Morphological and Molecular Diversity, Phylogeography and Ethnobotany of *Prunus africana* (Hook. f.) Kalkman in Ethiopia

By

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ABSTRACT

Morphological and Molecular Diversity, Phylogeography and Ethnobotany of *Prunus africana* (Hook. f.) Kalkman in Ethiopia

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This dissertation reports diversity, phylogeography and ethnobotany of *Prunus africana* (Hook. f.) Kalkman in Ethiopia. *P. africana* is an economically important, but endangered tree species of Africa. Five quantitative morphological traits were studied *in situ* on 21 natural populations of *P. africana* over its distribution range in Ethiopia, and the following mean values were found: total height (19.3m), bole height (7.4m), diameter at breast height (70.2cm), bark thickness (20.3mm), and fresh bark mass (159.6kg). ANOVA based on the five traits revealed that there was significant variation among populations ($P < 0.001$), which could be due to environmental and/or genetic or age structure differences. Pearson's correlation analysis revealed significant positive correlations among all traits (except bole height vs. bark thickness) ($P < 0.01$). Furthermore, all traits (except bark thickness) showed significant negative correlations with altitude ($P < 0.05$). Six nSSRs and five cpSSRs were used to study molecular genetic diversity and structure of the 21 *P. africana* populations. A total of 89 nSSR and 14 cpSSR alleles, and 16 chloroplast haplotypes were found. The study showed that *P. africana* in Ethiopia maintains high levels of diversity in both nSSR ($H_T = 0.725$) and cpSSRs ($h_T = 0.703$). AMOVA revealed that most (88.05%) of the nuclear genetic variation occurs within populations; whereas nearly half (47.8%) of chloroplast genetic variation occurs among populations. There was moderate nuclear ($F_{ST} = 0.122$) and high chloroplast ($G_{ST} = 0.478$) genetic differentiation among

populations ($P < 0.001$). Distance-based clustering (PCoA and UPGMA) and individual-based population assignment methods as well as comparison of observed and permuted differentiation indices revealed geographic pattern for nSSR diversity, but no geographic pattern for cpSSR diversity, which could be due to differences in the effect of genetic drift and/or the mechanism of gene flow between cpDNA and nDNA. However, Mantel test indicated significant positive correlation between geographic and genetic distances for both nSSR ($Rxy = 0.126$) and cpSSR ($Rxy = 0.107$) ($P = 0.001$). The ethnobotanic study confirmed the multipurpose character of *P. africana*, and six major use categories (medicinal, construction and carpentry, fuel/firewood, beverage preparation, apiculture, and traditional rituals) were determined for the species. Significant genetic differentiation in more than 95% of the population pairs suggests that almost all the populations deserve conservation, but as there are often limitations of resources to conserve such a large number of populations, prioritization may be needed. Thus, based on a weighted-score population prioritization matrix that integrates genetic, morphological, conservation status, and ethnobotanic criteria; Kuni, Jimma, and Assela are the top three priority populations for conservation of the species.

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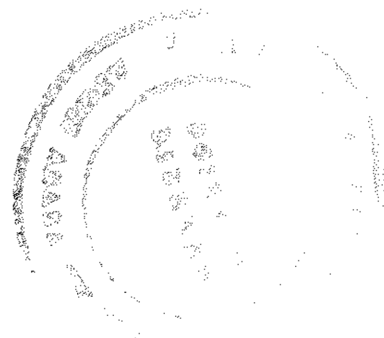

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LIST OF ACRONYMS

AMOVA = analysis of molecular variance

ANOVA = analysis of variance

a.s.l. = above sea level

b.s.l. = below sea level

CITES convention on international trade in endangered species of wild fauna and flora

cpSSR chloroplast simple sequence repeats

DBH = diameter at breast height

IUCN = international union for conservation of nature

nSSR nuclear simple sequence repeats

PCoA principal coordinate analysis

PCR = polymerase chain reaction

RAPD randomly amplified polymorphic DNA

SSR = simple sequence repeats

UPGMA un-weighted pair group methods with arithmetic average

USAID United States agency for international development

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CHAPTER 1: INTRODUCTION

1.1. Diversity and Ethiopia in brief

The high geographical and climatic diversity in Ethiopia have given rise to many and varied ecosystems. These ecosystems have species richness and high percentage of endemism. More than thirty-nine percent of the internationally recognized biodiversity hotspot area, the Eastern Afromontane Biodiversity Hotspot, is found in Ethiopia (BirdLife International, 2012).

Ethiopia contains a significant number of the world's broad ecological regions with its remarkable geological history, broad latitudinal spread (3° and 15°N) and enormous altitudinal range (from 116 m b.s.l. at Afar depression to 4620 m a.s.l. at mountaintops of Ras Dashen). The Great Rift Valley cuts diagonally across the country from northeast to south, creating a vast depression that separates the two major highland systems of the country. Much of the area of Ethiopia is dominated by highland plateaus that are interrupted by deep gorges and valleys, which are formed by large rivers and their tributaries. Geological events have produced the extreme landscape that partly affects patterns of rainfall and provides altitudinal gradients in ambient temperatures, offering a variety of ecosystems.

The variety of habitats in Ethiopia supports a rich variety of different species, which contributes to the overall biological diversity of the country. Biological diversity or biodiversity has been defined by the Convention on Biological Diversity (CBD) as "the variability among living organisms from all sources including *inter alia*, terrestrial; marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species, and of

ecosystems". A diverse biological wealth is provided by the varied ecosystems of the country: as pattern of diversity strongly associates with environmental gradients, including gradients of precipitation, temperature, seasonality, evapotranspiration, soil, and topography (Givnish, 1999; McCain, 2007). The huge biodiversity the country hosts today is also partly attributed to the proximity of the country to Asian continent (USAID, 2008).

Ethiopia is rich in biodiversity including human. There are more than 80 population groups in the country. Each group has its own indigenous knowledge accumulated through time and passed from generation to generation. Studying and understanding the indigenous knowledge of these people can have a contribution to the proper and sustainable utilization of natural resources of the country. Traditional people around the world possess unique knowledge of plant resources on which they depend for food, medicine and general utility (Martin, 1995). Particularly, multipurpose plant species have considerable contribution to the livelihood of local populations. Unfortunately, most of these multipurpose species are facing a decline of their populations due to the growing demand of their products for household consumption as well as for local, regional and international trade. Therefore, assessing the use pattern of these species is essential to develop a sustainable participatory conservation strategy for them.

1.2. Statement of the problem

Prunus africana is an economically important, but endangered tree species of Africa. Several aspects of the species such as diversity, phylogeography and ethnobotany are studied in most parts of its distribution range. However, Ethiopian populations were not fully covered in the previous studies, though the species is widely distribution in

the country. Therefore, unless such aspects of the species are thoroughly studied over its distribution range in the country, development of efficient management strategies for conservation, domestication and sustainable utilization of the species would not be possible.

1.3. Hypotheses of the study

The major hypotheses of the study are:

1. There could be genetic differentiation among different populations of *P. africana* due to limited gene flow as populations are highly fragmented and sparsely distributed in the country.

2. *Prunus africana* could have migrated from Ethiopia to the other distribution range countries of Africa.

3. Different communities in Ethiopia could use *P. africana* for different purposes as reported from other distribution range countries of the species because traditional people around the world possess unique knowledge of plant resources on which they depend for food, medicine and general utility.

1.4. Objectives of the study

1.4.1. General objective

The general objective of the research was to study diversity, phylogeography and ethnobotany of *P. africana* populations established at different altitudes and geographical locations over its distribution range in Ethiopia.

1.4.2. Specific objectives

The research was specifically aimed to:

- Assess genetic diversity within and among populations of the species using nuclear and chloroplast microsatellite markers.
- Investigate pattern of genetic diversity of the species in relation to its distribution.
- Infer the phylogeography of *P. africana* in Ethiopia.
- Verify the Ethiopian tree seed zone system for *P. africana*.
- Assess variation in quantitative morphological traits among populations of the species.
- Investigate association between morphological traits of the species and environmental factors.
- Survey indigenous knowledge on *P. africana* from different parts of Ethiopia.
- Categorize populations of *P. africana* in their order of priority for conservation.

CHAPTER 2: LITERATURE REVIEW

2.1. Assessment of genetic diversity

Genetic diversity is a level of biodiversity that refers to the variation among alleles of genes in different members of populations of a species (Steffen *et al.*, 2009). Genetic variation is reflected in differences among individuals for many characters from DNA sequences and proteins to behavioral and morphological traits. Each species that persists has a characteristic genetic diversity. The current magnitude and distribution of genetic diversity within a species depends on the effects and interactions of several evolutionary forces (mutation, selection, migration, and genetic drift) over the long evolutionary history of the species. In order to conserve and use genetic diversity, its extent and distribution should first be assessed and determined. Diversity can be evaluated at the phenotypic, genotypic as well as physiological levels. Assessment of phenotypic variation focuses on morphological traits: those characteristics that define the shape and appearance of individuals. Some of these traits can be considered as genetic if their expression in related individuals is heritable. The genetic variation among individuals at different levels can be investigated by employing a variety of genetic markers.

A genetic marker is a measurable character that can detect variation in a DNA sequence. Three types of genetic markers namely: morphological, biochemical (protein/allozyme) and molecular (DNA) have been developed to assess genetic variation among individuals.

The traditional way of determining variation within and between populations was by assessing morphological differences among individuals. Morphological measures

have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus they are available for immediate use. However, morphological determinations need to be taken by an expert in the species, they are subject to changes due to environmental factors and may vary at different developmental stages and their number is limited.

Biochemical (protein/allozyme) markers are analyzed by electrophoresis and revealed by histochemical stains specific to the proteins being assayed. Detecting polymorphisms in protein markers has the advantages of being co-dominant, technically simple, and inexpenssive. However, protein markers are also limited by being influenced by the environment and their expression changes in different developmental stages.

Molecular (DNA) markers are developed to detect polymorphisms in nuclear or organellar DNAs. As molecular markers concern the DNA molecule itself, they are considered to be objective measures of variation. They are not subjected to environmental influences; tests can be carried out at any time during developmental stages; they have the potential of existing in unlimited numbers. Many different types of molecular markers with different properties have been developed, but the most commonly used ones are RFLP, RAPD, AFLP, ISSR, SSR and SNP (Maheswaran, 2004). For instance, RAPD, AFLP, ISSR and SSR were employed to study the following Ethiopian forest tree species: *Hagenia abyssinica* (Bruce) J.F. Gmel. – RAPD (Kumlign Asmare, 2005), ISSR (Tilye Feyissa *et al.*, 2007), AFLP and SSR (Taye Bekele *et al.*, 2009); *Prunus africana* – RAPD (Hailu Atnafu, 2007); *Cordia africana* Lam. – AFLP and SSR (Abayneh Derero *et al.*, 2011); *Juniperus procera* Hochst. ex Endl. – AFLP (Demissew Sertse *et al.*, 2011).

A genetic marker is described as good marker if it is polymorphic, reproducible in any laboratory experiment, co-dominant, evenly distributed throughout the genome, discriminating, not subjected to environmental influences, selectively neutral, and inexpensive; however as no single type of molecular marker fulfills all these criteria, the nature of the question being addressed, technical demand, operational cost, manpower, facilities available should be weighed to choose the suitable marker (Maheswaran, 2004).

For this research, both nuclear and chloroplast SSR markers as well as quantitative morphological traits were used. SSR markers are used since they became increasingly popular in plant population genetics due to their hyper-variability, locus-specificity and co-dominance nature (Squirrell *et al.*, 2003). The time and cost involved in developing species-specific primers from genomic libraries or sequence databases has been cited as the major drawback of the SSR markers (Nybom, 2004). However, transfer of primers developed for a particular species to related taxa avoids the laborious and time consuming process of cloning new microsatellite (SSR) markers for a species. This approach was employed in the present study as species-specific SSR primers have not been developed for *P. africana*. With regard to the morphological quantitative traits of *P. africana* used in this study, there is no data to what extent the traits can be influenced by environmental factors. Presumably, environmental influence on the traits, at least on some, could not be low. In *Prunus avium* L... genotype by site interaction was quite high for stem height but was low for girth increment (Muranty *et al.* 1998). Hitherto, the objective of this study was not to determine heritability of the traits but to assess whether there are differences in the traits among populations over the distribution range of the species in the country.

Studying genetic diversity of species has several applications in different sectors such as conservation (e.g. conservation prioritization, regulation of threatened species, and identification of taxonomic units), agriculture (e.g. germplasm improvement), and medicine (e.g. personalized medicine). The existing genetic variation is the result of continuous changes in natural selection and adaptability to changes in the various forms of environment through both phyletic and phylogenetic evolution (Endashaw Bekele. 1986). Genetic diversity in domestic species and their wild relatives enables researchers to develop improved varieties of animals and plants for human needs. Diversity in wild species is a potential resource; species that might not have known direct economic value today may turn out to be economically important in the future.

To ensure future adaptability of species and to allow for selection and breeding, intraspecific genetic variation must remain available (FAO, FLD and IPGRI, 2004). An understanding of the patterns of variation within and among populations of trees is essential for devising optimum genetic management strategies for their conservation and sustainable utilization (Dawson and Powell, 1999). A prerequisite for the efficient use of genetic resources in any planting program is a detailed understanding of the extent and distribution of genetic variation available within the species.

Trees provide a wide range of products, including food, fodder for livestock, and medicines for both people and livestock. *P. africana* is one of such tree species that deserve diversity study for its conservation, domestication and utilization.

2.2. Importance of ethnobotanic study

Ethnobotany studies the relationship between humans and plants in all its complexity and is generally based on a detailed observation and study of the use a society makes

of plants, including all the beliefs and cultural practices associated with this use (Ghorbani *et al.*, 2006). Ethnobotanic studies show that cultural attitudes and perspectives on the use and application of biological resources within communities constitute a valuable component in conservation, domestication and improvement of plant-based products (Omonhinmin, 2012).

Ethnobotanic knowledge plays a great role in drug discovery, and thus a specifically designated field of research called Ethnopharmacology has been developed. Ghorbani *et al.* (2006) define Ethnopharmacology as "the interdisciplinary scientific exploration of biologically active agents traditionally employed or observed by man". According to Fabricant and Farnsworth (2001), most useful drugs derived from plants have been discovered by follow up of ethnomedical uses. Fabricant and Farnsworth (2001) reported that from a total of 122 compounds scientifically identified from 94 species of plants. 80% were used for the same or related ethnomedical purposes.

Ethnobotanic information can reduce the enormous effort needed to discover drugs from plants by random search. For example, according to Fabricant and Farnsworth (2001), the bioactive constituent affinin was identified from *Heliopsis longipes* (A. Gray) Blake in less than two weeks based on an ethnomedical report of the use of the plant as an analgesic (local anesthetic).

Some wild plant resources are severely threatened by habitat loss and species selective overexploitation. In the context of conservation and sustainable use of wild plant resources, ethnobotany can contribute to the scientific base for management decisions by identifying practices that are either positive or negative to the conservation of the resources. If an ethnobotanic study reveals utilization of plant resources non-sustainably, intervention actions can be taken to ensure the survival of

the species. Alternatively, if the ethnobotanic study identifies practices that have positive contribution to the conservation of the species, actions to encourage such practices can be taken.

2.3. Botanic description of *P. africana*

The following botanical description is extracted from Kalkman (1965). *P. africana* (Hook. f.) Kalkman (synonym *Pygeum africanum* Hook.f) (family Rosaceae) is a medium to large canopy tree with 30–40 m height and up to 1 m diameter. Young trees have smooth, reddish bark whereas older trees have dark, platy, resinous bark. It has the rare ability to regenerate its bark as long as the vascular cambium is not destroyed (Stewart, 2003a). Leaves are simple, alternate, oval or lance shaped, 5-15 x 2-6 cm. They are evergreen but some fall prior to fruit development. Inflorescence is 10 to 30-flowered raceme and is composed of small, white or greenish, hairy and fragrant flowers. The tree produces flowers with male and female parts. Fruits are spherical, 5 to 8 mm wide and 9 to 11 mm long, bitter, pinkish-brown, turning to dark-red or reddish-brown pulp as they get ripe. The fruit is a drupe, each with a single seed. Leaves, twigs, fruits, and bark emit a "cherry" odor when crushed, which is characteristic of the genus *Prunus*.

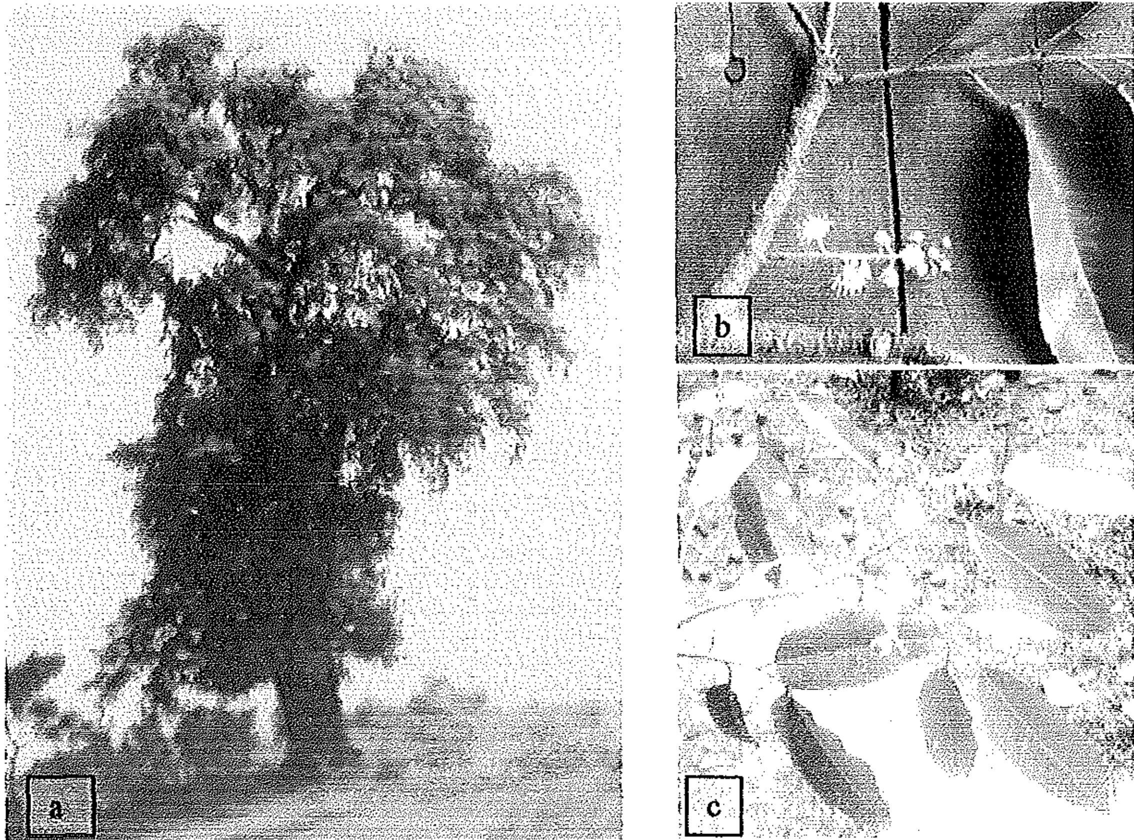

Figure 1. *Prunus africana*: (a) tree (b) raceme with flowers and (c) twig with leaves and fruits (Photos taken by Ziyin Mihretie, 2012).

Prunus africana is known by several names including African cherry, red stinkwood, African plum, and bitter almond (English). In Ethiopian languages, it is also known by the names *Tikur inchet* (Amharic), *Bouraio*, *Buraya*, *Homi* and *Mukoraja* (Oromifa), *Beru* (Gimira), *Arara* (Hadere), *Mrchiko* (Sidama) and *Garba* or *Onsa* (Wolayeta language) (Azene Bekele, 2007).

2.4. Reproductive biology of *P. africana*

Commonly the onset of flowering appears to be after 10 years, but sometimes it may occur at a lower age (Hall *et al.*, 2000). Flowering and fruiting frequency seems variable; Dowsett-Lemaire (1985) reported that individual trees of *P. africana* fruited

in alternate years in Malawi (*ca* 10°30'S). In South Africa, fruiting is also reported to be irregular (Breitenbach, 1965; Geldenhuys, 1981). The cause of the irregular flowering frequency for the species is not known. In terrestrial orchids, the irregular flowering patterns were reported to be caused by a complex of biotic and abiotic factors, which can act in both ways and it depends on the species and its habitat (Kindlmann and Balounova, 2001).

According to Hall *et al.* (2000), three zones have been identified in relation to reproductive seasonality of *P. africana*; 'year-round' equatorial zone (within 5° of the equator), 'northern' seasonal zone (north of latitude 5°N), and 'southern' seasonal zone (south of latitude 5°S). In the equatorial zone, there is no strong flowering seasonality; some individuals are flowering almost every month. With few exceptions, north of 5°N the flowering season corresponds to the November-January period. South of 5°S, flowering tends to coincide with cool and dry conditions from April to October. Fruits develop within 4 to 6 months of pollination (Sacande *et al.*, 2004).

Though there is lack of data from well designed studies, evidences from different sources suggest that pollination in *P. africana* is mediated by animals. Hall *et al.* (2000) consider the role of wind to be negligible based on Hamilton's (1972) observation that pollen grains falling to the soil surface are poorly dispersed. According to Hall *et al.* (2000), the fragrant character of the flowers also suggests insect pollination. Of course, the report of Fichtl and Admasu Adi (1994) that bees (Hymenoptera: Apidae) forage for nectar and pollen in Ethiopia supports the above suggestion.

The potential seed dispersal agents of *P. africana* are birds and monkeys (Sunderland and Nkefor, 1996; Hall *et al.*, 2000; Farwig *et al.*, 2006). Sunderland and Nkefor

(1996) reported two potential dispersal agents of the species in Cameroon: the primate *Cercopithecus preussii* and the bird *Andropadus montanus*. In Kenya, Farwig *et al.* (2006) observed 36 frugivorous species including birds (*Andropadus gracilirostris*, *Pycnonotus barbatus* and *Turtur tympanistria*) and primates (*Cercopithecus mitis*, *Cercopithecus ascanius* and *Colobus guereza*) feeding on *P. africana* fruits and potentially dispersing the seeds. Despite the presence of such seed dispersal agents, Berens (2010) found that the mean seed dispersal distance of the species was 5 m in the Kakamega Forest (Kenya).

Doubts over whether *P. africana* seed is strictly recalcitrant have been expressed (Were and Munjuga, 1998; Legesse Negash, 2004) and the occurrence of germination inhibitors in the pericarp of fresh seeds has been suggested (Geldenhuys, 1981). Nevertheless, for practical purposes the seed is considered recalcitrant and unless carefully stored only a small proportion remains viable after as short a period as three weeks (Sunderland and Nkefor, 1996).

Vegetative propagation through cuttings from juvenile plants of *P. africana* has been achieved with varying degrees of success in different media (Tchoundjeu *et al.*, 2002). Rooting success in an experiment in Cameroon was higher (80%) with a sawdust medium than with sand (72%) or a 1:1 mix of the two (71%).

In terms of seedling growth, light was observed to be a significant factor in Cameroon: under 70% shade, seedlings became weak and pale whereas at 40% shade normal internode length was found (Sunderland and Nkefor, 1996).

2.5. Distribution and ecology of *P. africana*

Prunus africana is the only species in the genus *Prunus* that is native to Africa (Hedberg. 1989). As shown in its distribution map (Figure 2), *P. africana* is geographically widespread species growing in the highland forests in mainland Africa (Angola. Cameroon. Democratic Republic of Congo. Ethiopia. Kenya, Malawi, Nigeria. Somalia, South Africa, Sudan, Swaziland, Tanzania, Uganda, Zimbabwe) and outlying islands (Bioko, Grande Comore, Madagascar, Sao Tome) (Kalkman, 1965).

In the tropics, *P. africana* is found between 1200-3000 m a. s. l., but further south, where cooler latitudes compensate for altitude, it occurs at lower elevations (Cunningham, 2006). *P. africana* is geographically associated with mean annual rainfall from 500-700 mm (high latitudes) to over 3000 mm (low latitudes) and optimal conditions for the species appear to be temperatures of 11-19 °C and 17-23 °C in the coolest and warmest months respectively (Hall *et al.*, 2000).

Prunus africana is restricted to those parts of Africa that experience temperate climatic conditions and with a moisture supply sufficient to meet potential evapo-transpiration during the growing season. It is high temperature and/or insufficient rainfall during the warmest months of the year that essentially limit *P. africana* to the montane regions of Africa (Hall *et al.*, 2000).

Prunus africana occurs in forests transitional between lowland and Afromontane, and in a range of Afromontane forest types. In the various Afromontane forest types, the abundance of *P. africana* varies widely but the species is sufficiently prominent to

have been used as a plant community descriptor: *Prunus* Zone of the Montane Forest Belt (Hamilton, 1974), *Pygeum* Moist Montane Forest (Spinage, 1972).

Figure 2. Distribution map of *Prunus africana* (Source: Hall *et al.*, 2000).

In Ethiopia, *P. africana* populations are highly fragmented and sparsely distributed in the former Gojjam, Gondar, Shewa, Arsi, Bale, Harerge, Wollega, Illubabor, Kefa and Sidama areas (Hedberg, 1989).

Prunus africana forms symbiotic associations with arbuscular mycorrhizal fungi (Tesfaye Wubet *et al.*, 2003). A new species of fungi was isolated from *P. africana* seeds and named *Diplodia rosulata* sp. nov (Abdella Gure, 2004). Pathogenic fungi

were also reported from nursery and seedling studies (Breitenbach, 1965; Mwanza *et al.*, 1999).

2.6. Phylogeography of *P. africana*

Phylogeography is an integrative field of science that uses genetic information to study the geographic distribution of genealogical lineages, especially those found within species (Avise, 2000). Deciphering spatial and temporal components of population structure and interpreting the evolutionary and ecological processes responsible are major goals of phylogeography.

With regard to the processes leading to the current distribution of *P. africana*, different suggestions have been made based on extant stands (Aubreville, 1976 cited in Kadu *et al.*, 2011; Kalkman, 1988) and DNA studies (Muchugi *et al.*, 2006; Kadu *et al.*, 2011; 2013). Aubreville (1976 cited in Kadu *et al.*, 2011) suggested a Laurasian origin of *Prunus* with subsequent movement through the Middle East into north-east of Africa; whereas Kalkman (1988) proposed a Gondwanian origin of *Prunus* with northward movement along a path starting in regions corresponding to Australia, South America and Africa.

The role of fossil record in inferring the phylogeography of *P. africana* has been limited as there are only few reports from its distribution range. Fossil pollen grains of *Prunus* have been reported from younger deposits (< 40 000 years old) on Mount Kilimanjaro, Tanzania, and on Mount Kenya (Coetzee, 1967; Van Zinderen Bakker and Coetzee, 1972). The occurrence of pollen of *Prunus* comparable to *P. africana* was also reported from Ugandan sediments of nearly the same age as the earlier reports (< 43,000 years old) (Marchant *et al.*, 1997).

The phylogeography of *P. africana* has become the subject of some recent studies using DNA markers. Based on random amplified polymorphic DNA (RAPD) study, Muchugi *et al.* (2006) concluded that both long-distance seed dispersal and migration via the southern migratory tract (SMT) could be responsible for the occurrence of *P. africana* in outlying islands and the distant West African mountain massifs. They supported Laurasian origin of *Prunus* with subsequent movement through the Middle East into north-east of Africa, and proposed the Eastern Rift Valley in Kenya as a probable barrier to gene flow. Kadu *et al.* (2011) provided significant insights into the population history of *P. africana* within mainland Africa and its neighboring islands based on cpSSR markers. They suggested an early split of Madagascar population from the main lineage speculating possibly unique dispersal events facilitated by birds or the southern Monsoon drift or the Mozambique current. For the colonization of West Africa, Kadu *et al.* (2011) suggested former existence of a migration corridor from east to west and proposed two migration scenarios: (i) a split during southward migration of *P. africana* at the southern fringe of the Ethiopian highlands with migration of Albertine Rift Valley populations to West Africa; or (ii) the independent colonization of West Africa via a north-western migration corridor and subsequent colonization of western Uganda from West Africa. They suggested the upper river Nile basin and the Lake Victoria basin as key barriers to dispersal in the early population history of the species, which doesn't agree with the earlier suggestion of the Rift Valley as a probable barrier to gene flow. However, another work of Kadu *et al.* (2013) based on nSSR agrees with the suggestion of the Rift Valley as a probable barrier to gene flow. Kadu *et al.* (2013) explained the disagreement to be due to the dislocation of a historical immigration barrier to a more recent barrier to gene flow over several hundreds of kilometers. Finally, Kadu *et al.* (2013) concluded that the

biogeography of *P. africana* is multifaceted and has been determined by rare long-distance dispersal events coupled with constant migration at intermediate geographical ranges and strong gene-flow barriers.

2.7. Ethnobotany of *P. africana*

Prunus africana has many traditional uses in its range countries. In Cameroon, branches are used for making axe, hoe and ceremonial spear handles (Nsom and Dick, 1992; Cunningham and Mbenkum, 1993; Stewart, 2001); timber is used for roof support, bridge decks, fuel wood (Stewart, 2001), window and door frames (Iverson, 1993); the bark is used as traditional medicine for human ailments (Nsom and Dick, 1992; Cunningham and Mbenkum, 1993; Stewart, 2001) and ailments of domestic animals (Stewart, 2001); leaves and seeds are also used as traditional medicines (Stewart, 2001); fruits and leaves used as wildlife food (Cunningham and Mbenkum, 1993; Stewart, 2001); flowers used for honey production (Stewart, 2001). Ugandans use the timber for mortars and pestles, beehive supports, building poles, bean stakes (Cunningham, 1996), firewood, charcoal, furniture, flooring, paneling, carving, building poles and posts, and utensils (Lambert, 1998). Kenyans use the timber for house building and for furniture (Beentje, 1994); the bark as traditional medicine to treat fevers (Kokwaro, 1976) and gonorrhea (Lindsay, 1978), as purgative (Beentje, 1994); and leaves to treat stomach pain (Kokwaro, 1976). In South Africa, the bark is used as a traditional medicine for chest pain (Van Wyk *et al.*, 1997) and intercostal pain (Hutchings *et al.*, 1996). In Ethiopia, the bark is used to treat wound (Moa Megersa *et al.*, 2013; Sintayehu Tamene, 2011), ear infection and toothache (Mirutse Giday *et al.*, 2009), Ascariasis and Gonorrhea (Fisseha Mesfin *et al.*, 2009), and leaves to treat eye infection (Nigussie Amsalu, 2010) and Tonsillitis (Ragunathan and

Mequente Solomon, 2009). The tree has also other benefits including erosion control, provision of shade or shelter, windbreak, soil fertility improvement, and as ornamental plant (Legesse Negash, 2002).

In modern medicine, the bark of *P. africana* is highly valued for its remedy against benign prostatic hyperplasia (BPH) (non-cancerous enlargement of the prostate), which is common in men over the age of 50 (Tyler, 1994). The bark extract was patented in 1966 (Debat, 1966) and processed to provide treatment for prostate gland hypertrophy (Longo and Tira, 1981; Catalano *et al.*, 1984). According to Cunningham (2006), patents for new products based on *P. africana* bark or bark extract has been proliferated with nine new patents taken out since 2000. The extract from the bark contains several pharmacologically active compounds including phytosterols (e.g. β-sitosterol), pentacyclic triterpenes (oleanolic and ursolic acids) and ferulic esters (n-docosanol and n-tetracosanol) (Longo and Tira, 1981; Catalano *et al.*, 1984), which may interfere with the development of BPH (Stewart, 2003b). According to Cunningham *et al.* (1997), an annual international trade of *P. africana* bark extract for the treatment of benign prostatic hyperplasia worth approximately US$220 million in the final pharmaceutical product. *P. africana* is a potential resource for Ethiopia to have high share in the international market of medicinal plants (Endashaw Bekele, 2007): farmers can benefit greatly through the domestication and cultivation of the tree (Legesse Negash, 2002).

2.8. Threats and conservation status of *P. africana*

Cunningham *et al.* (1997) estimated the worldwide annual export of barks collected by felling of trees from natural stands to be about 4,000 tonnes. The natural resource base is most exploited and under the greatest threat in Cameroon (Cunningham and

Mbenkum, 1993) and Madagascar (Walter and Rakotonirina, 1995 cited in Cunningham *et al.*, 1997). Exploitation is also high, though less intensive, in Kenya (Cunningham *et al.*, 1997) and on the island of Bioko (Equatorial Guinea) (Sunderland and Tako, 1999). According to Cunningham *et al.* (1997), accurate exploitation figures for other countries are not available, but are considered to be comparatively low. Though valuable genetic resources of *P. africana* might have been lost as some of the populations are heavily over-exploited in parts of its distribution range, it is not in danger of extinction at the species level (Dawson *et al.*, 2000). Problems with the sustainability of the bark harvest have resulted from a lack of knowledge of sustainable harvest levels and from the huge demands on wild populations (Stewart. 2003b). In addition to over-exploitation through commercial use, local use, deforestation, habitat fragmentation, wildfires, invasive alien species as well as climate change are among the threats of the species at different range countries (Jimu, 2011). Modeled distribution of *P. africana* indicates that the species is likely to be affected negatively by climate change (Mbatudde *et al.*, 2012a; Vinceti *et al.*, 2013).

In Ethiopia, though it is not known to what extent the bark of *P. africana* is commercialized, it is clear that the species is one of the victims of deforestation as the forest resources of the country have been seriously threatened by deforestation (Reusing. 2000). In the former times, Montane forests were the main constituents of the natural vegetation in the Ethiopian highlands (Breitenbach, 1963). However, in the last few decades, most of the Afromontane forests have been cleared and only a very small proportion of the original vegetation remains (Friis, 1992; Demel Teketay and Granström, 1995).

Due to the severity of the threats to *P. africana*, some international and national conservation measures have been taken. Internationally, the species is included in Appendix II of the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) and the IUCN (International Union for Conservation of Nature) Red List of threatened species. In national levels, policies have been established in various African countries aiming to ensure sustainable utilization and management of *P. africana* though enforcement issues and control problems persist (Vinceti *et al.*, 2013). In Ethiopia, two field gene-banks have been established at Debre Tabor and Lepis and *P. africana* is being conserved there (http://www.ibc.gov.et/biodiversity/conservation/fgbs/forest-field-genebanks), and the Oromia state government has included *P. africana* in the list of prohibited tree species (Anonymous, 2003). In Cameroon and Kenya, *P. africana* planting programs have enjoyed some success indicating potential for *ex situ* conservation if coupled with sustainable managed harvesting (Muchugi *et al.*, 2006).

2.9. Diversity of *P. africana*

Previous studies using RAPD markers on *P. africana* from Ethiopia, Kenya, Cameroon, Uganda, and Madagascar showed the existence of high genetic diversity in the species (Barker *et al.*, 1994; Dawson and Powell, 1999; Muchugi *et al.*, 2006; Hailu Atnafu, 2007). Dawson and Powell (1999) included a population of *P. africana* from Ethiopia in their study and they found the population to be the most diverse (H_e = 0.137) of all the 10 populations considered in their study. Hailu Atnafu (2007) studied the genetic variation of six natural population of *P. africana* from Ethiopia using RAPD markers and showed the existence of high genetic diversity (H_e) in the Tepi (0.307), Lepis (0.290) and Chilimo (0.297) populations. Farwig *et al.* (2008)

found higher values of genetic diversity (H_e) ranging from 0.73 to 0.83 in Kenya using nSSR markers. Kadu *et al.* (2011) found high total gene diversity (h_T = 0.886) from 32 populations in nine African countries excluding Ethiopia using cpSSR markers. In another study using nSSR markers, Kadu *et al.* (2013) also found high genetic diversity (H_e) ranging from 0.430 (South Africa; Mpumalanga) to 0.827 (Kenya: Kibri forest). In terms of phytochemical content, Martinelli *et al.* (1986) found a significant difference between bark extracts of the species from mainland Africa and Madagascar. Similarly, Kadu *et al.* (2012) reported a significant variation in the concentration of bark constituents among 20 *P. africana* populations. However, the concentration of bark constituents originating from different populations did not show a very distinct geographical pattern (Kadu *et al.*, 2012). Gachie *et al.* (2012) also reported the existence of variation in terms of crude bark extract mean yields, chemical composition, and specific compounds among different *P. africana* populations in Kenya.

Studies on morphological traits of *P. africana* are limited. Nevertheless, there are some studies on morphological traits mainly aimed at estimating bark yields of *P. africana* trees at a particular site (Cunningham and Mbenkum, 1993; Betti and Ambara, 2011). A survey carried out in Cameroon on a 15 year old enrichment planting site (Ntingue) with trees up to 17 m high showed that diameter at breast height of *P. africana* varied considerably (7.9 - 42.3 cm), with a mean of 15.8 cm (n = 49 trees) (Cunningham and Mbenkum, 1993). Another study carried out on Mount Cameroon forest (Cameroon) estimated the mean mass of stem barks of *P. africana* trees with DBH ≥ 30cm to be 99.86 kg based on the equation, $V = 0.00004*D^{1.916}$, which links the volume (V) of fresh bark to the diameter (D) of each *P. africana* tree (Betti and Ambara, 2011).

CHAPTER 3: MATERIALS AND METHODS

3.1. Population sampling

Population sampling was carried out over the distribution range of *P. africana* in Ethiopia, which included three regional states and one city administration; namely: Amhara, Oromia, SNNP, and Addis Ababa (Table 1 and Figure 3). The area geographically spans between 35°32' (Gore) to 41°49' (Gara Muleta) longitude East and 5°53' (Kibre Mengist) to 11°50' (Debre Tabor) latitude north. The geographic distance between populations ranges from 26 (Addis Ababa - Menagesha) to 700 km (Gara Muleta - Gore) (Table 8). Twenty one populations were selected from the distribution range of the species in the country based on geographic location (degree of isolation of populations), availability of trees (expected population size) and availability of logistical support for sampling (access to transportation). The specific sites from where samples were collected in each locality are described in Appendix 1. The altitudinal range of the populations is between 1584 m (Harenna) and 2859 m (Debre Tabor) above sea level. Geographic coordinate, altitude and type of habitat for each locality are presented in Table 1.

Table 1. Description of 21 sampling localities of *Prunus africana* in Ethiopia.

	Locality	Code	Lat (N)	Long (E)	Altitude (m)	SZ	Habitat
1	Addis Ababa	AA	9°03'	38°46'	2335-2587	20.3	Park & Campus
2	Agere Mariam	AG	5°53'	38°16'	2239-2271	24.2	Farm
3	Amanuel	AM	10°31'	37°34'	2228-2298	20.1	Farm
4	Asella	AS	7°56'	39°08'	2390-2638	21.1	Farm
5	Bedele	BD	8°28'	36°22'	2026-2103	23.2	Farm
6	Bonga	BO	7°16'	36°15'	1752-1841	23.3	Grazing field
7	Bulki	BU	6°17'	36°49'	2454-2485	23.3	Church forest
8	Chilimo	CH	9°04'	38°08'	2403-2447	20.4	State forest
9	Denkoro	DE	10°49'	38°44'	2522-2678	20.2	Farm
10	Debre Tabor	DT	11°50'	38°00'	2716-2859	20.1	Church forests
11	Gara Muleta	GM	9°09'	41°49'	2423-2536	21.2	Communal forest
12	Gore	GO	8°09'	35°32'	2011-2089	23.1	Grazing field
13	Harenna	HA	6°38'	39°42'	1584-1601	24.2	National park
14	Ingibara	IN	10°56'	36°56'	2624-2737	20.1	Communal forest
15	Jimma	JI	7°42'	36°48'	1808-1880	23.3	Coffee farm
16	Kibre Mengist	KM	5°53'	38°59'	1768-1823	24.2	Farm
17	Kuni	KU	9°00'	40°50'	2339-2501	21.2	Communal forest
18	Lepis	LP	7°18'	38°48'	2209-2225	21.1	Farm
19	Menagesha	MN	8°58'	38°33'	2294-2411	20.3	State forest
20	Nekemte	NK	9°06'	36°36'	2182-2269	20.4	Farm
21	Wof Washa	WW	9°46'	39°46'	2568-2656	20.2	State forest

Lat = latitude, Long = longitude, and SZ = seed zones according to Aalbæk (1993).

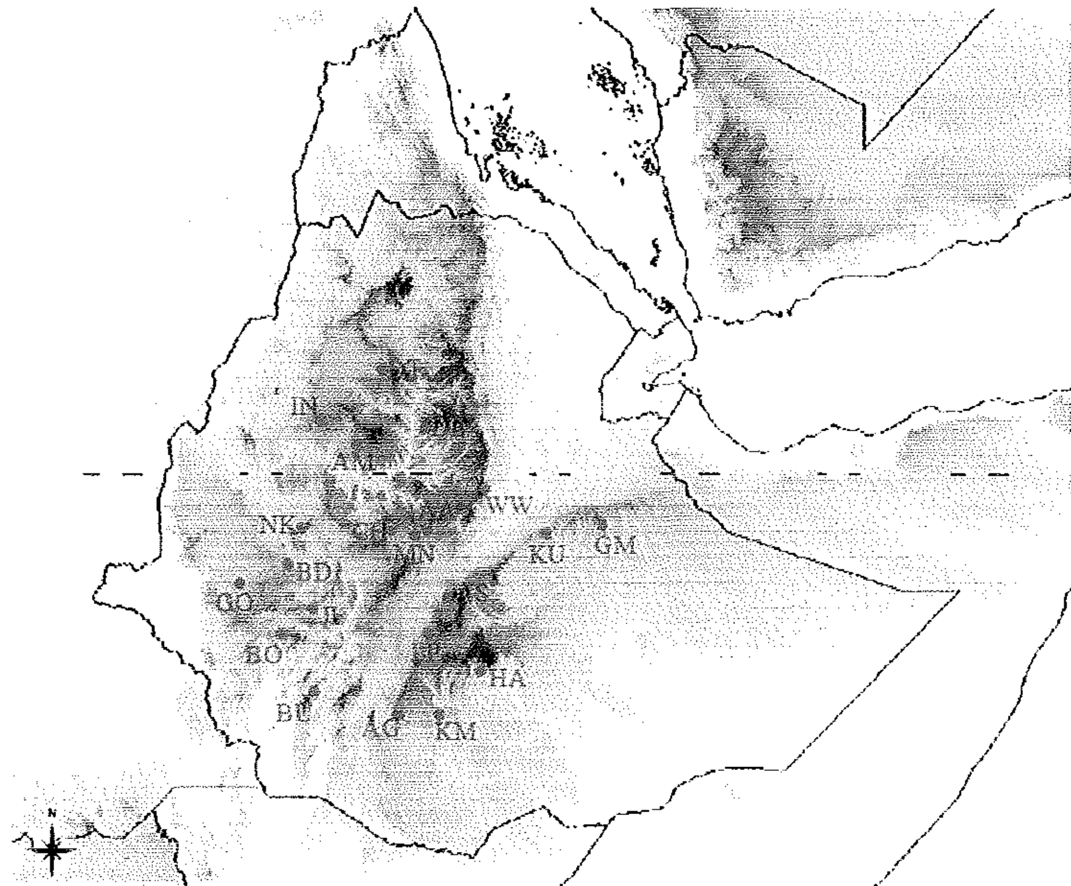

Figure 3. Map showing 21 sampling localities of *Prunus africana* in Ethiopia (See Table 1 for population abbreviations).

3.2. Quantitative morphological data collection and analyses

A total of 210 trees with stem diameter at breast height (DBH) greater than or equal to 30 centimeters were sampled from twenty one populations of *P. africana*. Five economically important quantitative morphological traits; namely: total height, bole height, DBH, bark thickness, and bark mass were measured *in situ* for each tree. The altitude, where each plant was found, was also measured with an altimeter.

Heights were measured using geometric methods (West, 2009) as illustrated in Figure 4 and described as follows: A tree of height **h** = AC, was standing on the ground. A straight stick of known length **l** = BC was positioned vertically at the base of the tree.

Height of the tree was determined by standing at a convenient distance away from the tree and holding a graduated ruler DF in a position that the line of sight OC to the base of the tree was coincided with the zero mark of the ruler. Without moving head up or down, the distance r = FE was read from the ruler, which coincided with the line of sight OB to the top of the stick against the tree. The distance t = DF was also read from the ruler, which coincided with the line of sight OA to the tip of the tree. Using geometric principles, the height of the tree was calculated from these measurements as **h = tl/r**.

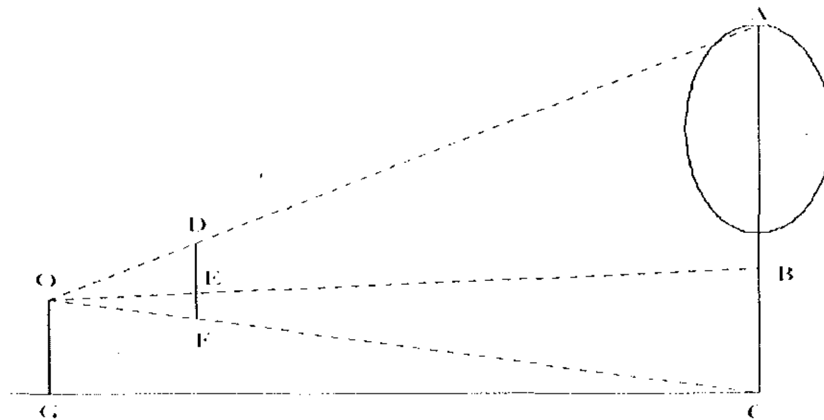

Figure 4. Principle of tree height measurement using trigonometric methods (West, 2009).

DBH was determined by measuring the girth of the stem at a height of 1.3m vertically above ground from the base of the tree with a tape measure. To obtain DBH, girth measurement was divided by the mathematical constant pi (π), which is the ratio of the circumference of any circle to its diameter and has a value of approximately 3.142.

Bark thickness of standing trees was measured with a ruler and a screw driver based on the principles applied on bark gauge (Cunningham, 2001) as follows. The screw

driver was pushed through the bark until the resistance of the underlying wood was felt. A rubber band was wound around the shaft of the screw driver adjacent to the outer surface of the bark and then part of the screw driver that had been inserted was measured with a ruler after it was pulled out of the bark. Four bark thickness measurements were made at right angles around the stem at a height of 1.3m and then their average was taken.

Bark mass per tree was calculated using the equation **Mb = 0.0405216 ($D^{1.916}$)**, where Mb is mass of fresh bark in kg and D is the diameter of the tree at breast height in cm. The equation was developed by Betti and Ambara (2011) for *P. africana* in Cameroon.

Descriptive statistics was used to calculate means and standard deviations of the quantitative morphological traits assessed. One way analysis of variance (ANOVA) and Dunnett T3 post hoc test (for multiple comparisons of means) were carried out to investigate variation in quantitative morphological traits among the populations surveyed. Pearson's correlation analysis was done to investigate the existence of correlations among the quantitative morphological traits and altitude. SPSS version 16.00 (SPSS Inc., 2007) was used for the analyses.

3.3. Sample collection for microsatellite (SSR) investigation

Leaf samples were collected from trees found at different habitat types including state forests, communal forests, church forests, communal grazing fields, public parks, and crop farms. From each population, young leaves from 10 trees were collected and dried in zip-lock plastic bags with silica gel. To decrease the chance of sampling closely related individuals within a population, trees normally a minimum of about

100 m and a maximum of about 5 km apart were sampled though the former criterion was not fulfilled in two of the populations, namely Denkoro and Wof Washa due to scarcity of trees.

3.4. DNA isolation

Total genomic DNA was isolated from 40 to 60 mg of dried leaf samples using triple cetyltrimethyl ammonium bromide (CTAB) extraction technique modified by Borsch *et al.* (2003). However, in this study, the second extraction was used as the quality of the DNA was better than the first extraction whereas its quantity was better than the third extraction. The isolated DNA samples were run in 1% agarose gel electrophoresis to check if the required quality and quantity of DNA was obtained from the extraction. Concentration and purity of the DNA samples was further determined using ND-1000 spectrophotometer (NanoDrop, USA).

3.5. Microsatellite (SSR) markers

A total of 11 microsatellite (SSR) markers originally developed for other species were used (Appendix 2). Nuclear DNA was studied at six microsatellite loci originally characterized in peach [*Prunus persica*, primer pairs U3 (UDP9-403) and U5 (UDP96-018); Cipriani *et al.*, 1999; and P2 (PS12A02): Sosinski *et al.*, 2000] and wild cherry (*Prunus avium*, primers pairs EMPaS01, EMPaS06 and EMPaS10; Vaughan and Russell, 2004). These microsatellite markers were previously transferred to *Prunus africana* (Cavers *et al.*, 2009; Kadu *et al.*, 2013).

For the investigation of chloroplast DNA variation, five microsatellite loci originally characterized in Japanese plum (*Prunus salicina*, primer pairs TPSCP1, TPSCP5 and TPSCP10: Ohta *et al.*, 2005) and *Sorbus aucuparia* (primer pairs rps16pm2 and trnT-

Lpm1; Chester *et al.*, 2007) were used. These markers were also previously transferred to *Prunus africana* by Kadu *et al.* (2011).

3.6. DNA amplification and genotyping

PCR reactions were performed in a 10 µL volume containing 1µL (10–70 ng) total genomic DNA, 2.3 µL KAPA2G™ Buffer A (KAPABIOSYSTEMS), 0.05 mM of each dNTP, 0.21 mM of each primer, one of the two being 5′ labeled with a fluorescence dye, 0.45 U KAPA2G™ Fast DNA polymerase (KAPABIOSYSTEMS), and autoclaved de ionized water. Amplifications were run on a PTC-100 thermo cycler using the following heating profile: a first step initial denaturing at 95 °C for 3 min followed by 35 cycles, each consisting of 30 s denaturing at 94 °C, 30 s annealing at a specific temperature (54 ^{0}C for all nSSR primers, 49 ^{0}C for trnT-Lpm1, 50 ^{0}C for TPSCP5 and rps16pm2, 53 ^{0}C for TPSCP1, and 55 ^{0}C for TPSCP10), and 5 s extension at 72 °C. The last cycle was ended by an extra 30 s at 72°C to complete extension.

PCR amplified DNA fragment size was determined by capillary gel electrophoresis using a CEQ8000 sequencer (Beckman-Coulter, USA). A volume of 1 µL PCR product in 35 µL sample loading solution with 0.5 µL DNA size standard (400bp) was run in the sequencer which separates fragments using polyacrylamide gel in a capillary system and generates electropherograms. Genotyping of amplified fragments was carried out from the readings of electropherograms produced for each sample.

Some *P. africana* DNA samples of Kadu *et al.* (2011) were amplified and genotyped along with the current samples as standards in order to compare and see how the present data fit into previously published result.

3.7. Nuclear SSR data analyses

The microsatellite data set was checked for the presence of genotyping errors and null alleles using the program MICRO-CHECKER (van Oosterhout *et al.*, 2004). Deviations from Hardy-Weinberg equilibrium (HWE) were assessed using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012). Genotypic disequilibrium between loci was tested using Arlequin 3.5 (Excoffier and Lischer, 2010).

Number of observed alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), unbiased expected heterozygosity (H_e), fixation index (F), number of private alleles (A_p), and percentage of polymorphic loci (PPL) per population were calculated using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012). Allelic richness (R_s) was calculated using FSTAT 2.9.3.2 (Goudet, 1995).

Phylogeographic signal was tested using SPAGeDi 1.4b (Hardy and Vekemans, 2002), which evaluates the contribution of the stepwise mutation in the differentiation pattern by comparing observed R_{ST} with R_{ST} obtained after 1000 allele size permutations (pR_{ST}). If stepwise mutations do not contribute to differentiation, F_{ST} and R_{ST} values are equal, but R_{ST} is expected to be significantly higher than mean permuted R_{ST} under a phylogeographic pattern if stepwise mutations contribute to differentiation.

Dendrograms were produced using un weighted pair group method arithmetic average (UPGMA) based on Cavalli-Sforza and Edwards (1967) chord distances after creating 1000 bootstrapped matrices in MICROSATELLITE ANALYSER (MSA) (Dieringer and Schlotterer, 2003). The computer programs NEIGHBOUR and CONSENSE in the PHYLIP 3.63 package (Felsenstein, 1989) were used for tree construction.

Additionally, principal co-ordinate analysis was conducted using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012) to explore multivariate relationships among inter-individual Nei's standard genetic distance (Nei, 1972).

Analysis of molecular variance (AMOVA) was carried out using Arlequin 3.5 (Excoffier and Lischer, 2010) to investigate population differentiation at various levels of subdivisions defined based on geographical units, tree seed zones, and individual-based population clusters using both F_{ST} and R_{ST} in separate analyses based on 10 000 permutations.

The model-based Bayesian clustering method implemented in STRUCTURE 2.3 (Pritchard *et al.*, 2000) was used to infer clusters by assigning individual multi-locus genotypes probabilistically to a user defined number of K clusters. The admixture model without incorporation of population information was used assuming correlated allele frequencies using K values ranging from 2 to 21, run lengths of 800 000 iterations with a burn-in period of 200 000. Five runs per K were performed on the total data set. The most likely number of clusters was inferred using the ΔK statistic of Evanno *et al.* (2005) implemented in STRUCTURE HARVESTER 0.6.93 (Earl and von Holdt, 2012).

Pair-wise population comparisons based on Nei's unbiased genetic distance (Nei, 1978) and different indices of population differentiation F_{ST} (Wright, 1943), G''_{ST} (Meirmans and Hedrick, 2011) and D_{est} (Jost, 2008), as well as correlation analysis between genetic and geographic distance matrices (Mantel, 1967) to test the hypothesis of isolation by distance were computed using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012).

3.8. Chloroplast SSR data analyses

Seven single loci, namely TPSCP1, TPSCP5, TPSCP10, rps16pm2a, rps16pm2b, trnT-Lpm1a, and trnT-Lpm1b were combined to construct multi-locus haplotypes using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012). The last four loci are indels (insertion/deletion) of 5–26 base pairs found in the flanking regions of the loci rps16pm2 and trnT-Lpm1 (Kadu *et al.*, 2011). In constructing multi-locus haplotypes, the present data were combined with the data set of Kadu *et al.* (2011), comprising haplotypes from the main block of the specie's geographic range across sub-Saharan Africa, to see how the current data fit into the previously published result.

The number of haplotypes per population (N_a), the effective number of haplotypes (N_e), the number of private haplotypes (N_p) and haplotype diversity (H_e) were calculated using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012).

Haplotype relationships were inferred by constructing a statistical parsimony network following a two-step strategy according to Bänfer *et al.* (2006). This procedure accounts for the (presumably) different mutation rates underlying indel and microsatellite variation. First, haplotype data from the indel variation were employed to construct a backbone network using TCS 1.18 (Clement *et al.*, 2000). Second, the network was enlarged by adding the variation at microsatellite loci manually at the respective positions of the backbone. Haplotypes were coded following Kadu *et al.* (2011).

Total haplotype diversity (h_T) and average within population haplotype diversity (h_S) were calculated according to Pons and Petit (1995; 1996) using the software PERMUT (http://www.pierroton.inra.fr/genetics/labo/Software). To test for the

existence of phylogeographic pattern in haplotype diversity, the two measures of differentiation N_{ST} and G_{ST}, which are based on ordered and unordered alleles, respectively, were compared. Significance was tested based on 1000 permutations.

Analysis of molecular variance (AMOVA) was carried out to determine the proportion of haplotype variation within individuals and among populations. Mantel test was done on genetic and geographic distance matrices to test the hypothesis of isolation by distance. Both AMOVA and Mantel test were computed using GenAlEx 6.501 (Peakall and Smouse, 2006; 2012) and significances were tested based on 9999 permutations.

3.9. Ethnobotanic data collection and analyses

Ethnobotanic data were collected from October 2011 to May 2012 from 20 localities (Table 1, excluding Addis Ababa). In total, 100 informants (5 per locality) belonging to the following five ethnic groups: Oromo, Amhara, Awi, Goffa and Keffa were interviewed. First, local authorities including kebele administrators, agricultural development agents and elders were communicated to explain the researchers' intention and request recommendation for informants (traditional healers and people with most probable knowledge about the plant). Based on recommendations, informants were contacted and briefed about the aims of the study and gave verbal prior informed consent. Informants were ensured of the anonymity of their personal information provided to increase the chances that they would provide genuine responses. Interviews were conducted individually to prevent informants from being influenced by each other and to respect their individual medical secrets. The whole plant or a branch with fresh leaves, flowers and fruits of *P. africana* was shown to the informants and asked to identify, name and describe it in their mother language, or

they were asked whether they knew the plant by telling the local name, showing pictures and giving descriptions of the plant to them. Semi-structured interviews were undertaken using the pre-prepared interview guide (Appendix 13) and conversations were held with the help of local assistants. With most of the informants interviews were carried out in Amharic language, but for some (who can't speak Amharic) questions were translated into their local language with the assistance of native interpreters. Questions were asked in a stepwise manner by first asking relevant data on their age, address, level of education and occupation. Following that, informants were asked to share their knowledge on the plant. Questions asked during the interviews were related to the importance of the plant; medicinal and non-medicinal uses: parts used, method of preparation, route and dose of administration, traditions and stories about the plant (Appendix 13). Qualitative and quantitative data analyses methods were employed to describe and present the information collected.

CHAPTER 4: RESULTS

4.1. Quantitative morphological traits

The mean values for quantitative morphological traits measured *in situ* on 21 populations of *P. africana* are shown in Table 2. The five quantitative morphological traits had the following mean values over all populations: total height (19.3 ± 6.1 m), bole height (7.4 ± 3.7 m), diameter at breast height (DBH) (70.2 ± 28.5 cm), bark thickness (20.3 ± 3.2 mm), and fresh bark mass (159.6 ± 124.3 kg) (Table 2). The highest mean values per population for the five quantitative morphological traits- total height (29.2 m), bole height (13.8 m), DBH (109.1 cm), bark thickness (23.3 mm), and fresh bark mass (333.6 kg) - were recorded in Bulki, Bulki, Lepis, Assela, and Jimma, respectively; whereas the lowest corresponding mean values were in the following populations: total height (14.4 m) and bark mass (51.7 kg) in Wof Washa, DBH (39.9 cm) and bark thickness (14.6 mm) in Nekemte, and bole height (3.9 m) in Ingibara. Mean values of morphological traits may vary according to ages of populations but it is difficult to infer ages of populations based on these mean values of morphological traits because of two reasons. First, inferences of population ages based on different traits (e.g. for height and DBH) cannot be the same. Second, we are not sure to what extent environmental factors affect the traits. For example, I have observed that trees inside forests tend to be taller and thinner than trees on open areas.

Table 2. Mean values (with standard deviations) for quantitative morphological traits measured *in situ* on 21 populations of *Prunus africana* in Ethiopia.

Population	Total height (m)	Bole height (m)	DBH (cm)	Bark thickness (mm)	Bark mass (kg)
BU	**29.2** (6.7)	**13.8** (3.3)	64.8 (14.6)	20.9 (1.5)	124.7 (50.9)
JI	24.7 (5.4)	6.0 (1.3)	106.1 (33.9)	20.9 (3.2)	**333.6** (197.5)
LP	24.4 (4.2)	10.6 (2.7)	**109.1** (17.6)	22.8 (1.9)	331.8 (99.6)
GO	23.0 (5.6)	8.9 (2.8)	74.8 (15.5)	22.2 (1.9)	162.3 (63.0)
BD	22.0 (7.4)	7.9 (1.9)	81.6 (19.90	21.7 (3.4)	194.9 (95.4)
AG	21.7 (8.4)	10.4 (3.8)	69.9 (19.9)	20.0 (4.3)	146.2 (77.7)
KM	21.4 (8.1)	7.9 (1.8)	72.9 (39.3)	20.0 (2.5)	184.3 (194.2)
BO	19.6 (5.8)	9.3 (2.1)	63.8 (23.3)	20.0 (4.1)	128.7 (86.7)
HA	19.6 (2.4)	11.6 (1.8)	66.7 (17.8)	18.8 (1.5)	133.8 (62.6)
CH	18.4 (4.0)	7.5 (2.5)	59.9 (21.3)	20.8 (2.6)	112.9 (77.1)
KU	18.1 (6.6)	9.0 (6.5)	80.4 (24.9)	19.9 (0.9)	195.4 (108.0)
DT	17.9 (3.3)	5.2 (1.6)	66.8 (23.5)	21.6 (2.4)	139.2 (92.2)
MN	17.4 (3.3)	6.4 (2.5)	48.2 (28.2)	20.5 (2.0)	86.1 (124.2)
NK	17.2 (4.9)	5.7 (4.2)	**39.9** (14.3)	**14.6** (4.7)	52.1 (38.1)
AS	16.9 (2.6)	5.2 (1.6)	98.3 (21.4)	**23.3** (3.0)	276.6 (109.4)
AA	16.5 (2.8)	5.7 (2.4)	48.9 (13.8)	19.8 (4.1)	74.4 (41.4)
DE	16.5 (3.8)	5.5 (2.5)	87.3 (14.9)	21.5 (2.2)	216.9 (62.9)
AM	15.5 (3.5)	4.4 (1.7)	71.2 (22.6)	19.5 (1.5)	154.9 (97.3)
GM	15.2 (4.4)	5.1 (1.4)	64.2 (25.8)	20.4 (0.7)	132.7 (95.6)
IN	15.0 (4.9)	**3.9** (2.4)	57.8 (31.3)	17.4 (1.5)	118.1 (147.5)
WW	**14.4** (2.9)	4.7 (2.4)	41.3 (7.0)	19.2 (1.5)	**51.7** (17.9)
Average of all samples	19.3 (6.1)	7.4 (3.7)	70.2 (28.5)	20.3 (3.2)	159.6 (124.3)

One-way analysis of variance (ANOVA) revealed that there was statistically significant variation ($p = 0.000$) among the populations of *P. africana* in the quantitative morphological traits assessed (Table 3). Further analysis by Dunnett T3 post hoc test showed that 9.0%, 20.0%, 11.9%, 6.2% and 9.5% of the population pairs (a total of 210 pairs) were significantly different in their mean total height, bole height, DBH, bark thickness, and bark mass, respectively ($P < 0.05$) (Table 4).

Pearson's correlation analysis revealed significant positive correlations among all quantitative morphological traits, except between bole height and bark thickness ($p < 0.01$). Furthermore, all quantitative morphological traits, except bark thickness, showed significant negative correlations with altitude ($p < 0.05$). However, bark thickness correlated positively with altitude though not significant ($r = 0.039$, $p > 0.05$) (Table 5).

Table 3. Analysis of variance (ANOVA) results for five quantitative morphological traits among 21 populations of *Prunus africana* in Ethiopia.

Morphological trait	Sum of Squares	df	Mean Square	F	P
Total height	2919.067	20	145.953	5.566	0.000
Bole height	1426.067	20	71.303	9.188	0.000
DBH	72283.457	20	3614.173	7.017	0.000
Bark thickness	706.381	20	35.319	4.881	0.000
Bark mass	1239959.791	20	61997.990	5.889	0.000

Table 1. Dunnett T3 multiple comparison results for 5 quantitative morphological traits of 21 populations of *Prunus africana* in Ethiopia. (Only significant mean differences at the 0.05 level are depicted: H = total height, B = bole height, D = DBH, T = bark thickness and M = bark mass). For population acronyms see Table 1.

	AA	AG	AM	AS	BD	BO	BU	CH	DE	DT	GM	GO	HA	IN	JI	KM	KU	LP	MN	NK	WW
AA				D,M					D,M						D			D,M			
AG																		D,M			
AM																					
AS														T					D	D,T,M	D,M
BD																				D	D
BO			B	B														D,M			
BU	H,B		H,B	H,B	B									T				D,M			
CH							B											D,M			
DE							H,B							T						D,M	D,M
DT						B	H,B							T				D,M			
GM						B	H,B							T				D,M			
GO													T	T				D,M		D,T,M	D,M
HA	B		B	B	B				B	B	B							D,T, M			
IN		B				B	H,B						B					T			
JI			H				B						B							D	D
KM			B				B						B								
KU																					
LP	H		H,B	H,B					H,B	B	H,B			H,B	B				D,M	D,T,M	D,T,M
MN							H,B						B								
NK							H,B														
WW						B	H,B						B		H			H,B			

Table 5. Pearson's correlations among quantitative morphological traits of *Prunus africana* trees in Ethiopia (r is below diagonal and P is above diagonal).

	Total height	Bole height	DBH	Bark thickness	Bark mass	Altitude
Total height		0.000	0.000	0.001	0.000	0.000
Bole height	0.667**		0.000	0.180	0.003	0.000
DBH	0.573**	0.242**		0.000	0.000	0.032
Bark thickness	0.234**	0.093	0.406**		0.000	0.572
Bark mass	0.555**	0.203**	0.980**	0.322**		0.037
Altitude	-0.283**	-0.330**	-0.148*	0.039	-0.144*	

** Correlation is significant at 0.01 and * at 0.05 level (2-tailed t-test).

4.2. Nuclear microsatellite markers

4.2.1. Genetic diversity within populations

A total of 89 alleles were revealed at the six microsatellite loci all over the populations (mean number of alleles per locus was 14.83). The most variable locus was EMPAS10 with 27 alleles, while the least variable one was U3 with only 2 alleles (Appendix 3). Eight of the populations (DT, HA, IN, LP, AG, WW, GM and AM) were fixed for a single allele at locus U3. The total gene diversity (H_T) for the species was 0.725; whereas the mean within population gene diversity (H_S) was 0.640. The observed (H_o) and expected (H_e) heterozygosities ranged from 0.433 (Gara Muleta) to 0.833 (Addis Ababa) and 0.452 (Denkoro) to 0.732 (Addis Ababa) respectively. Allelic richness (R_s) ranged from 2.667 (Wof Washa) to 7.167 (Nekemte) with an

average of 5.063. The mean value for the number of effective alleles (N_e) was 3.408 with the highest being in Bedele (4.479) and the smallest in Denkoro (1.992). Most of the populations had negative fixation index (F); only four populations (NK, CH, AM and GM) had positive values.

Private alleles were detected in more than 65 percent of the populations (Table 6), signaling regional divergence among the populations of *P. africana* in Ethiopia. Bedele had 3; Nekemte, Bulki, Amanuel, Gore and Harenna each had 2: Addis Ababa, Bonga, Menagesha, Asella, Kuni, Debre Tabor, Lepis and Gara Muleta each had 1 private allele. The frequencies of the private alleles were generally ≥ 0.05. The observed number of alleles, effective number of alleles, observed and expected heterozygosities, fixation indices, and percentages of polymorphic loci; all averaged over loci are shown in Table 6.

Though some populations showed heterozygote deficiency or excess, only one highly significant deviation from HWE ($P < 0.001$) was detected (population DE; Appendix 4). Linkage disequilibrium between loci within populations was also detected in some populations at some loci (Appendix 5).

Table 6. Genetic diversity indices based on six nSSRs for 21 populations of *Prunus africana* in Ethiopia.

Population	N_a	N_e	H_o	H_e	F	R_s	A_p	PPL
AA	6.167	3.902	**0.833**	**0.732**	-0.216	6.167	1	100.0
MN	5.500	4.092	0.717	0.704	-0.079	5.500	1	100.0
CH	5.167	3.646	0.617	0.672	0.025	5.167	0	100.0
WW	**2.667**	2.280	0.633	0.498	**-0.356**	**2.667**	0	83.3
IN	4.333	3.299	0.683	0.639	-0.121	4.333	0	83.3
AM	5.000	3.563	0.600	0.627	**0.004**	5.000	2	83.3
DT	4.167	3.099	0.750	0.619	-0.283	4.167	1	83.3
DE	3.333	**1.992**	0.550	**0.452**	-0.180	3.333	0	100.0
BO	6.000	4.277	0.717	0.731	-0.034	6.000	1	100.0
BD	6.333	**4.479**	0.717	0.718	-0.059	6.333	**3**	100.0
NK	**7.167**	4.064	0.667	0.718	0.005	**7.167**	2	100.0
BU	5.667	3.460	0.683	0.709	-0.020	5.667	2	100.0
GO	5.833	4.057	0.750	0.687	-0.141	5.833	2	100.0
JI	5.667	3.443	0.683	0.686	-0.058	5.667	0	100.0
AS	5.833	3.532	0.800	0.702	-0.191	5.833	1	100.0
KU	4.500	3.185	0.783	0.682	-0.230	4.500	1	100.0
KM	5.500	3.038	0.717	0.617	-0.206	5.500	0	100.0
HA	4.333	3.499	0.683	0.608	-0.203	4.333	2	83.3
LP	5.167	3.378	0.600	0.602	-0.020	5.167	1	83.3
AG	4.500	3.062	0.617	0.592	-0.086	4.500	0	83.3
GM	3.500	2.218	**0.433**	0.484	0.025	3.500	1	83.3

N_a = Number of observed alleles, N_e = number of effective alleles, H_o = observed heterozygosity, H_e = unbiased expected heterozygosity, F = fixation index, A_p = number of private alleles, PPL = percentage of polymorphic loci, and R_s = average allelic richness [N_a and R_s are the same because of equal sample size in each population (n=10)].

4.2.2. Genetic structure of populations

4.2.2.1. Regional pattern of genetic diversity

All the populations from southwestern (SW) part of Ethiopia were polymorphic at all the six loci (PPL = 100.0); whereas only 25.0%, 42.8% and 75.0% of the populations from northern, southeastern (SE) and central Ethiopia, respectively, were polymorphic at all the loci. The remaining percentages of populations from these regions had a PPL value of 83.3 (Table 6). Similarly, allelic richness (R_s) was above the average value for all the populations from SW Ethiopia; whereas it was above the average value only for 25.0%, 42.8% and 75.0% of the populations from north, SE and central Ethiopia, respectively. When counting the number of private alleles at regional level, populations from SW, SE, northern and central Ethiopia had a mean number of 1.67, 0.86, 0.75 and 0.50 private alleles, respectively.

4.2.2.2. Differentiation among populations

Overall differentiation among populations was highly significant for both the indices based on unordered alleles (F_{ST} = 0.122, P = 0.0001) and based on ordered alleles (R_{ST} = 0.186, P = 0.0001). Higher values were found for G''_{ST} (0.339, P = 0.0001) and D_{est} (0.247, P = 0.0001). Comparison of R_{ST} and pR_{ST} values revealed that the nuclear microsatellite diversity of *P. africana* had marginally significant phylogeographic signature with pR_{ST} = 0.125 and $R_{ST} > pR_{ST}$ (P = 0.047).

The relationship among populations based on a bootstrapped Cavalli-Sforza and Edwards (1967) chord distance matrix is shown in the UPGMA tree (Figure 5). Generally, clustering within the populations revealed geographic pattern with slight mismatches. All of the populations from northwestern side of the Great Rift Valley

(except DE, WW, MN and BU) grouped into one cluster; whereas populations from the southeastern side of the Great Rift Valley (except LP) aggregated into a separate cluster. However, when UPGMA analysis was done together with populations from other African countries (including the data set of Kadu *et al.*, 2013), all of the Ethiopian populations (except DE) were grouped together in a separate cluster (Appendix 10).

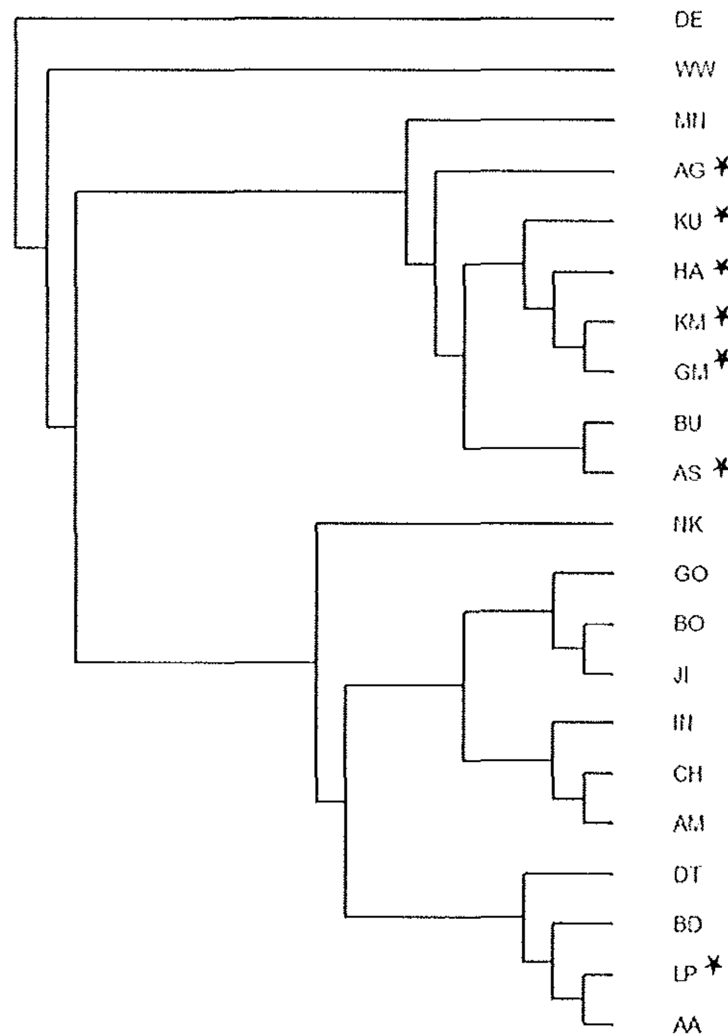

Figure 5. UPGMA dendrogram for 21 populations of *Prunus africana* from Ethiopia (Populations located southeast of the Great Rift Valley are marked with asterisk).

In the principal co-ordinate analysis (PCoA), 62.2% of the variation is explained by the first three principal axes; first axis (27.8%), second axis (20.4%) and third axis (14.1%). Generally, the principal co-ordinate analysis revealed phylogeographic pattern in the nuclear microsatellite diversity of *P. africana*. Most of the populations are distributed within the space of the first two principal coordinates in a similar pattern as their geographic distribution in the country. Along Principal Coordinate Axis 2, all of the populations occupying the right side of the plots were sampled from the northwestern side of the Great Rift Valley, while about 64 % of the populations occupying the left side of the plots were sampled from the southeastern side of the Great Rift Valley. Generally, populations sampled from near the central part of the country tend to occupy around the origin of the coordinates; whereas populations sampled far from the center of the country tend to occupy far from the origin of the coordinates (Figure 6). The population from Denkoro is clearly separated from the rest of the populations. Similar to the result of UPGMA clustering, when PCoA was done using the combined data (including the data set of Kadu *et al.*, 2013) all of the Ethiopian populations were grouped together in a separate cluster from other African populations (Appendix 11).

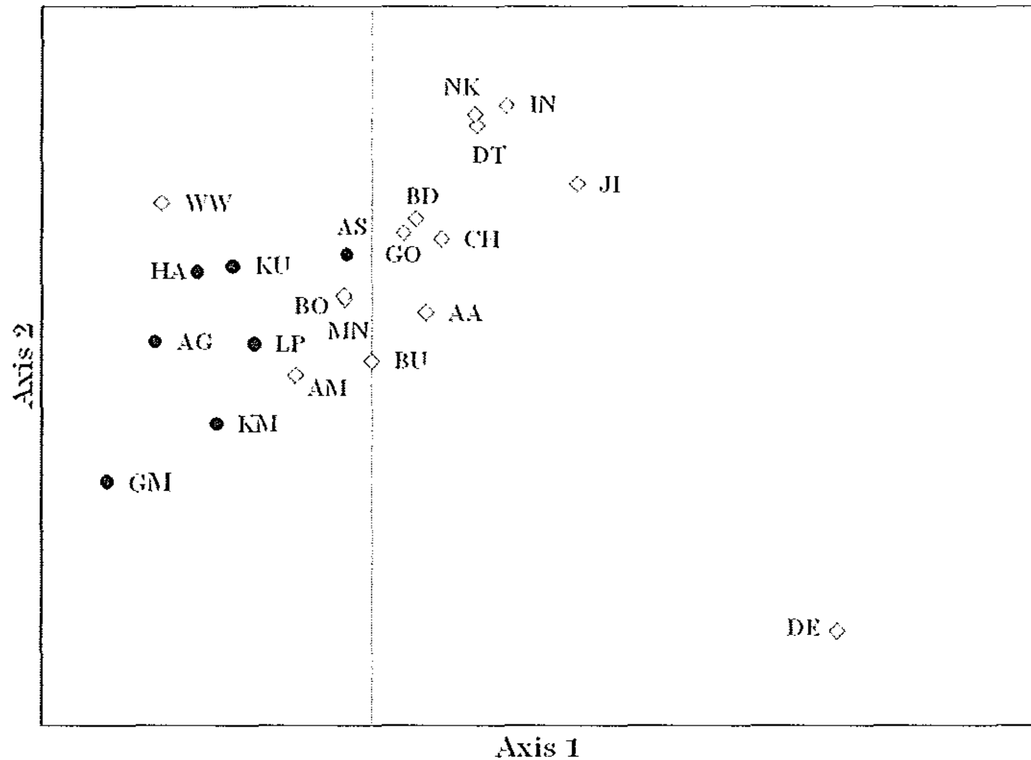

Figure 6. Principal Co-ordinate Analysis showing the multivariate relationships of 21 *Prunus africana* populations from Ethiopia. Populations southeast of the Great Rift Valley are marked with dots and populations northwest of the Rift Valley are marked with diamonds.

Generally, individual-based population assignment using admixture analysis in STRUCTURE revealed low assignment coefficients for the specific clusters illustrating weak population clusters. On the other hand, STRUCTURE HARVESTER 0.6.93 (Earl and vonHoldt, 2012) identified the most likely group structure to be seven as Δln P(D) reached a maximum at K = 7 (Appendix 6 and 7). However, at K = 7 most of the clusters showed heavy admixture from other clusters (Figure 7). Similar to the results of UPGMA and PCoA analyses, all of the Ethiopian populations were grouped together in a separate cluster from other African populations (Appendix 12).

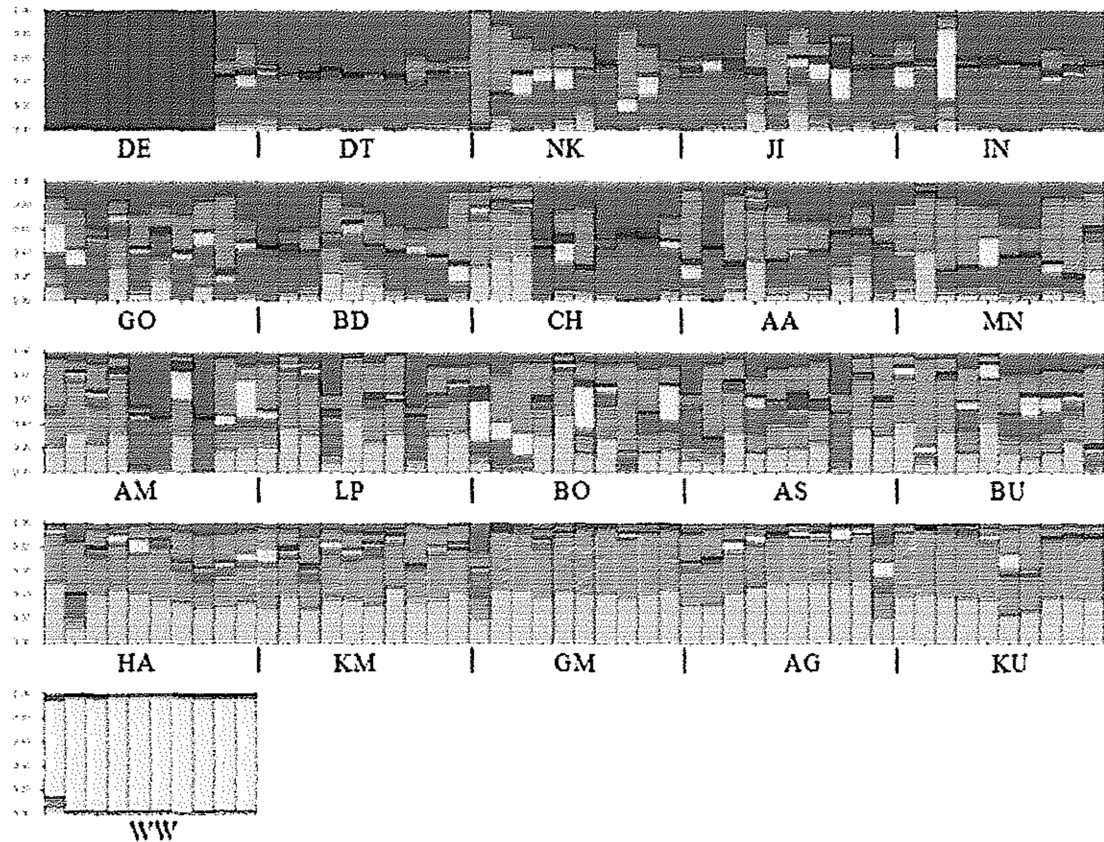

Figure 7. Results of individual population assignment (admixture model) performed with STRUCTURE for 21 *Prunus africana* populations from Ethiopia (K = 7).

When analysis of molecular variance (AMOVA) was done using F_{ST} as measure of differentiation, the molecular variance was partitioned into 11.95% among populations and 88.05% within populations. However, when R_{ST} was used as measure of differentiation by taking allele size and stepwise mutation into account, 18.0% of the variance was found among the populations. Analysis of populations based on geographical units, tree seed zones, and STRUCTURE based clusters using R_{ST} revealed significant differentiation among the groups. However, when F_{ST} was used there was no significant differentiation among the tree seed zones (Table 7).

Table 7. Partitioning of variation based on six nuclear microsatellite loci among 210 *Prunus africana* individuals in Ethiopia computed by analysis of molecular variance (AMOVA).

Source of variation	df	F_{ST} SS	F_{ST} VC	F_{ST} % variation	F_{ST} P	R_{ST} SS	R_{ST} VC	R_{ST} % variation	R_{ST} P
Among populations	20	143.01	0.26	11.95	***	10492.99	21.37	18.00	***
Within populations	399	768.15	1.93	88.05	***	38839.30	97.34	82.00	***
Among 7 clusters	6	71.57	0.12	5.42	***	6258.26	12.96	10.75	**
Among populations within clusters	14	71.44	0.16	7.21	***	4234.73	10.26	8.51	***
Within populations	399	768.15	1.93	87.37	***	38839.30	97.34	80.74	***
Among seed zones	9	68.13	0.02	0.84	NS	7502.06	13.54	11.32	**
Among populations within seed zones	11	74.88	0.24	11.16	***	2990.93	8.73	7.30	***
Within populations	399	768.15	1.93	88.00	***	38839.30	97.34	81.38	***
Among geog. units	3	30.44	0.03	1.56	*	3243.09	6.36	5.30	*
Among populations within geog. units	17	112.57	0.23	10.70	***	7249.90	16.46	13.70	***
Within populations	399	768.15	1.93	87.74	***	38839.30	97.34	81.01	***

df = degree of freedom, SS = sum of squares, VC = variance components, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and NS = non-significant ($P \geq 0.05$)

4.2.2.3. Pair-wise population comparisons

Pair-wise population matrix of Nei's unbiased genetic distances and geographic distances for 21 *P. africana* populations is presented in Table 8. The highest genetic distance (1.040) was between Denkoro and Wof Washa populations; whereas the smallest distance (0.010) was between Chilimo and Bedele populations. Pair-wise comparisons of populations using the three indices of population differentiation [F_{ST} (Table 9), G''_{ST} (Appendix 8) and D_{est} (Appendix 9)] also showed that the highest genetic differentiation was between Denkoro and Wof Washa; whereas the lowest was between Chilimo and Bedele populations, which are in agreement with Nei's genetic distance values. The pair-wise comparisons of populations using F_{ST}, G''_{ST} and D_{est} revealed that 95.7%, 95.7% and 96.2% of the population pairs (a total of 210 pairs), respectively, were significantly differentiated ($P < 0.05$) (Table 9, Appendices 8 and 9).

4.2.2.4. Correlation between genetic and geographic distance matrices

The Mantel test for isolation by distance revealed a significant positive correlation between genetic distance and geographic distance matrices for 21 *P. africana* populations in Ethiopia based on six nuclear microsatellite loci ($Rxy = 0.126$, $P < 0.001$) (Figure 8).

Table 2. Pair-wise population matrix of Nei's unbiased genetic distance (above the diagonal) and geographic distance in km (below the diagonal) among 21 *Prunus africana* populations in Ethiopia.

	DE	DT	NK	JI	IN	GO	BD	CH	AA	MN	AM	LP	BO	AS	BU	HA	KM	GM	AG	KU	WW
DE		0.706	0.689	0.471	0.634	0.546	0.582	0.489	0.545	0.599	0.484	0.614	0.541	0.740	0.567	0.812	0.545	0.698	0.773	0.978	**1.040**
DT	138		0.164	0.209	0.130	0.148	0.078	0.158	0.183	0.260	0.238	0.247	0.220	0.436	0.497	0.332	0.350	0.494	0.324	0.418	0.527
NK	302	340		0.123	0.103	0.117	0.091	0.048	0.299	0.191	0.220	0.292	0.130	0.260	0.320	0.250	0.303	0.428	0.364	0.3	0.328
JI	406	478	157		0.120	0.249	0.125	0.147	0.196	0.265	0.283	0.289	0.148	0.224	0.316	0.356	0.422	0.483	0.382	0.315	0.446
IN	197	153	207	360		0.122	0.110	0.100	0.298	0.255	0.202	0.306	0.181	0.234	0.376	0.273	0.397	0.451	0.403	0.438	0.355
GO	459	491	158	148	345		0.101	0.134	0.293	0.196	0.100	0.162	0.125	0.348	0.294	0.214	0.189	0.295	0.215	0.365	0.300
BD	368	415	75	98	281	98		**0.01**	0.062	0.115	0.094	0.089	0.048	0.170	0.201	0.126	0.157	0.269	0.172	0.223	0.470
CH	205	308	168	211	246	303	205		0.145	0.118	0.073	0.173	0.104	0.102	0.184	0.102	0.155	0.260	0.258	0.222	0.437
AA	196	321	238	263	290	369	272	70		0.212	0.148	0.151	0.066	0.202	0.203	0.238	0.205	0.275	0.270	0.197	0.641
MN	207	324	215	239	281	344	246	47	26		0.119	0.155	0.123	0.199	0.227	0.171	0.196	0.193	0.164	0.230	0.421
AM	132	154	190	324	83	345	263	173	209	203		0.105	0.062	0.267	0.229	0.130	0.051	0.039	0.154	0.188	0.336
LP	391	512	314	225	453	372	298	210	195	187	382		0.100	0.266	0.276	0.127	0.078	0.183	0.058	0.251	0.365
BO	480	543	207	77	415	126	134	288	341	316	389	281		0.194	0.044	0.141	0.071	0.164	0.121	0.165	0.254
AS	324	451	307	258	412	397	310	167	131	132	335	79	326		0.151	0.082	0.281	0.279	0.278	0.186	0.524
BU	546	631	314	158	517	251	248	342	375	354	478	246	126	315		0.238	0.205	0.296	0.282	0.247	0.392
HA	477	608	438	341	567	489	420	321	288	289	491	124	387	157	321		0.125	0.154	0.164	0.196	0.358
KM	549	670	444	315	605	457	407	366	353	346	538	159	339	229	244	115		0.083	0.079	0.179	0.320
GM	385	513	573	575	570	**700**	604	405	335	359	490	391	647	325	636	364	479		0.172	0.228	0.360
AG	551	662	402	259	580	393	356	354	356	344	521	168	271	247	166	179	79	534		0.312	0.237
KU	306	442	465	467	478	590	494	297	227	251	396	293	540	221	536	291	402	109	447		0.478
WW	163	300	355	399	336	499	400	195	136	160	255	294	476	215	505	348	440	235	462	145	

Table 3. Pair-wise population matrix of F_{ST} values for 21 *Prunus africana* populations in Ethiopia (F_{ST} values below the diagonal and probability, P based on 9999 permutations, above diagonal).

	DE	DT	NK	JI	IN	GO	BD	CH	AA	MN	AM	LP	BO	AS	BU	HA	KM	GM	AG	KU	WW
DE		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
DT	0.207		***	***	***	***	**	**	***	***	***	***	***	***	***	***	***	***	***	***	***
NK	0.182	0.064		*	*	**	*	NS	***	***	**	***	*	***	***	***	***	***	***	***	***
JI	0.156	0.074	0.050		**	***	**	**	**	***	***	***	*	***	***	***	***	***	***	***	***
IN	0.192	0.060	0.050	0.054		**	**	*	***	***	**	***	**	***	***	***	***	***	***	***	***
GO	0.168	0.062	0.049	0.073	0.054		**	**	***	***	**	***	**	***	***	***	***	***	***	***	***
BD	0.168	0.046	0.042	0.050	0.051	0.046		NS	NS	*	*	*	NS	**	**	**	***	***	**	**	***
CH	0.162	0.065	0.036	0.057	0.050	0.054	**0.029**		**	**	NS	**	*	*	**	**	**	***	***	***	***
AA	0.161	0.067	0.072	0.061	0.083	0.075	0.037	0.054		***	**	***	NS	***	***	***	***	***	***	***	***
MN	0.173	0.082	0.060	0.074	0.078	0.063	0.047	0.051	0.062		**	**	*	**	***	***	***	***	***	***	***
AM	0.169	0.086	0.073	0.087	0.076	0.051	0.049	0.045	0.060	0.055		*	NS	***	***	**	*	NS	**	***	***
LP	0.198	0.091	0.089	0.092	0.100	0.067	0.050	0.070	0.063	0.065	0.056		*	***	***	**	**	***	*	***	***
BO	0.160	0.073	0.048	0.054	0.064	0.050	0.034	0.047	0.037	0.048	0.042	0.053		**	NS	**	*	***	**	**	***
AS	0.191	0.110	0.070	0.068	0.075	0.086	0.057	0.047	0.061	0.062	0.082	0.086	0.060		**	**	***	***	***	***	***
BU	0.168	0.117	0.078	0.081	0.097	0.078	0.061	0.062	0.060	0.066	0.075	0.087	0.034	0.054		***	**	***	***	***	***
HA	0.224	0.107	0.081	0.102	0.093	0.077	0.058	0.053	0.078	0.067	0.062	0.063	0.061	0.048	0.080		***	**	***	***	***
KM	0.183	0.109	0.089	0.111	0.114	0.071	0.063	0.064	0.071	0.071	0.041	0.049	0.045	0.086	0.072	0.062		**	**	***	***
GM	0.246	0.164	0.136	0.150	0.152	0.115	0.107	0.109	0.108	0.091	0.046	0.094	0.085	0.111	0.113	0.085	0.061		***	***	***
AG	0.224	0.108	0.102	0.109	0.119	0.079	0.069	0.089	0.086	0.068	0.070	0.044	0.058	0.090	0.090	0.073	0.050	0.091		***	***
KU	0.221	0.110	0.079	0.084	0.109	0.091	0.067	0.071	0.062	0.069	0.070	0.085	0.057	0.062	0.071	0.073	0.069	0.101	0.098		***
WW	0.287	0.166	0.116	0.140	0.130	0.113	0.139	0.141	0.160	0.133	0.128	0.138	0.101	0.150	0.128	0.135	0.126	0.160	0.108	0.146	

* $P < 0.05$; ** $P < 0.01$;** $P < 0.001$; NS, non-significant ($P \geq 0.05$)

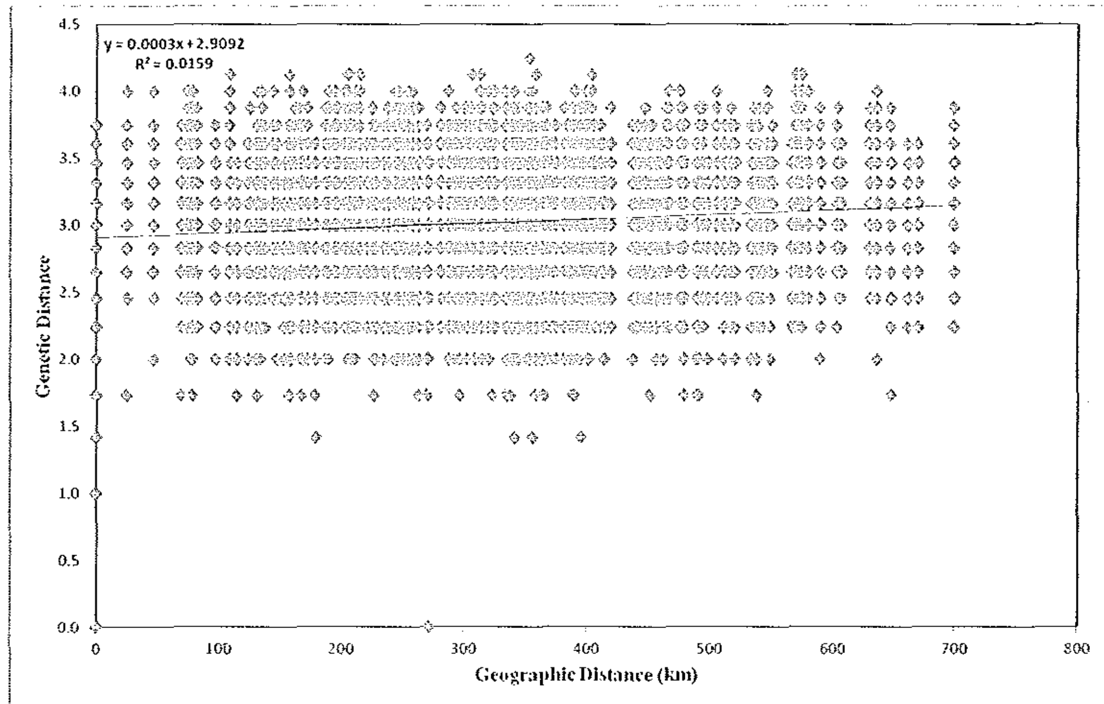

Figure 8. Correlation between geographic and genetic distance matrices of 21 *Prunus africana* populations in Ethiopia.

4.3. Chloroplast microsatellite markers

4.3.1. Chloroplast DNA variation

Even though three cpDNA microsatellites (TPSCP1, TPSCP5 and TPSCP10) and four cpDNA indels (rps16pm2a, rps16pm2b, trnT-Lpm1a and trnT-Lpm1b) were considered in generating data from 210 individuals of *P. africana* in Ethiopia, only the three cpDNA microsatellites were found to be polymorphic (Appendix 3). The four cpDNA indels were monomorphic. The most variable loci were TPSCP5 and TPSCP10 with four alleles each, followed by TPSCP1 with two alleles. Thus, a total of 14 alleles were produced from all the seven loci including the four monomorphic cpDNA indels. The monomorphic loci were not excluded from further analysis for the sake of consistency in comparing the present data with previously published results.

A total of 20 alleles generated at the seven chloroplast loci from 792 individuals of *P. africana* (including 582 from Kadu *et al*., 2011) were used to construct multi locus haplotypes. Thirty-two multi locus haplotypes were produced from the combination of 20 alleles (Table 11). Half of the haplotypes were found in Ethiopian populations with the predominant haplotype being HT1h (frequency of 46.2%). HT1i was the second most frequent haplotype (frequency 30.5%). The least frequent (frequency 0.48%) haplotypes were HT1p, HT1q, HT1v, HT1w, HT1x, and HT1y. Number of haplotypes (N_a) per population ranged from one to five with an average of two. Ten of the Ethiopian haplotypes were private to single populations, while the remaining six of them occurred in two or more populations. Lepis (LP) and Bulki (BU) each had two private haplotypes (LP: HT1k and HT1s; BU: 1v and 1w). Agere Mariam (AG) was the only population that did not share haplotype with other populations.

Fifteen populations harbored two to five haplotype, while the remaining six populations contained only single haplotype. Of the later group of populations, Bedele, Chilimo, Debre Tabor, and Nekemte were fixed for HT1h; Menagesha for HT1i and Agere Mariam for HT1u. The highest haplotype diversity (H_e) of 0.822 was found in the Amanuel population, followed by the Bulki population (H_e = 0.800). The characteristics of chloroplast haplotypes in each population are shown in Table 10.

Table 10. Haplotype diversity measures for 21 populations of *P. africana* in Ethiopia.

Population	**n**	**Haplotype**	N_a	N_e	N_p	H_e
AM	10	1a, 1g, 1h, 1i	4	**3.85**	0	**0.822**
BU	10	1a, 1h, 1i, 1v, 1w	5	3.57	2	0.800
LP	10	1i, 1k, 1r, 1s	4	2.94	2	0.733
JI	10	1g, 1h, 1q, 1t	4	2.78	1	0.711
GO	10	1h, 1i	2	2.00	0	0.556
KU	10	1h, 1i	2	1.92	0	0.533
IN	10	1h, 1i	2	1.92	0	0.533
BO	10	1g, 1h	2	1.92	0	0.533
KM	10	1c, 1i, 1r	3	1.85	1	0.511
GM	10	1h, 1i	2	1.72	0	0.467
AS	10	1h, 1i, 1p	3	1.52	1	0.378
WW	10	1h, 1i, 1y	3	1.52	1	0.378
AA	10	1h, 1i	2	1.47	0	0.356
HA	10	1t, 1x	2	1.22	1	0.200
DE	10	1h, 1i	2	1.22	0	0.200
BD	10	1h	1	1.00	0	0.000
CH	10	1h	1	1.00	0	0.000
DT	10	1h	1	1.00	0	0.000
MN	10	1i	1	1.00	0	0.000
NK	10	1h	1	1.00	0	0.000
AG	10	1u	1	1.00	1	0.000

n = sample size, N_a = number of haplotypes, N_e = effective number of haplotypes, N_p = number of private haplotypes, and H_e = haplotype diversity.

Table 4. Haplotype construction and frequency in 21 *Prunus africana* populations revealed by four cpDNA indels (loci 1-4) and three cpDNA microsatellites (loci 5-7). Alleles are represented as one for the presence of fragment and two for the absence of fragment in the case of the indels (loci 1-4) and number of nucleotide repeats in the case of microsatellites (loci 5-7).

Backbone Nt	1																									2		3			4	5
Nt subdivision	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x	y	a	b	a	b	c	a	a
Indels*																																
1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2
2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1
3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
MS§																				b												
5	9	10	10	10	11	8	9	9	9	9	9	9	8	8	8	10	10	10	10	9	9	9	9	9	9	8	10	9	9	9	9	10
6	9	9	9	9	9	9	10	10	9	8	8	8	8	8	8	10	10	8	8	10	11	11	11	9	9	8	8	9	10	9	9	9
7	9	9	11	12	12	11	9	10	10	9	10	11	9	10	11	10	11	10	9	11	10	11	9	11	12	9	9	9	9	10	11	11
Population†																																
AA								2	8																							
BD								10																								
CH								10																								

*Indel loci 1,2,3,4 are rps16pm2a, rps16pm2b, trnT-Lpm1a, and trnT-Lpm1b; §MS-Chloroplast microsatellite loci 5, 6, 7 are TPSCP1, TPSCP5, and TPSCP10; †See Table 1 for population codes.

Table 11. (Continued).

DT								10																								
KU								6	4																							
GO								5	5																							
HA																				9				1								
IN								4	6																							
JI							3	5									1			1												
KM			2						7									1														
LP									5		2							1	2													
MN									10																							
NK								10																								
BO							6	4																								
DE								9	1																							
AG																					10											
AS								1	8							1																
BU	1							4	3													1	1									
WW								8	1																1							
GM								7	3																							
AM	2						3	2	3																							
Total	3	0	2	0	0	0	12	97	64	0	2	0	0	0	0	1	1	2	2	10	10	1	1	1	1	0	0	0	0	0	0	0

4.3.2. Relationship and geographic distribution of haplotypes

The relationship between 32 haplotypes is demonstrated by the genealogical network in Figure 9. The backbone of the network consists of five main haplotypes (denoted HT1-HT5). Sixteen of the haplotypes were found in Ethiopia. Ten of the Ethiopian haplotypes (HT1p-HT1y) were not found in other countries. Five of the sixteen haplotypes (HT1a, HT1c, HT1g, HT1h and HT1i) were shared with 'east' (excluding Uganda) and southern African populations but only one haplotype (HT1k) was shared with a West African population (Equatorial Guinea) (Figure 10). All of the haplotypes found in Ethiopia were members of the HT1 family.

In the present study, the predominant haplotype was HT1h, which occurred in 16 Ethiopian populations, mainly on the northwestern side of the Rift Valley, and in two non-Ethiopian populations (1 Kenyan and 1 Tanzanian). The second most frequent haplotype was HT1i, which occurred in 13 populations on both sides of the Rift Valley in Ethiopia and in two non-Ethiopian populations (1 Kenyan and 1 Tanzanian) (Figure 10).

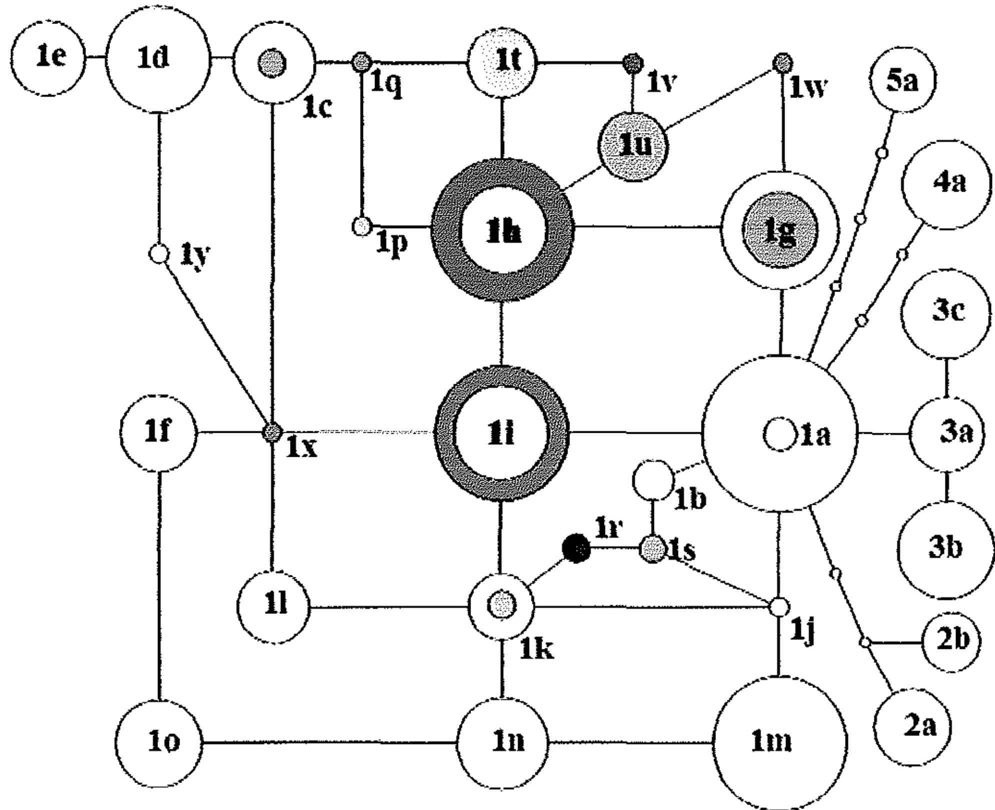

Figure 9. Genealogical network of 32 haplotypes of 53 *Prunus africana* populations from 10 African countries. Non-white colors represent haplotypes found in Ethiopia, and haplotypes shared with populations of other African countries are represented by concentric circles. The size of the haplotypes is drawn proportional to their frequencies. Very small white circles represent hypothetical intermediate haplotypes.

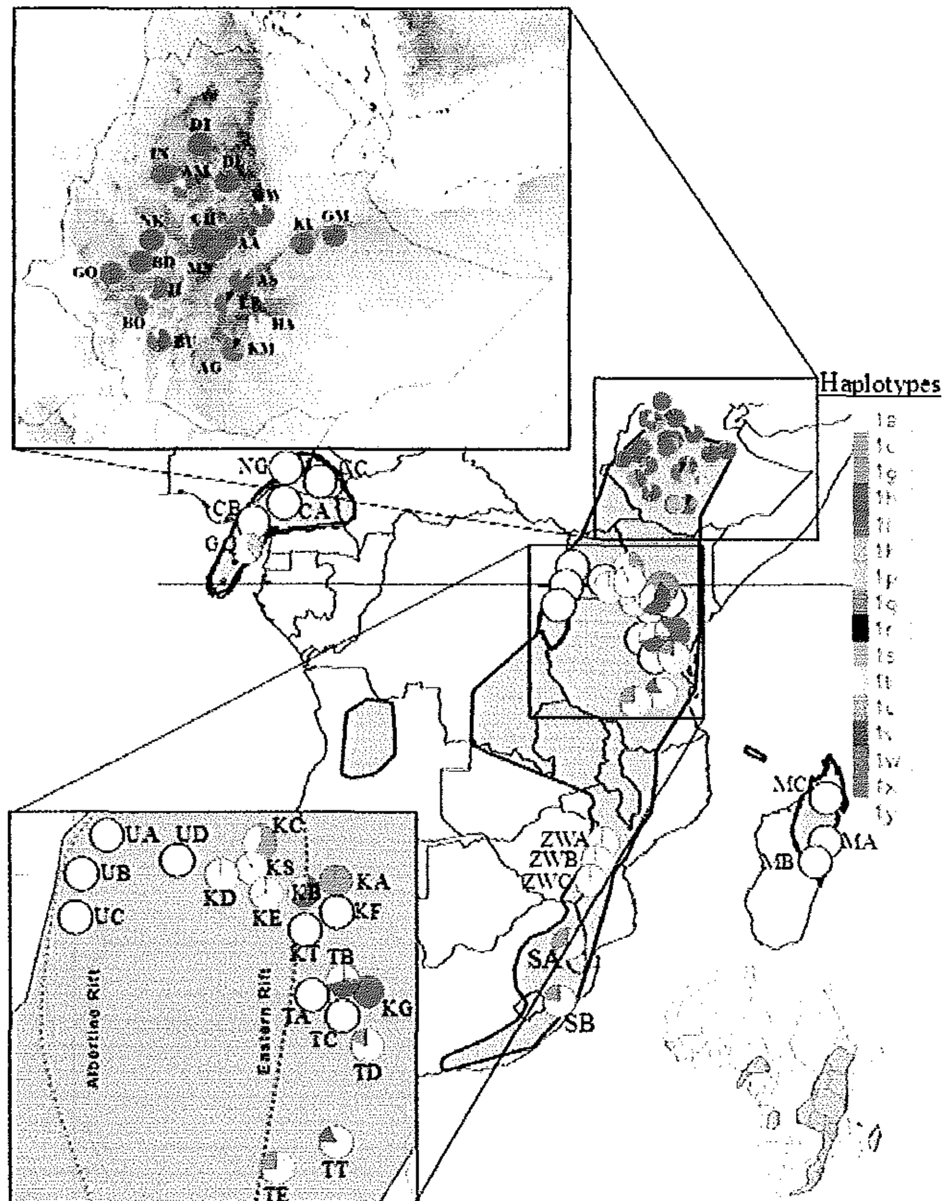

Figure 10. Distribution of 16 haplotypes of *Prunus africana* found in Ethiopian populations (marked with non-white colors). Codes for non-Ethiopian populations: GQ=Moka, KA=Chuka, KB=Kinale, KC=Kapcherop, KD=Kakamega, KE=Londiani, KG=Taita, KS=Kibiri forest, SA=Mpumalanga, SB=KwaZulu-Natal, TB=Kilimanjaro, TD=Shume Magamba, TE=Kidabaga, TT=Udzungwa, ZWA=Nyanga, ZWB=Chimanimani, ZWC= Chipinge, NG=Ngel Nyaki Forest Reserve, CB= Lower Mann's Spring, CA=Ngashie-Mt Oku, CC=Ngashie-Mt Oku, MC=Antsahabiraoka, MA=Marovoay, MB=Lakato forest, UA=Kibale Forest, UB=Kalinzu Forest, UC=Bwindi Forest, UD=Mabira Forest, KF=Ol Danyo Sambuk, KT=Lari, TC=Kindoroko Catchment, TA=Meru Catchment and for Ethiopian populations see Table 1.

Total haplotype diversity (h_T =0.703, SE = 0.0572) was about two times higher than the mean within population diversity (h_S = 0.367, SE = 0.0629), which suggests genetic differentiation among populations. Indeed, analysis of molecular variance (AMOVA) revealed highly significant genetic differentiation among the populations (G_{ST} = 0.478, P = 0.0001). The haplotype variation was partitioned into 47.8% among populations and 52.2% within populations (Table 12).

The measure of genetic differentiation based on ordered alleles (N_{ST} = 0.483, SE = 0.0836) was higher than unordered allele measure of genetic differentiation (G_{ST} = 0.478, SE = 0.0903). However, comparison of observed N_{ST} (0.483) with mean permuted N_{ST} (0.473) revealed that the chloroplast microsatellite diversity of *P. africana* had no significant phylogeographic pattern in the investigated populations ($P > 0.05$). Nevertheless, Mantel test for isolation by distance revealed a significant positive correlation between geographic and Nei's genetic distance matrices (Rxy = 0.107, $P < 0.001$) (Figure 11).

Table 12. Partitioning of haplotype variation among 210 *Prunus africana* individuals in Ethiopia computed by analysis of molecular variance (AMOVA).

Source of variation	df	SS	Variance components	% of variation	P
Among populations	20	37.25	0.17	47.8%	0.0001
Within populations	189	34.70	0.18	52.2%	
Total	209	71.952	0.35	100%	

df = degree of freedom, and SS = sum of squares

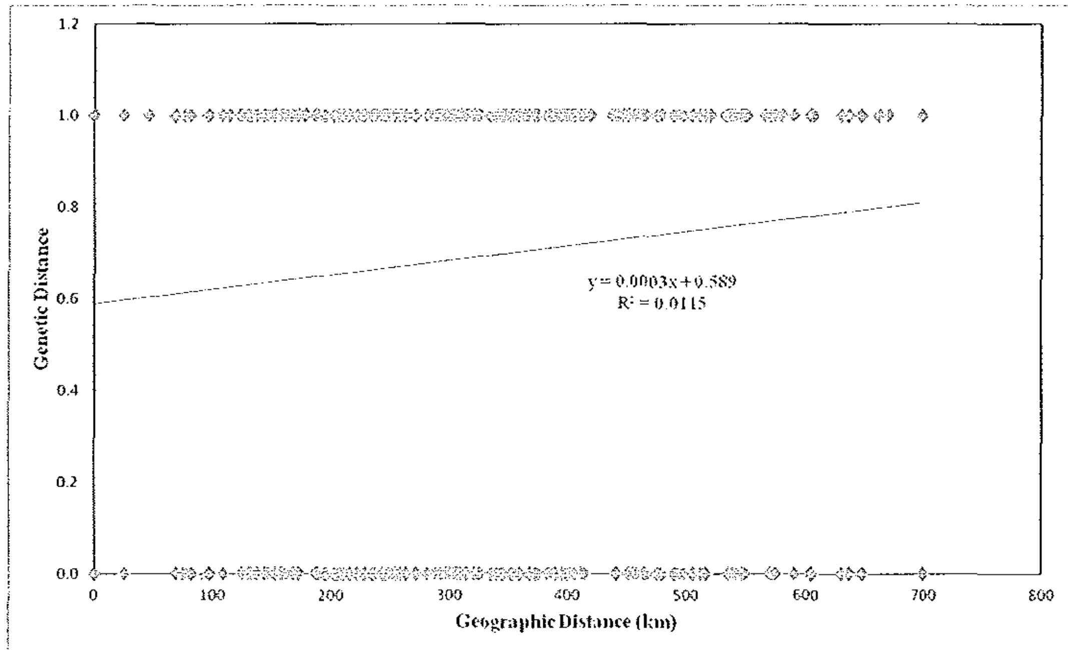

Figure 11. Correlation between geographic and genetic distance matrices of 21 *Prunus africana* populations in Ethiopia based on seven cpSSR loci.

4.4. Ethnobotany of *P. africana*

The ethnobotanic survey on *P. africana* in the 20 localities over its distribution range in Ethiopia revealed the multipurpose character of the species; people in different parts of the country use the plant for different purposes and thus six major use-categories were recorded for the species.

4.4.1. Local nomenclature of *P. africana*

Prunus africana is known by several names in different parts of Ethiopia (Table 13). Its common name in Amharic is Tikur inchet. However, it is also known by other Amharic names including Homa by the people around Amanuel and Debre Tabor towns, and Koma by the people around Denkoro forest. However, the common name Tikur inchet is used for other tree species in some localities of the Amhara region like

Amanuel, Debre Tabor and Wof Washa. The use of this common name can lead to confusion in such areas. In Oromiya, *P. africana* is called by several names including Burayu by the people around Chilimo forest, Menagesha forest, and Asella town; Homi by the people around Bedele, Gore and Nekemte towns; Haleba or Keteba by the people around Kuni town; Mechelo by the people around Gara Muleta; Suke by the people around Agere Mariam town, Harenna forest, Kibre Mengist town, and Lepis village; and Omo by the people around Jimma town. The people around Bonga town also call *P. africana* by the name Omo in Keffa language. Okanse in Goffa language is the name given to *P. africana* by the people around Bulki town. The Awi people around Injibara town call *P. africana* by the name Damtse.

Table 13. Nomenclature of *Prunus africana* in different localities of Ethiopia based on responses of five informants in each locality.

Local Name	Language	Locality
Burayu	Oromifa	Asella
		Chilimo Forest
		Menagesha Forest
Damtse	Awi	Ingibara
Homa	Amharic	Amanuel
		Debre Tabor
Homi	Oromifa	Bedele
		Gore
		Nekemte
Keteba/Haleba	Oromifa	Kuni
Koma	Amharic	Denkoro Forest
Mechelo	Oromifa	Gara Muleta
Okanse	Goffa	Bulki
Omo	Keffa	Bonga
Omo	Oromifa	Jimma
Suke	Oromifa	Agere Mariam
		Harenna Forest
		Kibre Mengist
		Lepis
Tikur inchet	Amharic	Addis Ababa
		Wof Washa Forest

4.4.2. Medicinal uses of *P. africana*

People in different parts of Ethiopia claimed that *P. africana* is used for the treatment of several types of health problems for both human and livestock (Table 14). Several traditional medicines were reported to be prepared from fresh, dried, or powdered leaf or bark of *P. africana* alone or mixed with other ingredients. Informants reported that those traditional medicines are commonly prepared in the form of juice, decoction, paste or powder for oral, nasal or dermal administration.

Reported traditional treatments for human health problems

Strong cough, asthma, Mich, Tikusat (fever), snake bite, stomach pain in children, head lice, wound, bed wetting in children, and menstruation problem (over bleeding) were reported to be treated by *P. africana*. To treat **strong cough**, one Melekiya (15ml) of bark decoction with sugar is taken orally for three days, or one-third of a Melekiya of the juice of crushed leaf bud in water is taken orally for three days, or small amount of juice of crushed leaf in water with salt is taken as a drink for one day, or bark is boiled together with coffee and a cup of decoction is taken orally, or about 5cc of dried leaf decoction is taken orally twice a day for two days. For **asthma** treatment, small amount of leaf bud decoction is taken orally for three days. For **Mich** (Sunstroke), one cup of juice of leaves crushed together with leaves of Tena Adam (*Ruta chalepensis* L.) and Tejesar (*Cymbopogon citratus* Stapf) in water is administered orally for three days, or leaf crushed together with leaves of Bisana (*Croton macrostachyus* Hochst. ex Delile) is smelled. For **Tikusat** (fever), one cup of juice of crushed leaves together with leaves of Sensel (*Justicia schimperiana* T.Anderson) in water is taken orally for one day and body is washed by the crushed leaves. For **snake bite**, small amount (part of a Melekia that immerses half of the

small finger's internodes) of juice of crushed bark in water is taken orally for one day. For **stomach pain in children**, small amount of juice of crushed leaf bud in water is given orally for one day. To destroy **head-lice**, hair is washed with leaves crushed in water. For **wound** healing, powdered root-bark mixed with butter is applied on the wound in the evening and the powder is sprinkled on the wound in the morning, or powder of dried leaf mixed with lemon juice is applied on the wound twice a day for three days. **Bed wetting in children** is said to be treated by exposing part of the child's body below the neck to the smoke produced by putting twigs on a burning charcoal. Oral administration of about five milliliter of the filtrate of dried leaf powder in cold water for one day was claimed as a treatment when a woman encounters over bleeding due to **menstruation.**

Reported traditional treatments for livestock health problems

Livestock health problems reported to be treated by *P. africana* are wound on cattle, horse, mule and donkey; eye illness in cattle; fungal disease on calf's skin; stomach-ache in cattle; and shivering in cattle. It was also claimed that *P. africana* is used for increasing milk production in cow, and for fattening of ox. For **wound** treatment on cattle, horse, mule and donkey, the powder of dried bark mixed with salt is sprinkled on wound, or juice of crushed leaf or bark is applied on wound until it heals. To treat **eye illness** in cattle, juice of the crushed leaf is applied on the sick-eye for three days. **Fungal disease** on calf's skin was said to be treated by applying juice of crushed bark on infected skin and administering half a bottle of the juice orally.

Table 14. Reported traditional medicinal uses of *Prunus africana* to treat health problems in both human and livestock in Ethiopia.

	Health problem	% Inf	Part used	Application route
Human	Strong cough	8	bark, leaf	oral
	Mich (Sunstroke)	4	leaf	oral[1], nasal[2]
	Wound	4	root-bark, leaf[3]	topical
	Snake bite	4	bark	oral
	Asthma	3	leaf	oral
	Stomach pain in children	2	leaf	oral
	Tikusat (fever)	3	leaf[4]	oral, topical
	Head-lice	2	leaf	hair
	Bed wetting in children	2	twig	topical
	Menstruation problem	3	leaf	oral
Livestock	Wound on cattle, horse, mule and donkey	13	leaf, bark	topical
	Eye illness in cattle	4	leaf	topical
	Fungal disease on calf's skin	2	bark	topical, oral
	Stomach-ache in cattle	2	bark[5]	oral
	Shivering in cattle	1	bark[6]	topical
	Urination difficulty in cattle, horse, mule and donkey	2	leaf	nasal
	Reduced milk production in cow	2	leaf	oral
	Skinniness of ox	2	bark	oral

% Inf = Percentage of informants, [1]Mixed with *Ruta chalepensis* L. and *Cymbopogon citratus* Stapf; [2]mixed with *Croton macrostachyus* Hochst. ex Delile; [3]mixed with lemon juice; [4]mixed with *Justicia schimperiana* T.Anderson; [5]mixed with *Albizia schimperiana* and *Millettia ferruginea*; [6]mixed with *Echinops kebericho* and frankincense.

To treat **stomach-ache** in cattle, two cups of juice of the bark crushed together with barks of Sessa (*Albizia schimperiana* Oliv.) and Birbira (*Milletia ferruginea* Hochst.) is given orally for 3-4 days. Bark of *P. africana*, Kebercho (*Echinops kebericho* Mesfin), frankincense and hen's feces are smoked near the animal as a treatment to **shivering** in cattle. When **urination difficulty** occurs in cattle, horse, mule and donkey, one glass of juice of crushed leaf in water is added through nose openings for one day. To **increase milk** production, one glass of juice of crushed leaf bud in water is given for one day to a cow that gives small amount of milk. To **fatten** a skinny ox, one glass of juice of crushed bark in water with salt is given orally for one day.

4.4.3. Non-medicinal uses of *P. africana*

Other than its medicinal use, *P. africana* was reported to have several uses in house construction, carpentry, making plowing tools, bridge construction, apiculture, local alcoholic drink preparation, wedding ceremonies and other traditional rituals (Table 15).

According to the informants, the wood of *P. africana* is very strong and resistant to pest attack. It is thus highly sought for construction works such as bridges and houses. It is used for making pillars, beam for roof, and doors. However, people around Injibara town do not use the wood for house construction as they believe that it attracts lightening if used for house construction.

The wood has a general use for handles of tools such as axe and hoes. It is also used for making timber, mortar, coffin, grain storage barrel and bed. Branches or young stems are used for making "Mensh" (Ethiopian traditional tool used during the threshing process of cereals and grain legumes) and walking sticks. Branches or small

sized stems are used for making "Kenber" (yoke), "Mofer", "Erf" and "Digir", which are the components of a traditional plowing apparatus that is pulled by a pair of oxen.

Figure 12. A debarked *Prunus africana* tree near Injibara town. The bark is removed to kill the tree so that mortars made from the dried stem will not crack (Photo taken by Ziyin Mihretie, 2012).

The wood is reported to be excellent for charcoal production. Dried twigs and branches are also reported to be suitable for firewood.

Prunus africana trees are reported to be useful in apiculture. It is said that beekeepers prefer *P. africana* trees for mounting traditional hives as they are suitable for this purpose. Barks, resins, as well as leaves are reported to be useful for smoking traditional hives in order to attract bees to the hive. Flowers are also reported to be important for bee foraging.

Leaves are used for making two types of local alcoholic drinks called "Areke" and "Tella". Some informants reported that in order to make Areke "stronger" small amounts of *P. africana* leaves are mixed with the leaves of Gesho (*Rhamnus*

prinoides L'Hér.), which is one of the major ingredients to make the local alcoholic drinks. Leaves are used for washing containers (large pots) that are used for the fermentation process of the local beer called Tella. The leaves are also used for baking bread that is used for making the beer.

In some localities, wedding ceremonies and traditional ritual practices take place under the shade of *P. africana* trees. In East Gojjam, there is a tradition of performing part of a wedding ceremony called "Homa-Koreta" under a tree of *P. africana*. The ceremony takes place in the morning of the wedding day. For the ceremony, the bride or bridegroom is taken to a nearby *P. africana* tree being carried on the back of a man (for bride) or on the shoulder of the man (for bridegroom) accompanied by several people. Usually, at least a tree of *P. africana* is found within a village as it is protected mainly for this purpose. Most of the invited guests, especially youngsters, participate in this ceremony. When the participants of the ceremony reach to the tree, they move round the tree thrice by singing a typical song called "Logaw-Shibo". Then, an earthenware cup called "Tsiwa" filled with "Difdif" is given to the bride or bridegroom to taste it thrice and break the Tsiwa by throwing it to the tree. If the Tsiwa is broken being crashed with the trunk of the tree, it is considered as a sign of good luck to the bride or bridegroom. Then a piece of "Injera" pasted with a type of sauce called "Awaze" is given to each participant. Then each participant of the ceremony takes leaves from the tree and gives it to the bride's or bridegroom's mother at home. The bride's or bridegroom's mother receives the leaves by carrying a traditional sieve called "Wonfit" on her head. Throughout this ceremonial activity the attendants chant, dance and sing songs related to the ceremony.

In some localities of Oromia Region, informants reported that there is a traditional ritual practice called "Kallu" under a large tree of *P. africana*. It is said that such a tree is demarcated and protected by making fences with stones surrounding it. Such a tree is respected and no one is allowed to cut it.

In spite of the fact that *P. africana* is reported to have such a wide variety of medicinal and non-medicinal uses, it is reported to be poisonous. According to the informants, leaves of *P. africana* are not useful as fodder to livestock; they kill if consumed. Especially, cut and wilted leaves are reported to be very toxic to sheep.

Table 15. Reported non-medicinal uses of *Prunus africana* in Ethiopia.

Use category	**Description of uses**
Construction and carpentry	Wood for house and bridge construction, pillar, door, beam for roof, handles of axe and hoe, mortar, coffin, grain storage barrel, bed, timber, "Mensh", walking stick, "Kenber", "Mofer", "Erf" and "Digir"
Fuel/firewood	Dried twigs and branches for firewood, and wood for charcoal
Apiculture	Tree for mounting traditional hives; bark, resin, as well as leaves for smoking traditional hives; flowers for bee foraging
Local alcoholic drinks	Leaves: mixed with leaves of Gesho (*Rhamnus prinoides)* for making "Areke", for washing pots that are used for "Tella" preparation, for baking bread for Tella
Traditional rituals	Part of wedding ceremony called "Homa-Koreta", and a traditional ritual practice called "Kallu" take place under *P. africana* trees

CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1. Discussion

5.1.1. Genetic diversity within populations

The present study showed that there is high genetic diversity in the 21 *P. africana* populations in Ethiopia as revealed by both nuclear and chloroplast microsatellite markers. Within population nuclear gene diversity (H_e = 0.452 - 0.732) is comparable to genetic diversity values of 25 *P. africana* populations in other eight African countries (H_e = 0.430 – 0.827; Kadu *et al.*, 2013), and in eight Kenyan populations (H_e = 0.73 – 0.83; Farwig *et al.*, 2008). However, this value is higher than genetic diversity values observed in other previous studies on *P. africana* based on RAPD markers [H_e = 0.020 – 0.137 (Dawson and Powell, 1999); H_e = 0.041 – 0.150 (Muchugi *et al.*, 2006); H_e = 0.150 – 0.307 (Hailu Atnafu, 2007)]. The RAPD based diversity of Ethiopian *P. africana* is higher than that of other populations assessed by RAPD markers. The differences in the level of genetic diversity between RAPD based and microsatellite based studies could be partly due to the nature of the genetic markers and/or sampling strategies used; microsatellite markers give higher within population diversity value than RAPD markers (Nybom, 2004). The mean within population chloroplast haplotype diversity in the present study (h_S = 0.367) is higher than the result of an African-wide *P. africana* study in 32 populations from nine countries (h_S = 0.234; Kadu *et al.*, 2011), and it is still higher than the total haplotype diversity of five *P. africana* populations in three countries (h_T = 0.242; Mbatudde *et al.*, 2012b).

The study revealed that there are differences in the levels of genetic diversity among the populations investigated. The highest within population nuclear genetic diversity (H_e) was recorded in the Addis Ababa population, while the lowest value was in Denkoro. Gara Muleta and Wof Washa populations had also relatively lower genetic diversity values. The highest nuclear genetic diversity in the Addis Ababa population could be partly due to pooled effect of gene flow mediated by humans. As Addis Ababa is the capital city of Ethiopia, there is high rate of human migration from different parts of the country to the city. Thus, there are possibilities that some of the *P. africana* trees sampled for the present study could have been established from seeds or seedlings, which were accidentally or purposefully brought from other parts of the country.

Generally, populations in the southwestern part of the country had relatively higher genetic diversity. A similar pattern of genetic diversity distribution was found in *Cordia africana* Lam. (Abayneh Derero, 2007). This relatively higher difference in the level of genetic diversity among the populations could be explained by range edge effect; Denkoro, Gara Muleta and Wof Washa populations are the northeastern limits of the range of *P. africana*, which is primarily distributed in southwestern part of Ethiopia extending to other African countries. If this northeastern part of the species range has been recently colonized, diversity levels could be lower due to founder effects or population bottlenecks during migration events (Austerlitz *et al.*, 2000). Recent re-colonization of northern Ethiopia by *Hagenia abyssinica* J.F.Gmel. from possibly south western part of the country was reported based on chloroplast haplotype and fossil pollen evidences (Taye Bekele *et al.*, 2009). According to Taye Bekele *et al.* (2009), fossil pollen evidences also indicate a northward re-colonization of some other tree species such as *Podocarpus falcatus* A.Cunn. ex Parl., *Juniperus*

procera Hochst. ex Endl. and *Olea* L. species in Africa. Unfortunately, there is no such pollen fossil evidence for *P. africana* to supplement the genetic data and make a strong conclusion on the colonization path of the species.

5.1.2. Genetic structure of populations

Genetic differentiation among populations

Significant genetic differentiation among populations of *P. africana* was revealed in both nDNA and cpDNA studies. The genetic differentiation among populations in cpDNA was higher than in nDNA, as indicated by the G_{ST} value of cpSSRs (0.478) and F_{ST} value of nSSRs (0.122). The reasonable difference between F_{ST} and G_{ST} values suggests that historical gene flow via seeds alone moderately reduced the differentiation in nDNA. Thus, the difference between the levels of genetic differentiation in cpDNA and nDNA could be explained by (1) gene flow is higher for nDNA (both seed and pollen dispersal) than in cpDNA (only seed dispersal), and (2) the effect of genetic drift is stronger on cpDNA than on nDNA as the effective population size is lower in cpDNA than in nDNA (Latta, 2004; Pettit *et al.*, 2005). In support of the first explanation, Berens (2010) found that pollen dispersal distance exceeds seed dispersal distance by a factor of 23 in *P. africana*. Mbatudde *et al.* (2012b) also found that gene flow among populations of *P. africana* based on nuclear DNA data was significantly higher than that based on chloroplast DNA.

The level of nuclear genetic differentiation found in this study (F_{ST} = 0.122) is lower than reported for 25 natural populations of *P. africana* from other African countries using the same nSSR markers (F_{ST} = 0.27; Kadu *et al.*, 2013). The value of chloroplast DNA differentiation in the present study (G_{ST} = 0.478) is also lower than

reported for 32 natural populations of *P. africana* from other African countries using the same cpSSR markers (G_{ST} = 0.735; Kadu *et al.*, 2011). Higher values of differentiation indices in the previous studies could be due to the larger maximum geographical distance between the sampled populations (Nybom, 2004), which ranges from Madagascar to Equatorial Guinea: whereas the maximum geographical distance between the sampled populations in the present study is between Gore and Gara Muleta. Thus, the lower level of genetic differentiation among populations investigated in the present study could be due to absence of strong barrier to gene exchange as only a few migrants per generation are necessary to inhibit differences accumulating between populations (Mills and Allendorf, 1996) or the time after populations have been separated could not be large enough to accumulate such big levels of genetic differences.

Pair-wise comparison of populations using F_{ST} values revealed that 95.7% of the population pairs were significantly differentiated. The highest genetic differentiation was between Denkoro and Wof Washa populations. Non-significant differentiation was found only between the following 9 pairs of populations out of the total 210 pairs: BO-BD, CH-NK, CH-AM, CH-BD, AA-BD, AA-BO, BO-AM, and AM-GM. The lack of genetic differentiation among these populations suggests that there was gene flow at least during the establishment of sampled trees for this study. Most of the populations were presumably connected genetically in the past when the Afromontane forests were widely distributed in the country (Breitenbach, 1963). However, in the last few decades the distance between contemporary populations have been increased due to anthropogenic fragmentation of forest ecosystems (Friis, 1992; Demel Teketay and Granström, 1995: Reusing, 2000). Even at present, some remnant trees most likely exist and act as stepping stones for gene flow between some populations like

BO-BD and CH-NK. Homoplasy could also be another possible explanation for non-significant genetic differentiation between the populations, especially for population pairs such as AM-GM and BO-AM for which the geographic distance appears too large for gene exchange to take place (Whitlock, 2011).

The AMOVA analysis showed that most (88.05%) of the nSSR variation lies within populations, a result compatible with previous studies based on RAPD markers (Dawson, 1999; Muchugi, 2006; Hailu Atnafu, 2007). A high within population genetic diversity is a characteristic of species with long-lived individuals, overlapping generations, out breeding mating system and wide distribution range (Nybom, 2004; Aegisdottir *et al.*, 2009). Whereas, for cpSSR markers nearly half (47.8%) of the genetic variation lies among the populations. The different percentage of within and among population genetic variation values obtained for nuclear and chloroplast markers could, in combination with differences in gene flow by pollen and seed (Berens, 2010; Mbatudde *et al.*, 2012b), be due to differences in the impact of genetic drift on chloroplast and nuclear DNAs (Latta, 2004; Pettit *et al.*, 2005).

In this study, when F_{ST} was used as measure of differentiation, 11.95% of the variance was found among the populations. However, when R_{ST} was used as measure of differentiation by taking allele size and stepwise mutation into account, 18.00% of the variance was found among the populations, which suggests that mutation has played a significant role on the differentiation of populations. Still, a higher difference between F_{ST} and R_{ST} values and thus a significant effect of mutation on the regional differentiation of *P. africana* populations was revealed in Kadu *et al.* (2013).

The AMOVA analysis using R_{ST} among groups of populations classified based on geographical units, tree seed zones, and STRUCTURE-based clusters revealed

significant differentiation among the groups. However, there was no significant differentiation among the tree seed zones when F_{ST} was used for the analysis. The absence of significant genetic differentiation suggests that there was gene flow across the seed zones, which were delineated for Ethiopia by Aalbæk (1993) as a basis to regulate the use of tree reproductive material for all important species in the country.

One-way analysis of variance (ANOVA) showed that there was statistically significant variation among the populations of *P. africana* in the quantitative morphological traits assessed. Further analysis by Dunnett T3 post hoc test revealed that 9.0%, 20.0%, 11.9%, 6.2% and 9.5% of the population pairs were significantly different from each other in their mean total height, bole height, DBH, bark thickness, and bark mass, respectively. The significant divergence between populations in quantitative morphological traits could be due to genetic and/or environmental differences or it could be due to differences in age structures of the populations. Variable mean values of DBH were reported for *P. africana* populations at different age groups by different authors; 15.8 cm at 15 years (Cunningham and Mbenkum, 1993), 20.5 cm at 17 years, 27.5 cm at 18 years (Gachie *et al.*, 2012), and 52.2 cm at 18.5 years, 30.7 cm at 21.6 years, 11.7 cm at 15 years (Weru, 2012). Information on heritability of the morphological traits of *P. africana* is not available in the literature. However, height and DBH showed moderate to high narrow and broad sense heritabilities in *Prunus avium* (Muranty *et al.* 1998).

Pearson's correlation analysis revealed significant positive correlations among all quantitative morphological traits, except between bole height and bark thickness. Furthermore, all the quantitative morphological traits, except bark thickness, showed significant negative correlations with altitude, which supports the above suggestion

that environmental factors could be partly responsible for the differences between populations in morphological traits. However, there was no significant correlation between genetic diversity measures and quantitative morphological traits investigated in this study. This could be partly due to the genetic markers used and quantitative trait loci studied are individually inherited and probably no association exists.

Correlation between genetic and geographic distances

To investigate genetic relationship between populations, Nei's unbiased genetic distances were computed and the highest genetic distance was found between Denkoro and Wof Washa populations; whereas the smallest distance was between Chilimo and Bedele populations, which are in line with the values of pair-wise population comparisons using genetic differentiation indices (F_{ST}, G''_{ST} and D_{est}). Environmental barriers, historical processes and life histories may shape the genetic structure of populations (Gerlach and Musolf, 2000; Aegisdottir *et al.*, 2009). Moreover, populations in close proximity are genetically more similar than more distant populations as species' geographical distributions are typically more extended than an individual's dispersal capacity. Indeed, the Mantel test for isolation by distance revealed a significant positive correlation between geographic and Nei's genetic distance matrices of *P. africana* populations investigated in this study using both cpSSR and nSSR markers. A similar result was reported by Hailu Atnafu (2007) for six *P. africana* populations from Ethiopia using RAPD markers. Mbatudde *et al.* (2012b) also found significant correlations between geographic and genetic distances of *P. africana* populations for both cpDNA and nDNA.

Phylogeographic pattern

Phylogeographic analysis relies on interpreting patterns of congruence or lack of congruence between the geographical distribution of alleles and their genealogical relationships (Avise, 2000). A pattern of congruence is observed if closely related alleles are geographically restricted and occur in proximity to each other. Such congruence indicates a long-standing pattern of highly restricted gene flow. This pattern arises when scattering is reduced because the novel mutations remain localized within the geographical context of their origins. In the present study, comparison of R_{ST} and permuted R_{ST} (pR_{ST}) revealed that the nSSR diversity of *P. africana* had marginally significant phylogeographic pattern with $R_{ST} > pR_{ST}$ (P = 0.047) (Hardy and Vekemans, 2002); whereas the comparison of observed N_{ST} (0.483) with mean permuted N_{ST} (0.473) revealed no phylogeographic pattern (P > 0.05) for cpSSR diversity in the investigated populations of the species (Pons and Petit, 1996). One possible explanation for the lack of phylogeographic pattern in cpDNA differentiation is that genetic drift has a stronger effect on cpDNA than on nDNA (Latta, 2004; Pettite *et al.*, 2005) and thus it could have disrupted such patterns in cpDNA. The zero level of within population cpDNA haplotype diversity in 28% of the populations suggests that genetic drift has greatly affected the haplotype frequencies of the populations.

High proportion of cpSSR haplotypes (five of the sixteen) of *P. africana* from Ethiopian populations were shared with 'east' (excluding Uganda) and southern African populations, but only one haplotype was shared with a population from Equatorial Guinea, which could be due to homoplasy. The high proportion of

haplotype sharing with 'east' and southern African populations and a higher mean within population haplotype diversity in the Ethiopian populations supports the hypothesis that suggests southward migration of *P. africana* from the Ethiopian highlands. However, the absence of Ethiopian haplotypes in the 'Western' populations (in both West African and Ugandan populations) do not support either of the two migration scenarios of *P. africana* to West Africa proposed by Kadu *et al.* (2011) as: (i) southward migration of *P. africana* from the southern fringe of the Ethiopian highlands to Albertine Rift valley (Uganda) and then to West Africa or (ii) the independent colonization of West Africa directly from the Ethiopian highlands possibly using the Marra Mountains (Sudan) as stepping stones via a northwestern migration corridor and the subsequent colonization of western Uganda from West Africa.

5.1.3. Indigenous knowledge on *P. africana*

The present ethnobotanic study confirms the multipurpose nature of *P. africana*. Six major use categories (medicinal, construction and carpentry, fuel/firewood, beverage preparation, apiculture, and traditional rituals) were determined for the species. The findings are consistent with previous studies, which reported several uses of *P. africana* from different African countries (Nsom and Dick, 1992; Cunningham and Mbenkum, 1993; Cunningham, 1996; Lambert, 1998; Stewart, 2001). Especially, the three use categories- medicinal, construction and carpentry, and fuel/firewood- are most frequently reported from different distribution range countries of the species.

In this study, strong cough, asthma, Mich, Tikusat (fever), snake bite, stomach pain in children, head lice, wound, bed wetting in children, and menstruation problem (over bleeding) were reported to be treated by using different parts (mainly bark and leaf) of

P. africana trees. Similar human health problems previously reported to be treated by the plant are wound (Stewart, 2003b; Moa Megersa *et al.*, 2013; Sintayehu Tamene, 2011), menstruation problem and fever (Kokwaro, 1976; Stewart, 2003b), and stomach pain (Kokwaro, 1976). Bii *et al.* (2010) demonstrated the antifungal and antibacterial activity of *P. africana* using hexane and methanol stem bark extracts, which supports the claimed traditional medicinal uses of the plant.

Livestock health problems reported to be treated by *P. africana* are wound on cattle, horse, mule and donkey; eye illness in cattle; fungal disease on calf's skin; stomach-ache in cattle; and shivering in cattle. A similar livestock ailment reported in previous studies is wound (Stewart, 2003b; Sintayehu Tamene, 2011). The claim that bark of *P. africana* is used to treat fungal disease on calf's skin is supported by the scientific demonstration of the specie's antifungal activity by Bii *et al.* (2010).

In this study, besides its medicinal use, the species was reported to have several other uses in house construction, carpentry, making plowing tools, bridge construction, fuel wood, apiculture, local alcoholic drink preparation, wedding ceremony and traditional rituals. Similar uses reported earlier include house and bridge construction (Iverson, 1993; Beentje, 1994; Cunningham, 1996), apiculture and fuel (Stewart, 2003b).

5.1.4. Implications for conservation of *P. africana*

The presence of high within population genetic diversity, and the significant genetic differentiation revealed in more than 95% of the population pairs investigated in this study suggest that almost all the populations of *P. africana* deserve conservation, but as there are often limitations of resources to conserve such a large number of populations, prioritization for conservation may be needed. Thus, a weighted-score

population prioritization matrix that integrates genetic, morphological, conservation status, and ethnobotanic criteria was developed and used to prioritize the populations of *P. africana* for *in situ* and *ex situ* conservation of the species. This method is similar to that of Taye Bekele *et al.* (2011).

The following criteria were used to score populations for genetic and morphological traits, conservation status of the populations as well as ethnobotanic values of the species in the localities:

A. Within population diversity of each population (H_e) was scored on a scale from 1 to 5 relative to the mean diversity ($H_e = 0.642$) for all the investigated populations as follows: (1) $H_e < 0.508$, (2) $0.508 \leq H_e < 0.564$, (3) $0.564 \leq H_e < 0.620$, (4) $0.620 \leq H_e < 0.676$, and (5) $0.676 \leq H_e$.

B. Genetic differentiation was scored on a scale from 1 to 5 based on the mean average genetic distance (Nei, 1978) from a population to all others (AGD): (1) AGD < 0.200, (2) $0.200 \leq$ AGD < 0.300, (3) $0.300 \leq$ AGD < 0.400, (4) $0.400 \leq$ AGD < 0.500, and (5) $0.500 \leq$ AGD.

C. Bark mass was scored on a scale of 1 to 5 as the average mass of fresh bark (BM) of all sampled trees in a population: (1) BM < 108.1, (2) $108.1 \leq$ BM < 164.5, (3) $164.5 <$ BM < 220.8, (4) $220.8 \leq$ BM < 277.2, and (5) $277.2 \leq$ BM.

D. Current conservation status was qualitatively assessed by observation of the pressure from surrounding communities and the current level of legal protection on a scale from 1 to 4: (1) Well-protected, not threatened; (2) fair protection, but vulnerable; (3) open accessible and endangered; and (4) open accessible and gravely endangered.

E. Ethnobotanic value was scored on a scale of 1 to 5 based on six use-categories of the plant in a locality. Use-categories had the following values: medicinal (1.5); construction and carpentry (0.5); fuel/firewood (0.5); apiculture (1); local alcoholic drinks (0.5): and traditional rituals (1).

Table 16. Relative weights of different criteria (%) to prioritize *Prunus africana* populations in Ethiopia for *in situ* and *ex situ* conservation.

Criterion	*In situ* conservation	*Ex situ* conservation
A. Diversity (H_e)	45%	50%
B. Genetic distance (AGD)	25%	40%
C. Bark mass (BM)	10%	10%
D. Conservation status	10%	-
E. Ethnobotany	10%	-

The criterion H_e ensures the inclusion of populations with high genetic diversity while average genetic distance (AGD) avoids redundancy by choosing genetically separated populations for both *in situ* and *ex situ* conservations (Taye Bekele *et al.*, 2011). The current conservation status of the populations and the ethnobotanic values of the plant in the localities are important criteria for *in situ* conservation but not applicable to *ex situ* conservation. Similarly, the morphological criterion (bark mass) is important for *ex situ* conservation, domestication, and utilization programs. Based on these criteria, total scores were calculated for each population giving different weight to each criterion (Table 16). The values from all criteria were summed and populations were ranked according to their total score (Table 17).

Table 17. Summary of prioritization results of 21 *Prunus africana* populations in Ethiopia for *in situ* and *ex situ* conservation.

a. Prioritization for *in situ* conservation								b. Prioritization for *ex situ* conservation					
Pop*	H_e	AGD	BM	CS	ET	Sum	Rank	Pop*	H_e	AGD	BM	Sum	Rank
KU	45	15	6	7.5	4	77.5	1	KU	50	24	6	80	1
JI	45	10	10	5.0	7	77.0	2	JI	50	16	10	76	2
AS	45	10	8	7.5	5	75.5	3	AS	50	16	8	74	3
NK	45	10	2	10.0	7	74.0	4	GO	50	16	4	70	4
GO	45	10	4	7.5	7	73.5	5	BU	50	16	4	70	4
BU	45	10	4	2.5	8	69.5	6	NK	50	16	2	68	5
BO	45	5	4	7.5	7	68.5	7	AA	50	16	2	68	5
BD	45	5	6	5.0	7	68.0	8	MN	50	16	2	68	5
IN	36	10	4	10.0	5	65.0	9	BD	50	8	6	64	6
MN	45	10	2	2.5	4	63.5	10	BO	50	8	4	62	7
AA	45	10	2	2.5	-	59.5	11	IN	40	16	4	60	8
AM	36	5	4	7.5	7	59.5	11	DT	30	24	4	58	9
KM	27	10	6	10.0	5	58.0	12	LP	30	16	10	56	10
LP	27	10	10	7.5	2	56.5	13	DE	10	40	6	56	10
CH	36	5	4	2.5	7	54.5	14	AM	40	8	4	52	11
AG	27	10	4	7.5	5	53.5	15	KM	30	16	6	52	11
DT	27	15	4	2.5	4	52.5	16	CH	40	8	4	52	11
HA	27	10	4	2.5	7	50.5	17	AG	30	16	4	50	12
DE	9	25	6	7.5	2	49.5	18	HA	30	16	4	50	12
WW	9	20	2	5.0	2	38.0	19	WW	10	32	2	44	13
GM	9	10	4	10.0	4	37.0	20	GM	10	16	4	30	14

* Pop=population codes follow Table 1; H_e = gene diversity, AGD = average genetic distance between one population and the rest, BM = bark mass, CS = conservation status, and ET= ethnobotany.

The top three priority populations for *in situ* conservation are Kuni, Jimma and Asella. Nekemte population follows in fourth position (Table 17a). Kuni, Jimma and Asella are still the top three priority populations for *ex situ* conservation; Gore and Bulki equally follow in fourth position (Table 17b).

In order to get better insights into the conservation units that can best maintain evolutionary processes and the potential for evolutionary change in the future, consideration of both genetic and ecological information is important (Endashaw Bekele, 1986; Crandall *et al.*, 2000). However, for ethnobotanically important species

consideration of its ethnobotanic importance must be considered. Furthermore, biochemical evaluations are also important because the chemical constituents and amounts may vary based on local conditions. Ecological factors, such as frequency dependent mating, pollinator interactions (Cavers *et al.*, 2003) as well as regeneration status, should also be taken into consideration. Furthermore, as Crandall *et al.* (2000) recommends, management of populations for conservation should consider the following three principles. First, management should aim to preserve adaptive diversity and evolutionary processes across the geographic range of a species. Second, management actions might depend on the severity and nature of recent disturbance on the conservation unit. Third, when possible, management recommendations should be made on the basis of adequate sampling and appropriate analyses. With regard to ecological factors, the present study lacks data but, as a starting point, identification of priority conservation units mainly based on molecular data can provide a valuable practical framework for the conservation of *P. africana* in Ethiopia.

From the ethnobotanic study, practices that have either positive or negative impact on the conservation of *P. africana* were identified. Some traditional practices such as using the tree for construction, carpentry and fuel are detrimental as long as sustainable use is not practiced; whereas other practices such as using leaves and barks for local medicine are not as such damaging to the plant. Still other traditional practices and beliefs such as avoiding the use of *P. africana* wood from house construction by people around Injibara fearing strike of the house by lightening, protecting trees for wedding ceremony in some localities of Amhara region and for the ritual practice called 'Kallu' in some localities of Oromia have positive contribution to the conservation of the species.

5.2. Conclusions

There is high level of genetic diversity in Ethiopian populations of *P. africana* though the species has been highly threatened by deforestation in the country. Nevertheless, there are differences in the level of genetic diversity among the investigated populations. Generally, populations in the southwestern part of the country have relatively higher genetic diversity.

The populations are significantly differentiated from each other in both nDNA and cpDNA. The differentiation among populations in cpDNA is higher than nDNA, which could be due to the differences in the effect of genetic drift and/or the mechanism of gene flow between cpDNA and nDNA. Most of the nuclear genetic variation lies within populations, whereas for that of chloroplasts nearly half of the variation lies among the populations. Significant nuclear genetic differentiation also exists among groups of populations classified based on geographical factors and the software STRUCTURE. However, there is no significant genetic differentiation among the populations collected from different tree seed zones of Ethiopia.

Prunus africana populations in Ethiopia are genetically differentiated through isolation by distance; populations in close proximity are genetically more similar than more distant populations as evidenced by significant correlations between genetic and geographic distances in both nSSR and cpSSR markers.

There is also statistically significant variation among the populations of *P. africana* in the assessed morphological traits. The significant divergence of populations in the morphological traits could be due to genetic and/or environmental differences or it could be due to differences in age structures of the populations.

Nuclear microsatellite diversity of *P. africana* has marginally significant phylogeographic pattern; but no pattern for cpSSR diversity in the investigated populations. The phylogeographic pattern in cpSSR could have been disrupted by genetic drift; as drift has a stronger effect on cpDNA than on nDNA.

The absence of Ethiopian haplotypes in the 'Western' populations (West African and Ugandan populations) does not support migration scenarios of *P. africana* from Ethiopia to 'West Africa'. However, the high proportion of haplotype sharing with 'east' (excluding Uganda) and southern African populations and a higher mean within population haplotype diversity in the Ethiopian populations supports the hypothesis that suggests southward migration of *P. africana* from the Ethiopian highlands.

Prunus africana has several uses in the country and six major use categories are determined for the species. Some traditional practices such as using the tree for construction, carpentry and fuel are detrimental; whereas other practices such as using leaves and barks for local medicines are not as such damaging to the plant. Still other traditional practices and beliefs such as protecting trees for wedding ceremony and rituals have positive contribution to conserve the species.

Based on the weighted-score population prioritization matrix, which is developed in this study, the top three priority populations for *in situ* as well as *ex situ* conservations are Kuni, Jimma, and Asella.

5.3. Recommendations

As the study revealed high within population genetic diversity and significant genetic differentiation among populations, nearly all the investigated populations of *P. africana* deserve conservation. However, as there are often limitations of resources to

conserve such a large number of populations, the weighted-score population prioritization matrix developed based on genetic and morphological data of the present study should be considered during the designing of strategies to conserve the species. Genotypes from the other populations should be introduced to those sites with high population priority; namely Kuni, Jimma, and Assela.

Provenance trials should be carried out to disclose whether the causes of the differences among populations in quantitative morphological traits are genetic and/or environmental or age structure differences.

The tree seed zone system of Ethiopia, which was delineated based on ecological factors needs adjustment for *P. africana* using genetic data from molecular studies and provenance trials.

Biomedical studies are needed to verify the claims on the medicinal values of *P. africana* for both humans and livestock. Prioritizing based on informants' consensus would help to focus the biomedical or pharmacological studies to some key health problems.

Community participatory conservation strategies should be designed to reduce the negative impacts of traditional practices on *P. africana* and to increase the benefits communities gain from the species.

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APPENDICES

Appendix I. Specific sites from where *Prunus africana* samples were collected in Ethiopia.

	Locality	Code	MD*	Specific site
1	Addis Ababa	AA	4	Sheger and Hamle 19 Parks, and 4kilo & 6kilo campuses of AAU
2	Agere Mariam	AG	1	Meressa village, 14km North of Agere Mariam town on the way to Gerba town
3	Amanuel	AM	5	Laydamot village, about 10km North of Amanuel town
4	Asella	AS	1	2km South East of Asella town
5	Bedele	BD	1	3km North West of Bedele town
6	Bonga	BO	2	Within 2km around Bonga town
7	Bulki	BU	0.5	Medhanealem church forest, 0.5km East of Bulki town
8	Chilimo	CH	2	South Eastern part of Chilimo state forest
9	Denkoro	DE	1	Bili village, about 4km South East of Denkoro state forest
10	Debre Tabor	DT	1	Eyesus and Lijitu Mariam churches in Debre Tabor town
11	Gara Muleta	GM	1.5	Gara Muleta escarpment, 4km North West of Girawa town
12	Gore	GO	2	Within 3km around Gore town
13	Harenna	HA	1	About 15km East of Angetu town within the Harenna forest
14	Ingibara	IN	1	Kolela mountain, about 5km South East of Injibara town
15	Jimma	JI	4	5km North West of Jimma town on the way to Agaro town
16	Kibre Mengist	KM	2	Within 3km around Kibre Mengist town
17	Kuni	KU	1	7km South west of Kuni town
18	Lepis	LP	1	2km West of Lepis town
19	Menagesha	MN	1	South Eastern part of Menagesha state forest
20	Nekemte	NK	2	About 5km North East of Nekemte town
21	Wof Washa	WW	0.3	North Western part of Wof Washa state forest

* MD - Maximum distance (km) between sampled trees

Appendix 2. Sequences of 11 microsatellite markers (6 nSSR and 5 cpSSR) used in this study.

nSSR	**Primer sequence (5'-3')**	**Repeat motif**[†]	
EMPAS01	F: CAAAATCAACAAAATCTAAACC R: CAAGAATCTTCTAGCTCAAACC	(GA)9(GA)11	
EMPAS06	F: AAGCGGAAAGCACAGGTAG R: TTGCTAGCATAGAAAAGAATTGTAG	(CT)12	
EMPAS10	F: GCTAATATCAAATCCCAGCTCTC R: TGAAGAAGTATGGCTTCTGTGG	(GA)28	
U3	F: CTGGCTTACAACTCGCAAGC R: CGTCGACCAACTGAGACTCA	(AG)22	
U5	F: TTCTAATCTGGGCTATGGCG R: GAAGTTCACATTTACGACAGGG	(AC)21	
P2	F: GCCACCAATGGTTCTTCC R: AGCACCAGATGCACCTGA	(GA)21	
cpSSR	**Primer sequence (5'-3')**	**Repeat motif**[†]	**Location**
TPSCP1	F: TTGAAAACGAATCCTAATG R: ATTTTCTTTTTCCTTTGTATTATC	(T)9	rpl16 intron
TPSCP5	F: TTTCTATCTCATTGGTCCTT R: ATTCGCTCTTGACAGTGAT	(T)8	atpB-rbcL intergenic
TPSCP10	F: GGTTTCTTTTGAGTTATTTGAG R: CTTTTTTCTTATTCTTCCCCAAC	(T)9	rps16 intron
rps16pm2	F: CAACTTGAGTTATGAGGATAC R: TCGGGATCGAACATCAATTGCAAC	(T)9(G)11(G)10	rps16 intron
trnT-Lpm1	F: CATTACAAATGCGATGCTCT R: CGCTATATTAATAGGTATGTT	(A)4(TA)2	trnT-L spacer

[†] In the species from which the markers were originally developed.

Appendix 3. List of alleles generated at six nuclear and seven chloroplast microsatellite loci of *Prunus africana* from 21 populations in Ethiopia.

	nSSR Primers									
	EMPAS10		EMPAS06		P2		EMPAS01		U5	U3
Allele (PCR fragment size)	134	170	190	212	144	164	230	250	246	142
	138	182	194	214	146	166	236	252	248	150
	140	186	196	216	148	168	240	254	250	
	142	190	198	218	150	170	242	256	252	
	144	192	200	220	152	172	244	258	254	
	146	194	202	222	154	174	246	260	256	
	148	196	204	224	156	176	248	262		
	150	202	206	226	158	178	249			
	152	204	208	230	160	180				
	154	206	210	248	162					
	156	208								
	160	210								
	164	212								
	168									
Total	**27**		**20**		**19**		**15**		**6**	**2**

	cpSSR Loci						
	TPSCP10	TPSCP5	TPSCP1	rps16pm2a	rps16pm2b	trnT-Lpm1a	trnT-Lpm1b
Allele [a,b]	9	8	9	2	1	1	1
	10	9	10				
	11	10					
	12	11					
Total	**4**	**4**	**2**	**1**	**1**	**1**	**1**

[a] number of nucleotide repeats for the microsatellite loci (TPSCP1, TPSCP5, and TPSCP10).

[b] 1 = presence and 2 = absence of fragment for indels (rps16pm2a, rps16pm2b, trnT-Lpm1a, and trnT-Lpm1b).

Appendix 4. Summary of chi-square tests for Hardy-Weinberg equilibrium at six nuclear microsatellite loci for 21 *Prunus africana* populations in Ethiopia.

		nSSR primers					
Population	Pop. Code	EMPAS01	EMPAS06	EMPAS10	U3	U5	P2
Addis Ababa	AA						
Bedele	BD						
Chilimo	CH						
Debre Tabor	DT						
Kuni	KU						
Gore	GO						
Harenna	HA						
Ingibara	IN			*			
Jimma	JI						
Kibre Mengist	KM						
Lepis	LP						*
Menagesha	MN						
Nekemte	NK						
Bonga	BO						
Denkoro	DE		*	**	*	***	*
Agere Mariam	AG						
Asella	AS						
Bulki	BU						
Wof Washa	WW					*	*
Gara Muleta	GM	*					
Amanuel	AM					*	

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

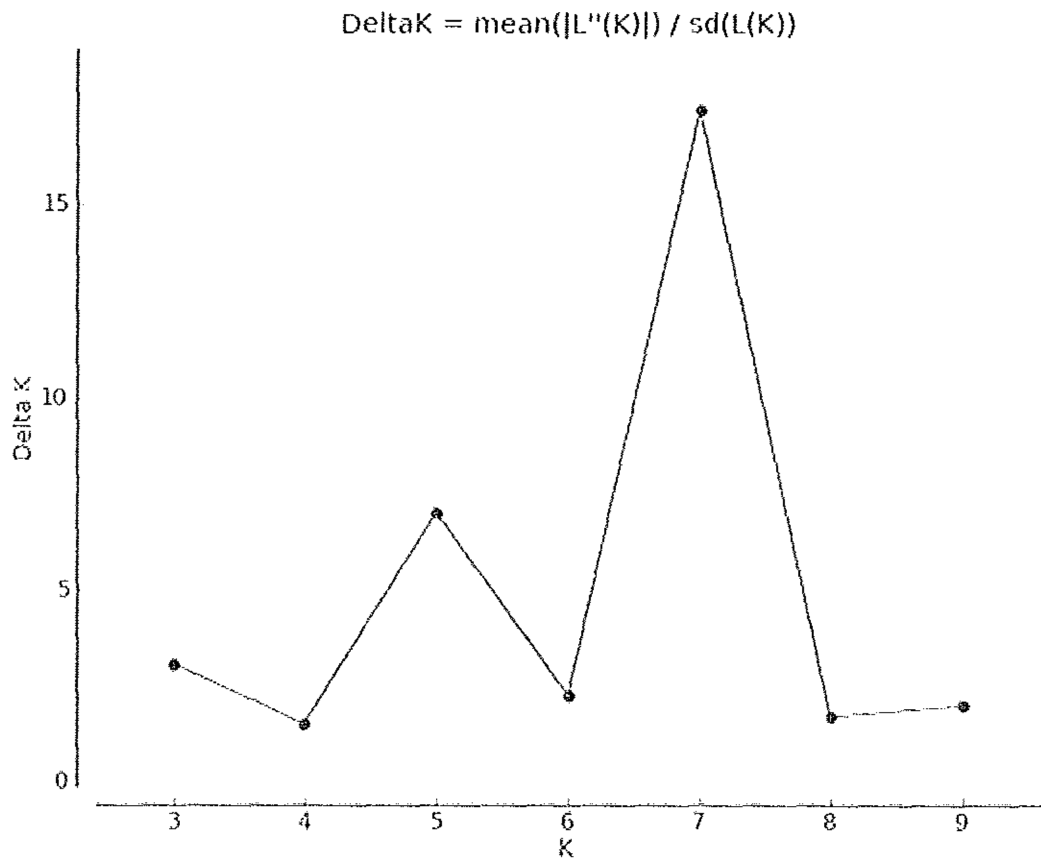
Appendix 5. Summary of linkage e disequilibrium tests among six nSSR loci for 21 *Prunus africana* populations in Ethiopia (Populations with significant linkage disequilibrium at $P < 0.05$ are depicted).

	EMPAS01	EMPAS06	EMPAS10	U3	U5
EMPAS01					
EMPAS06	CH,HA,AG				
EMPAS10	DT,BO,WW	KU,LP,DE			
U3			DE		
U5	KU,KM,WW	DE	BD,LP,DE,AS	KU,BO,DE	
P2	NK,BO,WW,GM	KU,DE,WW	DE,AG,WW,AM		LP,DE,WW

Appendix 6. The Evanno table output of STRUCTURE HARVESTER analysis.

K	Reps	Mean LnP(K)	St. dev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	5	-4463.220000	0.901110	—	—	—
3	5	-4700.480000	150.499375	-237.260000	461.580000	3.066989
4	5	-4476.160000	50.656717	224.320000	76.220000	1.504638
5	5	-4328.060000	22.614663	148.100000	158.120000	6.991924
6	5	-4338.080000	32.985027	-10.020000	74.160000	2.248293
7	5	-4273.940000	19.727975	64.140000	344.540000	17.464540
8	5	-4554.340000	62.620029	-280.400000	106.320000	1.697859
9	5	-4728.420000	74.805862	-174.080000	148.460000	1.984604
10	5	-4754.040000	70.396293	-25.620000	—	—

Appendix 7. The Evanno graph output of STRUCTURE HARVESTER analysis.



Appendix 8. Pair-wise population matrix of G''_{ST} values for 21 *Prunus africana* populations in Ethiopia (G''_{ST} values below the diagonal and probability, P based on 9999 permutations, above diagonal).

	DE	DT	NK	JI	IN	GO	BD	CH	AA	MN	AM	LP	BO	AS	BU	HA	KM	GM	AG	KU	WW
DE		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
DT	0.673		***	***	***	***	**	**	***	***	***	***	***	***	***	***	***	***	***	***	***
NK	0.655	0.230		*	*	**	*	NS	***	**	**	***	*	***	***	***	***	***	***	***	***
JI	0.543	0.279	0.153		**	***	**	**	**	**	***	***	*	***	***	***	***	***	***	***	***
IN	0.635	0.198	0.146	0.169		**	**	*	***	***	**	***	**	***	***	***	***	***	***	***	***
GO	0.590	0.220	0.153	0.297	0.177		**	**	***	***	**	***	**	***	***	***	***	***	***	***	***
BD	0.608	0.136	0.111	0.160	0.160	0.140		NS	NS	*	*	*	NS	**	**	**	***	***	**	**	***
CH	0.552	0.220	0.057	0.183	0.138	0.175	**0.011**		**	**	NS	**	*	*	**	**	**	***	***	***	***
AA	0.595	0.267	0.330	0.247	0.358	0.342	0.094	0.193		***	**	***	NS	***	***	***	***	***	***	***	***
MN	0.616	0.329	0.224	0.305	0.312	0.246	0.148	0.151	0.260		**	**	*	***	***	**	***	***	***	***	***
AM	0.551	0.309	0.270	0.333	0.263	0.147	0.136	0.099	0.212	0.167		*	NS	***	***	**	*	NS	**	***	***
LP	0.627	0.325	0.348	0.348	0.370	0.228	0.142	0.228	0.227	0.218	0.150		*	***	***	**	*	***	*	***	***
BO	0.587	0.292	0.154	0.185	0.239	0.167	0.062	0.133	0.097	0.155	0.098	0.158		**	NS	**	*	***	**	**	***
AS	0.681	0.473	0.300	0.275	0.299	0.388	0.217	0.141	0.260	0.250	0.327	0.336	0.241		**	**	***	***	***	***	***
BU	0.598	0.505	0.339	0.346	0.409	0.333	0.235	0.220	0.246	0.264	0.281	0.336	0.055	0.196		***	***	***	***	***	***
HA	0.714	0.406	0.315	0.408	0.345	0.288	0.192	0.151	0.318	0.240	0.188	0.190	0.211	0.146	0.305		***	**	**	***	***
KM	0.594	0.420	0.361	0.456	0.447	0.263	0.225	0.214	0.287	0.266	0.086	0.128	0.128	0.352	0.272	0.196		**	**	**	***
GM	0.685	0.548	0.496	0.531	0.514	0.398	0.376	0.356	0.393	0.301	0.080	0.275	0.279	0.389	0.396	0.248	0.157		***	***	***
AG	0.699	0.399	0.413	0.430	0.452	0.289	0.243	0.321	0.349	0.234	0.216	0.093	0.189	0.352	0.346	0.238	0.133	0.265		***	***
KU	0.762	0.462	0.340	0.359	0.465	0.405	0.274	0.273	0.260	0.283	0.251	0.322	0.215	0.249	0.295	0.273	0.255	0.339	0.381		***
WW	**0.799**	0.573	0.429	0.513	0.450	0.409	0.530	0.504	0.628	0.498	0.431	0.458	0.374	0.566	0.477	0.457	0.427	0.474	0.346	0.539	

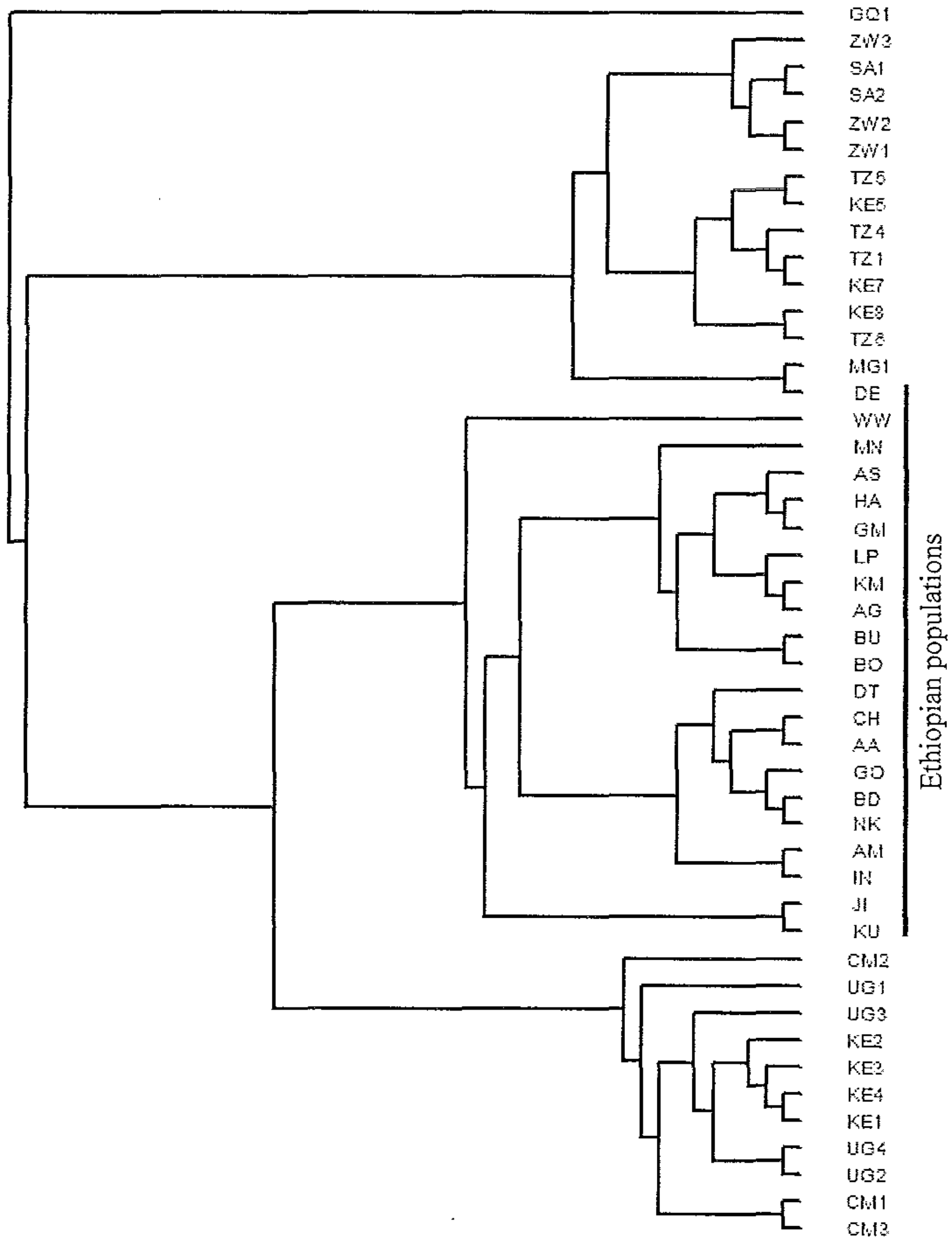
* $P < 0.05$; ** $P < 0.01$;** $P < 0.001$; NS, non-significant ($P \geq 0.05$)

Appendix 9. Pair-wise population matrix of D_{est} values for 21 *Prunus africana* populations in Ethiopia (D_{est} values below the diagonal and probability, P based on 9999 permutations, above diagonal).

	DE	DT	NK	JI	IN	GO	BD	CH	AA	MN	AM	LP	BO	AS	BU	HA	KM	GM	AG	KU	WW
DE		***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
DT	0.521		***	***	***	***	**	***	***	***	***	***	***	***	***	***	***	***	***	***	***
NK	0.526	0.166		*	*	**	*	NS	***	**	**	***	*	***	***	***	***	***	***	***	***
JI	0.403	0.201	0.113		**	***	**	**	**	***	***	***	*	***	***	***	***	***	***	***	***
IN	0.485	0.133	0.104	0.118		**	**	*	***	***	**	***	**	***	***	***	***	***	***	***	***
GO	0.449	0.154	0.113	0.224	0.125		**	**	***	***	**	***	**	***	***	***	***	***	***	***	***
BD	0.474	0.095	0.082	0.118	0.114	0.103		NS	NS	*	*	*	NS	**	**	**	***	***	**	**	***
CH	0.408	0.154	0.041	0.133	0.095	0.126	0.008		**	**	NS	**	*	*	**	**	**	***	***	***	***
AA	0.462	0.196	0.263	0.188	0.275	0.268	0.070	0.143		***	**	***	*	***	***	***	***	***	***	***	***
MN	0.479	0.244	0.171	0.234	0.233	0.184	0.110	0.109	0.201		**	**	*	***	***	***	***	***	***	***	***
AM	0.397	0.217	0.200	0.247	0.184	0.101	0.096	0.066	0.154	0.117		*	NS	***	***	**	*	NS	**	***	***
LP	0.469	0.226	0.261	0.256	0.266	0.159	0.099	0.159	0.163	0.154	0.098		*	***	***	**	*	**	*	***	***
BO	0.456	0.217	0.117	0.139	0.177	0.124	0.045	0.097	0.072	0.116	0.069	0.111		**	NS	**	*	***	**	**	***
AS	0.550	0.370	0.233	0.208	0.221	0.304	0.164	0.101	0.200	0.189	0.243	0.247	0.184		**	**	***	***	***	***	***
BU	0.463	0.402	0.268	0.270	0.318	0.258	0.180	0.164	0.190	0.202	0.207	0.249	0.040	0.146		***	***	***	***	***	***
HA	0.567	0.293	0.233	0.308	0.246	0.206	0.136	0.102	0.237	0.171	0.125	0.124	0.151	0.100	0.224		***	**	***	***	***
KM	0.436	0.307	0.273	0.353	0.335	0.187	0.162	0.149	0.212	0.192	0.055	0.082	0.090	0.262	0.198	0.129		**	**	**	***
GM	0.504	0.400	0.372	0.399	0.373	0.279	0.267	0.243	0.282	0.204	0.046	0.171	0.191	0.273	0.282	0.152	0.093		***	***	***
AG	0.547	0.285	0.315	0.325	0.336	0.206	0.174	0.231	0.261	0.165	0.144	0.058	0.133	0.259	0.256	0.157	0.084	0.162		***	***
KU	0.642	0.356	0.264	0.276	0.363	0.316	0.209	0.203	0.198	0.214	0.179	0.233	0.162	0.185	0.225	0.194	0.181	0.229	0.281		***
WW	0.651	0.425	0.312	0.382	0.316	0.289	0.406	0.372	0.506	0.372	0.298	0.316	0.267	0.436	0.353	0.315	0.291	0.305	0.222	0.405	

* $P < 0.05$; ** $P < 0.01$;** $P < 0.001$; NS, non-significant ($P \geq 0.05$)

Appendix 10. UPGMA dendrogram for 46 *Prunus africana* populations including the data set of Kadu *et al.* (2013). See Appendix 12 for abbreviations of non-Ethiopian populations and Table 1 for that of Ethiopian populations.



Appendix 11. Principal Co-ordinate Analysis showing the multivariate relationships of 46 *Prunus africana* populations including data set of Kadu *et al.* (2013). See Appendix 12 for abbreviations of non-Ethiopian populations and Table 1 for that of Ethiopian populations.