16	OPB 15	
Agere Mariam	*0.225*	*0.223*	*0.133*	*0.182* ± 0.037
Bedele	*0.220*	*0.116*	*0.184*	*0.175* ± 0.041
Chilimo	*0.344*	*0.256*	*0.291*	*0.297* ± 0.038
Lepisi	*0.195*	*0.352*	*0.325*	*0.290* ± 0.040
Tepi	*0.254*	*0.368*	*0.298*	*0.307* ± 0.037
Bulki	*0.114*	*0.193*	*0.207*	*0.150* ± 0.044
Mean over all populations				*0.234*

Table 5- 4. Primer-wise Nei gene diversity estimates, partitioning of the genetic variation into within and between populations (G_{ST}), and F_{ST} obtained from AMOVA.

Primer	Nei Gene Diversity estimate				AMOVA
	H_T	H_S	D_{ST}	G_{ST}	
OPC 10	0.285	0.205	0.08	0.281	*0.40*
OPC 16	0.335	0.254	0.081	0.240	*0.163*
OPB 15	0.343	0.254	0.089	0.261	*0.220*
Mean	$\overline{H}_T$	$\overline{H}_S$	$\overline{D}_{ST}$	$\overline{\overline{G}}_{ST}$	$\overline{F}_{ST}$
	0.320*± 0.054	0.236*± 0.038	*0.084*	0.262*± 0.055	0.257*

*Mean obtained by averaging per locus values across all loci.

Partitioning of the populations genetic diversity showed that genetic diversity within populations, $\overline{H}_S$=0.236, accounted for 73.7% of the total genetic diversity (Table 5.4). Genetic diversity among population accounted for 26.2%, indicating low differentiation among the populations of sampling. This result was reflected by Nei (1978) G_{ST} = 0.262, which measures the proportion of the genetic diversity attributable to population differentiation. Additionally, the analysis of molecular variance (AMOVA) calculated using the present/absent (RAPD) data for the six populations' revealed significant genetic difference (P < 0.00001) among the six populations of *P. africana* (Table 5.5). Of the total genetic diversity, 25.7% was attributed to population differences and to 74.3% individual differences within populations.

Table 5- 5. Analysis of molecular variance (AMOVA) calculated for the six populations of *P. africana* based on only polymorphic loci.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation Index
Among populations	5	65.44	1.320 Va	25.72%	F_{ST}: 0.257
Within populations	42	148.44	3.810 Vb	74.28%	
Total	47	213.88	6.174		

Nei's genetic distance calculated between pair of populations revealed the lowest genetic distance (0.061) between Agere Mariam and Lepisi, while the highest distance (0.213) was found between Chilimo and Agere Mariam (Table 5.6). The average genetic distance was 0.147, indicating the existence of considerable level of genetic differentiation among the populations. Genetic relationships among populations were further examined by UPGMA clustering analysis and represented by a dendrogram (Fig. 5.1). The genetic distance estimate between populations was tested for its goodness of fit for cluster analysis. The co-phenetic correlation between the genetic distance matrix and its co-phenetic value matrix was found to be 0.85, which indicates that its goodness of fit for cluster analysis is good, as described in Rolf (2000).

The resulting dendrogam separates the six populations into two primary clusters which somewhat reflects their geographic locations. The first cluster includes the southern populations (Agere Mariam, Lepisi, Bulki and Tepi) with the Tepi material separated first from the three populations by a genetic distance coefficient of 0.055, and then the Bulki material separated from the Agere Mariam and Lepisi populations by 0.04 genetic distance. The second cluster includes the Western populations (Bedele and Chilimo), that appears to be separated from the first cluster with 0.03 genetic distance. The mantel test computed using the genetic distance matrix among populations and geographical distances matrix, which was calculated from their map distances, between these populations revealed the relation of these two variables with correlation coefficient of $r=0.621$:$P< 0.003$.

Table 5- 6. Nei's (1978) Unbiased Measures of Genetic distance.

Population	Agere Mariam	Bedele	Chilimo	Lepisi	Tepi	Bulki
Agere Mariam	0.000					
Bedele	0.173	0.000				
Chilimo	0.213	0.081	0.000			
Lepisi	0.061	0.148	0.210	0.000		
Tepi	0.140	0.150	0.196	0.183	0.000	
Bulki	0.096	0.125	0.204	0.100	0.123	0.000

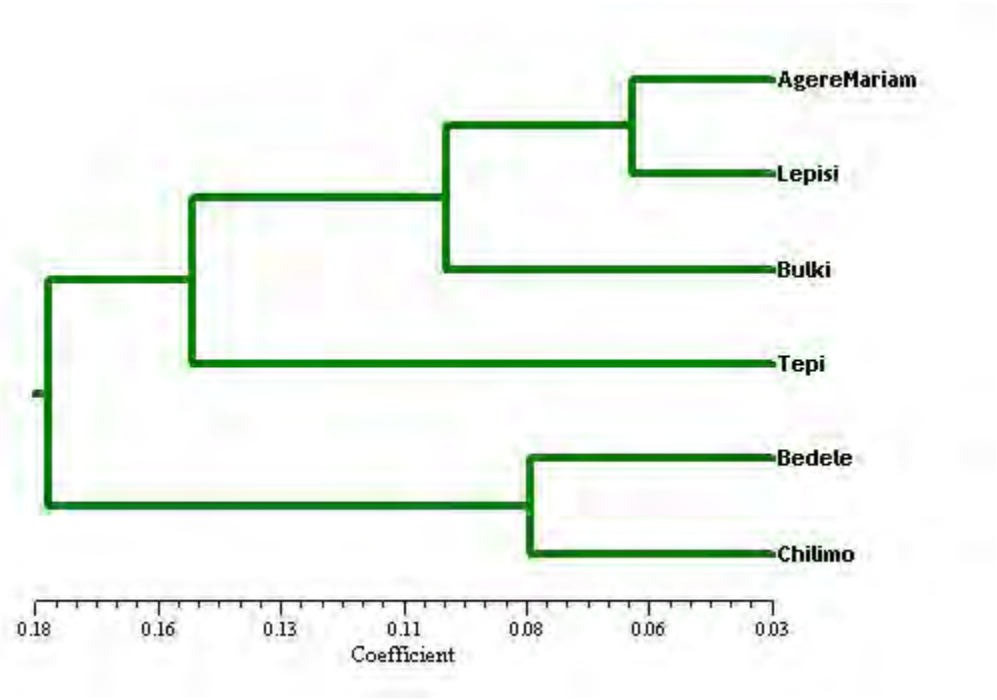

Figure 5- 4. Dendrogram based on Nei's (1978) genetic distances between the six populations of *Prunus africana*.

6. Discussion

6.1 RAPD Polymorphism

In the present study, the application of three RAPD primers to 48 individuals of *P. africana* generated a total of 34 scorable bands of which 28 (82.35%) were polymorphic loci across the whole material. Each of the three primers produced a considerable number of bands that show a degree of polymorphism both within each population and across the whole material. This result is in line with other RAPD studies on the same species sampled in different geographical regions, which generally reported substantial amount of polymorphic loci using a certain numbers of RAPD primers. For example, the study of Barker *et al*., (1994) on *P. africana* using RAPDs yielded 61 (85%) polymorphic loci using five primers. Dawson and Powell (1999) also obtained 48 clear polymorphisms from 10 primer combination on the same species employing the same markers.

The high percentage of polymorphic loci and the wide range of percentage of polymorphic loci detected among the sampled populations investigated in the present study suggest the existence of high genetic polymorphism in *P. africana,* and the powerful applicability of the RAPD technique to the assessment of genetic polymorphism, at least for this species. This is generally in agreement with Liu and Furnier (1993) who concluded that the overall utility of RAPD system measured in terms of generating polymorphic loci was very high, and that the system provides a very powerful tool for 'fingerprinting' individuals.

6.2 Genetic Diversity

For long-lived plants that have to cope with considerable temporal and spatial environmental heterogeneity genetic variability is of prime importance for species persistence. The results of previous studies indicated that, compared to annual plants, the long-lived forest trees maintain much more genetic variation (eg. Li *et al.,* 1992; Loveless, 1992; Hall *et al.,* 1996). Similarly, despite the current threatened state of the species, the level of overall within population diversity estimated by present RAPD data of *P. africana* as $\overline{H}_S$ was not as such very low (0.236 ± 0.0157).

The extent of overall within population genetic diversity observed in this study can be only loosely compared with other long-lived species RAPD studies due to differences in the number and range of individuals surveyed, number and type of primers used, and life-history traits. Nevertheless, the value observed for *P. africana* was much closer to the mean value of 37 RAPD studies (0.25) on long-lived perennials than to the mean value of 17 RAPD studies (0.2) on short-lived perennials in a review by Nybom (2004). The mean molecular variation reported in this review is probably somehow underestimated because sexually as well as clonally reproducing species were included.

Within population diversity, as revealed by partitioning of the overall genetic diversity ($\overline{H}_T$) into within and between population components and the AMOVA calculated in the present study, accounts most of the overall species diversity (~74%). This is most probably due to the outcrossing nature of the *P. africana* which tends to promote high level of variability to be maintained within populations of the species (Loveless and Hamrick, 1984).

However, the level of variation observed within each of the studied populations (H_W) was quite different in such a way that the overall species genetic variation is maintained only by some populations. Slightly more than two fold of difference in the extent of genetic diversity was found between populations with the higher (Tepi; H_W = 0.307) and lower (Bulki; H_W = 0.150) estimates. The diversity estimates recorded for each population somehow reflects the adverse consequences of population size depletion due to various human induced pressures on genetic diversity.

The value of RAPD variation estimated for each population was found to be increased in the order of Bulki, Bedele, Agere Mariam, Lepisi, Chilimo and Tepi. Interestingly, this is in accordance of the current population size of these populations which also relatively increase in the same order. Highest level of variability was detected for Tepi, which is also relatively the largest population containing numerous mature individuals compared to the rest. More or less similar level of variation to Tepi was recorded for the Chilimo and Lepisi populations, which have also comparatively large population size. On the other extreme, the smallest value was detected for the smallest population, Bulki, followed by Agere Mariam and Bedele which are also small sized populations. This might be due to the effect of small size of population that could allow the loss of genetic diversity due to genetic drift.

Population size may be critical for the maintenance of genetic variation (Frankham, 1996). In large populations, genetic drift is insignificant, but it becomes important in small populations and may be particularly pronounced after dramatic reduction in range size and fragmentation of habitats of a species (Ellstrand and Elam, 1993; Srikwan and Woodruff, 2000).

The degree of forest protection that has long been put in Lepisi population, and protection (although not a such strong) combined with the low level of human pressure (attributed to the relatively small human population size there) in Tepi and Chilimo populations, seems to help these populations to retain large population sizes. Consequently, the present analysis revealed relatively higher RAPD variation in these populations.

On the other hand, the large impact of human population on the forests in general and selective utilization of the species in particular should have contributed to the reduction in population size of Bulki, Agere Mariam and Bedele populations.

During sample collecting trip, it was observed that trees of the species are found in a relatively smaller number in these areas. In Bulki, only some trees of *P. africana* are found on agricultural and grazing lands in a very scattered manner. The local people in Agere Mariam still have almost free access in the remnant patchy forest areas and still selectively fell young and mature trees of the species for making farm and house hold implements and various constructions. The situation is more or less the same in Bedele, where the species is highly threatened and remnant trees are found in managed natural and planted forest coffee areas and in patchy natural forests adjoining Bedele and Dega. This is due to the joint effect of massive clearance of forest areas for agricultural expansion and selective failing of mature trees of the species for mainly making local timbers. These practices in general have led to the reduction in population size of trees of the species in those areas.

In small populations mating could occur between individuals that have one or more common ancestors in common. In other words, they are prone to inbreeding, which causes reduction of heterozygosity. If the population size continues to decrease, strong genetic drift will result, which subsequently would lead to a loss of genetic diversity (Ellstrand and Elam, 1993; Srikwan and Woodruff, 2000).

Hence, the pronounced effects of genetic drift owing to the smaller number of breeders that may contribute to the next generation in small sized populations could be the reason for the relatively low level of variation detected for the Bulki, Agere Mariam and Bedele populations.

The extent of diversity per population generated from the present data could be explained in terms of the maximum level of diversity to be attained for biallelic loci from eight individuals per population. Under this condition the maximum possible value for Nei's gene diversity to be attained is 0.54, and it is obtained when the allelic frequency of the null allele and presence allele is 0.494 and 0.506, respectively. However, the highest gene diversity recorded in the present case was 0.307, and the overall mean was 0.234 (43.3% of the maximum possible value). Using the same formula, Mulatu *et al*. (2006) reported an overall mean of 0.176 (10 individual per population) for Ethiopian niger (*Guizoita abyssinica*), an outcrossing annual crop.

Thus, although the level of variability detected for each populations vary extensively, the extent of genetic diversity in Ethiopian *P. africana* might be not as such very low for propagation, domestication and producing superior trees of greatest interest through breeding program, and for conservation of genetic resource of this tree crop. Moreover, the higher level of genetic

variation detected for populations from Tepi, Lepisi and Chilimo indicate the importance of due attention to these populations as these populations might have unique genetic properties.

6.3 Population Differentiation

The studied six populations exist as isolated from each other with a considerable geographical distances between which other land use systems, villages or cities, large valleys, and some times extensive tracts of forest without presence of the species found. Despite this situation of

geographical isolation, not as such high levels of population differentiation were found. The overall mean of population differentiation estimated from gene diversity as $\overline{\overline{G}}_{ST}$ in the present study indicate that differentiation among populations accounts only about a quarter of the overall species diversity. At the spatial scale examined these populations exhibits substantial genetic homogeneity, with most genetic variation (74%) found within populations. The F_{ST} value (0.257) obtained from AMOVA also revealed the same result, which is highly significant ($P<0.00001$).

Estimation of population differentiation based on $\overline{\overline{G}}_{ST}$ and F_{ST} in the present study revealed more or less similar result. This is in line with Nybom (2004) who indicated that $\overline{\overline{G}}_{ST}$ and F_{ST} obtained by AMOVA usually produce very similar estimates when applied to the same plant material using the same set of marker data.

Life history traits, such as breeding system, pollen and seed dispersal mechanisms, life cycle, population size and phenology, have been mentioned as the main determinant factors in the partitioning of genetic variation within and among populations in plants (Loveless and Hamrick, 1984). Recent analyses of among-population patterns of genetic variation have emphasized the correlates of the partition of among-population genetic variance with specifics of the breeding system (Hamrick and Godt 1997), pollination-mediated gene-flow (Sork *et al*., 1998) and seed dispersal (Hamrick *et al*., 1993; Hamrick and Godt 1997; Jordano and Godon, 2000).

The larger level of within population variability than to among-populations variability detected between the six *P. africana* populations would therefore seem as the result of a high level of genetic variability maintained by the particular life history trait of the species, which is long-

lived, outcrossing, animal-mediated mode pollen and seed dispersal, mid to late successional stage (Baired *et al.,* 1997; Munjuga *et al*., 2000) - all of which are factors associated with low genetic differentiation between populations. As previously verified with allozyme data (Hamrick and Godt, 1989), several RAPD based studies showed that long-lived, outcrossing, late successional taxa which are dispersed by animals tends to more diverse within populations, with less genetic differentiation between populations (Nybom and Bartish, 2000; Nybom, 2004).

However, the level of among-population variation revealed in the present study is relatively higher to those reported previously for *Prunus* spp. For example, at about the same scale of spatial sampling, Dawson and Powell (1999) reported the among-population component of 22% for four populations of *P. africana* in Madagascar using RAPD marker. Jordano and Godon (2000) and Mariette *et al.* (1997) also reported mean G_{ST} values of 0.19 for seven wild *P. mahaleb* populations in Spain, and 0.05 for six wild *P. avium* populations in France, respectively, based on RAPD marker.

Furthermore, the result of this study is well above when compared with figures reported for other outcrossing tree species sampled on a similar geographical scale, which generally indicates a low level of partitioning among populations (Leonardi and Mennozi, 1995; Yeh *et al.,* 1995), even in species with disjunct and restricted distribution (Nesbitt *et al*., 1995; Kumilign, 2005).

The relatively higher value of among population variation detected in the present study compared with those studies might probably be due to reproductive isolation of Ethiopian *P. africana* populations.

As indicated earlier (Table 5.1), the studied populations exist at different elevations (ranging from 1290m.a.s.l. (Tepi) to 2420m.a.s.l. (Bulki), and thus may have different flowering and fruiting periods. Because, tree populations growing at higher altitude normally flower and fruit later than populations of the same species growing at lower altitude (Shimidt, 2000). For insect-pollinated and animal dispersed species such as *P. africana*, this temporal segregation of reproductive phenophases would be an important component of reproductive isolation. First, insect-mediated pollen flow can reach potentially long distances (Stacy *et al*., 1996) whenever reproductive individuals in anthesis are coincident temporally (McCauley, 1997). For species like *Prunus*, typically showing very short anthesis periods at the individual level (Munjuga *et al*., 2000), non coincident flowering can severely limit the possibilities of gene flow among populations.

During the fruiting phenophase the situation is also similar. For example, despite extensive foraging areas of frugivorous animals, especially the larger-sized species, seed dispersal between populations can be very low if the fruiting phenologies are nonoverlapping (e.g. due to altitudinal differences) and the fruit is an important component of diet (Wheelwright, 1983). If frugivores are tracking the availability of ripe fruits, they would restrict seed movement to those populations where ripe fruits are available concurrently, especially if no other species is available (Herrera and Jordano, 1981). As two populations located at different elevation would show non overlapping flowering and fruiting periods, thus hindering the possibilities for gene flow between them. This can typically occur for populations sampled in this study that are located at different elevation ranges (Table 4.1).

In addition, factors involved in vicariance events could have an important effect in populations of long-lived tree and shrub species with the characteristic combinations of life-history and reproductive traits mentioned above, determining non uniform genetic make-up throughout a species range (Bossart and Prowell, 1998). Clumps of related seedlings could arise from seed caches, but the implications for genetic structure will depend on seedling mortality prior to reproduction. Even if fruiting periods of two populations suited at different areas show overlapping and extensive seed dispersal between populations occur, demographic bottlenecks acting during the recruitment stages that follow seed dispersal can cause disproportionately high mortality of dispersed seeds, established seedlings, and saplings (Jordano and Herrera, 1995; Schupp and Fuentes, 1995; Schupp, 1995) and, as it was reported for other *Prunus* species and many other tropical tree species (Schupp and Fuentes 1995), this can also be the case in *P. africana*. Thus, though additional evidences are required, such higher level of genetic differentiation detected in the present study may also be due to disproportional postdispersal mortality.

Moreover, as explained before while three of the six sampled populations (Tepi, Lepisi and Chilimo) in the present study have large population size, the remaining three (Bulki, Bedele and Agere Mariam) have very small size of population. Small populations promote divergence due to drift (Loveless and Hamrick, 1984; Ellstrand and Elam, 1993). The level of population differentiation revealed by both G_{ST} and F_{ST} in this study is thus a trade-off in populations of all sizes between drift and probably low gene flow effects.

In general, the present analysis revealed considerable level of population differentiation among the six populations of *P. africana* which was higher when compared to several values reported for other outcrossing tree species sampled at about the same geographic range. Either of the above mentioned factors, or a combination of them might account the present result. Additional studies could elucidate which factor(s) has (have) caused this higher level of differentiation.

6.4 Genetic Distances

The pair-wise analysis of populations using the Nei (1978) unbiased measure of genetic distance, calculated to complement the analysis based on G_{ST} and F_{ST}, also revealed considerable genetic differences between studied populations. Interestingly, although yet considerable, comparatively lower level of genetic difference was observed between geographically closely suited populations than distantly located. The dendrogram constructed based on UPGMA analysis also clearly indicates that the populations from the geographically different localities are distinct.

The mantle test carried out for testing if there is a correlation between geographical distance between populations and their average genetic distances revealed a positive correlation between these variables (r=0.621:P< 0.003), indicating the distribution of genetic variation between pairs of *P. africana* populations in Ethiopia is associated with geographical proximity. The positive correlation identified in this study between the genetic distances and geographical distances is not unusual. Few allozyme studies have indicated that tropical tree species have high levels of outcrossing and genetic differentiation increases among populations of with increasing geographical distances (Hamrick and Murawski, 1991; Hall *et al.,* 1994; Murawski *et al.,* 1994). This was also evident in RAPD-based studies for several outcrossing species (Ayres and Ryan, 1997; Brunell and Whitkus, 1997; Martin *et al*., 1999; Steven *et al*., 1999).

In reference to genetic distances and associated estimates (mantle test and UPGMA analysis) obtained in the present study and the outcrossing nature of the species, it seems that geographic closeness contribute a lot to genetic similarity between populations of neighboring regions probably due to a pollen and seed movement between them.

As indicated in the afromentioned discussion, gene flow (through pollen or seed dispersal) between populations of the species can be limited primarily if they are located on different elevations and thus show non overlapping flowering and fruiting periods. However, this factor may not be a limiting factor for most pairs of geographically closely sited populations sampled in this study. Because, the difference in elevation of most pairs of populations that appeared to be separated by relatively lower geographic and genetic distances is also very low.

For example, the genetic distance detected between the Bedele and Chilimo populations was found to be the least value than all values obtained for any combination of the two with other populations. Although the Bedele population is found relatively closer to Chilimo and Tepi populations by about the same geographical distances than other populations, its elevation (2005m.a.s.l.) is more close to that of the Chilimo (2200m.a.s.l.) than for Tepi (1292m.a.s.l.). The flowering and fruiting periods of the Bedele population can, therefore, possibly be coincident with those of the Chilimo than with Tepi because of their closer elevation range. This explanation can hold true for most of other pairs of populations.

Although the phenophase periods of two populations with closer elevations can be overlapped as discussed above, it still seems very unlikely as any of two populations are not closer enough to allow the reach of insect-pollinated pollen and/or animal dispersed seeds from one population to the other. Direct estimates pollen and seed dispersal in numerous taxa indicate that the vast majority of such movement is locally restricted (Schaal, 1980). Nevertheless, here, it may be worthwhile to consider the importance of rare, long-distance gene flow in populations cohesion. Genetic studies on some tropical tree species indicates that some insects can be very efficient vectors of pollen flow and may successfully disperse pollen over long-distance in heterogeneous habitats, but this depends on insect's physiology and the reward structure of the populations (Franki and Haber, 1983; Webb and Bawa, 1983). Further, some studies of plant paternity have suggested that long-distance gene dispersal between populations is not as infrequent as previously thought (Ellstrand *et al.,* 1989; Dow and Ashley, 1996).

Even if rare, such gene flow events may potentially have great evolutionary significance. Theoretical studies have shown that only a small amount of long-distance gene flow is needed to prevent population differentiation for neutral alleles (Slatkin and Maruyama. 1975; Saltkin, 1987). Hence, the relatively higher genetic similarity detected for geographically close populations might be due to the result of gene flow between them since the occurrence of such rare events is higher for geographically close populations with more or less closer elevation compared to other distantly spaced populations.

This explanation can give more sense if one takes the following point into consideration. Unquestionably, each of sampled population in the present study had much larger population size and wider area of coverage than the present, at least, during the periods of their parental generations. According to Lisanwork and Mesfin (1989), only a hundred years ago most of Ethiopian highlands were covered with continuous large forests. It was since then, that most of the country's continuous highland forest cover had cleared and shrinked into small and patchy forest areas. By taking into account the life span of the studied species, which can live hundreds of years; it might be possible to suggest that these neighboring populations had a much wider area of coverage and thus were at least very much closely spaced populations during, at least, the period of one or more parental generations of the present populations (sampled individuals). Therefore, observed result can be more meaningfully explained in terms of more similarity of sampled populations between which a considerable ancient gene flow had occurred than from the other populations.

6.5 Implication for Conservation

The extent of diversity per population generated from the present data for *P. africana* is generally low. This may indicate the need of conservation measures to be taken primarily for assuring future survival of the species. Because, while the relationship between low heterozygosity and fitness is complex and not yet clearly predictable (Brewer *et al.,* 1990; Haig,

1998), many studies show that reduced hetrozygosity can cause a decrease in fitness (Reed and Frankham, 1994; Schmid and Jensen, 2000; Altizer *et al*., 2003).

Conservation effort should focus on maintaining large populations in order to counteract the potential negative effects of drift and promote the maintenance of genetic diversity in these populations. Thereby, conservation effort might improve the population fitness (to cope with the short-term Challenges) in the short-term and (future environmental changes) population sustainability in the long-term. In the present study, compared to relatively larger populations, the level of RAPD variation was much lower in the smaller sized populations. This is probably due to reduction in population size resulting from various types of human pressures. Protection of such populations from further human induced pressure should be considered along with restoration of these areas to increase their population size before the situation reach the point impossible or much costly to replenish. More efficient protection of populations from human encroachment is equally important in those relatively larger and more or less protected populations. Because, in one way or another they are under still human induced pressures, such as browsing of seedlings by domestic animals and illegal failing of mature trees (as in Tepi), that may lead to threatening of their genetic variation and future regeneration.

In the context of conservation, as the result from F_{ST} indicated that populations are genetically significantly differentiated, both populations need to be considered as separate unit for conservation effort. The recommendation here is that as many populations as possible should be preserved. Conserving all of the populations may help to capture the ecological and geographic range of the species. Moreover, the remaining populations of the species are few and some of

them are already highly reduced in number of individuals. Conserving all remaining populations would reduce the risk of the species in those regions to become extinct as the result of environmental stochasticity (Soule, 1973). As populations are genetically significantly differentiated, it would also preserve a large evolutionary potential if all populations are preserved.

Conservation measures should also focus on the maintenance of genetic diversity among populations for the long-term sustainability of the species. Because populations of the species are adapted to their unique local conditions, in the long-run, continued evolution of the species depends on these unique populations. A major conservation effort is thus need to be taken protecting these evolutionary significant lineages. Removal of barriers to dispersal, for example through translocation of individuals, can allow the transfer of locally maladaptive genes into populations, thereby, leads to reducing population fitness (Haig, 1998).

In comparison of the two commonly employed conservation strategies (*ex-situ* and *in-situ*), while the existing human pressures do not seem to allow preservation of the species in its natural habitat, *in-situ* conservation practice (establishment of gene-reserve forest) is the best means of conserving the species. *In-situ* conservation, unlike *ex-situ* strategies, involves maintaining genetic resources in their natural habitats where they uniquely adapted, i.e. such reserved forests represent natural evolution of the site. Gene-reserve forest (*in-situ* gene reserves) would allow evolution to continue and increasing genetic diversity to be conserved (Endashaw, 1986).

The implementation of *in-situ* conservation strategy for *P. africana* may, however, require solving of several socio-economic problems in prior with the local people whose day to day lives directly or indirectly connected to the forest. Although some limitations may still existed, the Chilimo practice, in which the natural forest is owned and well protected by local community and they draw some benefits from the forest without destroying it, can be used as a model for other areas. In any case, *in-situ* conservation of this species, particularly in areas where trees are seriously depleted, will require higher human intervention for restoration and increasing the size of the population.

In order to complement the *in*-situ conservation strategy, *ex-situ* conservation effort through establishment of field genes banks in a near by area should also be considered in parallel. The *ex-situ* strategy could form a back-up in case of adversity that requires the need for restoration of the primary *in-situ* populations.

With limited resources that may be available for developing conservation programs, if populations are to be ranked, emphasis should be on populations with highest amount of polymorphic markers, assuming a marker fragment to represent unique genetic variation that is important for long-term evolutionary potential. One criterion used to classify a specific population as a genetic resource for conservation is its distinctness within a species. The decision to preserve a certain population as a genetic resource should be drawn from the determination of the mean genetic distance of this population to many other populations of the species (FAO *et al*., 2004).

Applying this to the analysis carried out here one would classify the following populations as important genetic resources: Chilimo 0.181, Tepi 0.158, and Lepisi 0.141. The present study also indicated that these populations are relatively rich in genetic diversity. Hence, the Tepi, Chilimo and Lepisi populations need to get focus for conservation.

Apart from high levels of marker polymorphism, when other aspects of the studied populations is considered, i.e. size of the population and regeneration status, the Tepi, Lepisi, and Chilimo populations carry larger population size (many individuals) with relatively different age structure, i.e. seedlings and juvenile, and mature individuals (as it was observed during field data collection). For *in situ* conservation, it is essential if there are as many trees as possible that produce seed, resulting in a new generation that is genetically diverse and able to adapt to changes in the environment (FAO *et al*., 2004). Furthermore, trees on these areas occur relatively within a protected natural forest. Therefore, to ensure that a maximum of the genetic variation detected in these populations will be available for future reforestation or other uses in the country and elsewhere, these populations need to be given priority for *in situ* conservation.

Ex-situ conservation effort through establishment of field genes banks should also give attention to these populations with higher variability. However, taking the significant genetic structuring (F_{ST}) between the studied populations into consideration, which is an indication of the presence of unique individuals with unique genes in these populations, it is recommended that *ex-situ* conservation effort of *P. africana* should consider all populations. More particularly, for Agere Mariam, Bedele and Bulki populations, which are left with very few numbers of individuals in a

very scattered manner on other land use types, implementing *ex-situ* conservation measures could be the most appropriate solution to preserve their germplasm.

7. Conclusions and Recommendations

From the results of the present study it can be noted that the level of genetic variation detected for six populations of Ethiopian Afromontane tree species, *Prunus africana* is not generally very

low when estimated per population basis. This may indicate the need to look for ways of maintaining and improving this variability. It is difficult to compare the various diversity estimates obtained in this study with other studies, since studies which have conducted to investigate these genetic parameters of the studied species in the country were lacking.

Partitioning of the total genetic diversity into its components (both by $\overline{\overline{G}}_{ST}$ and F_{ST}) revealed that much of the genetic variation (~ 74%) is found within populations. This might result from the high level of genetic variability maintained by the life history characteristics of the species. Although differentiation among these population is not as such high, it is highly significant (F_{ST} = 25.7: P<0.00001). The result implies the need of considering each population as separate entities for conservation purpose.

Analysis of genetic distance between pairs of populations and UPGMA, based on Nei's genetic distances revealed relatively lower genetic differences between geographically closely situated populations. This might probably be due to ancient gene flow (through pollen and/or seed movement) between them. The mantle test analysis confirmed the existence of a direct relationship between geographical and genetic distance of the material studied at this scale.

In summery, although preliminary, this study has shown the applicability of the RAPD method to the assessment of genetic diversity for conservation and management purposes. The ability of RAPD to 'fingerprint' populations of *Prunus africana*, and the resultant measurement of genetic diversity across these populations and individual, mean that the impact of population destruction can be assessed, and conservation priorities can be identified. The result of this study can provide,

at least for the studied stands, a genetic input to the management of Ethiopian *P. africana* for conservation purposes. The data may also have implications for evaluation of the species in provenance trials, and for the wide-range germplasm collection of the species throughout Africa planned by International Center for Research on Agroforestry (ICRAF) (Dullo *et al*., 1997).

Unfortunately this study assesses the extent and patterns of genetic variation between only six populations of *P. africana* using only three primers. The use of additional primers, as well as the investigation of other sets of populations, needs to be carried out before any conservation priority sites be established. Other techniques should also be considered to confirm the result from RAPD data.

Furthermore, demographic studies are also recommended to monitor any population number change and its impact on the magnitude of genetic variability of *P. africana* populations. The value of *P. africana* and the importance of Afromontane forest as a reservoir of biodiversity indicates that continued research on *P. africana* will be worthwhile.

8. References

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Declaration

I declare that this thesis is my original work and has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

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