ADDIS ABABA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

COMPARATIVE PHYLOGEOGRAPHY AND CONSERVATION GENETICS OF SOME AFRO-MONTANE PLANT SPECIES FROM ETHIOPIAN AND TROPICAL EAST AFRICAN MOUNTAINS AS INFERRED FROM AFLP

A Dissertation Presented to the School of Graduate Studies of Addis Ababa University in Partial Fulfilment of the Requirement for the Degree of Doctor of Philosophy in Biology (Applied Genetics)

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Table of Contents

Page

Acknowledgements i

Table of Contents iii

List of Tables v

List of Figures vi

List of Appendices viii

Abstract ix

1. Introduction 1

1.1. The afro-montane/-alpine ecosystem 1

1.2. Pleistocene climatic fluctuations and the afro-montane/-alpine flora 4

1.3. Characterization and Distribution of the Study Species 9

1.3.1. *Erica arborea* (Ericaceae) 10

1.3.2. *Hypericum revolutum* (Hypericaceae) 11

1.3.3. *Lobelia giberroa* (Lobeliaceae) 13

1.4. Amplified fragment length polymorphism (AFLP) and its power to address Phylogeographic histories of species 15

1.4.1. Basic principles of AFLP 15

1.4.2. Interpreting AFLP bands 18

1.4.3. Advantages and limitations of AFLP 18

1.4.4. AFLP Applications 19

1.5. Phylogeographic study 20

1.6. Conservation Genetics and Biodiversity 21

1.7. Objectives of the study 22

2. Material and Methods 24

2.1. Sampling 24
2.2. DNA isolation and AFLPs..24
2.2.1. DNA isolation 24
2.2.2. AFLPs 25
2.3. Visualizing and scoring AFLP bands 30
2.4. Data Analyses 30
3. Results and Discussions...33
3.1. *Erica arborea*...33
3.1.1. Results...33
3.1.2. Discussion..43
3.2. *Hypericum revolutum* 47
3.2.1. Results...47
3.2.2. Discussion..58
3.3. *Lobelia giberroa* 61
3.3.1. Results...61
3.3.2. Discussion..72
4. Conclusion 78
5. References..83
Appendices...93
Declaration...96

List of Tables

Table 1. Restriction sites for the restriction enzymes (symbols ↓↑include the actual cutting sites), adapters (double stranded) and pre-selective prime sequences (only single-stranded primers are shown) used in the AFLP analysis of *E. arborea*, *H. revolutum* and *L. giberroa*.27

Table 2. Selective primer sequences and the primer pair combinations (*Eco*RI with the corresponding *Mse*I) used for AFLP analysis for each species 29

Table 3. Locality, number of individuals (n) and gene diversity (D) of the investigated populations of *E. arborea*. 39

Table 4. Locality, number of individuals (n) and gene diversity (D) of the investigated populations of *H. revolutum*. 52

Table 5. Average gene diversity among populations for the two main groups of *H. revolutum*. 54

Table 6. Analysis of molecular variance (AMOVA) of the AFLP data for *H. revolutum*. 55

Table 7. Locality data, number of individuals (n) and gene diversity (D) of the investigated populations of *L. giberroa* 66

Table 8. Average gene diversity among populations and the number of private AFLP markers for the three major groups of *L. giberroa* 68

Table 9. Analyses of molecular variance (AMOVA) of the AFLP data for *L. giberroa*. 69

List of Figures

Fig. 1. Map of East Africa showing the Eastern and Western branches of the East African Rift System. 2

Fig. 2. Heavy oxygen isotope (^{18}O) record in deep sea sediments showing climatic oscillations during Quaternary (Hays *et al*., 1976). 4

Fig. 3. Schematic representation of range shifts of the three vegetation belts of the afro-alpine region in response to climate fluctuations during the Pleistocene. 8

Fig. 4. Distribution map of *E. arborea*. 11

Fig. 5. Distribution map of *H. revolutum*. 12

Fig.6. Distribution map of *L. giberroa*. 14

Fig. 7. Schematic representation of the basic principles of AFLP analysis 17

Fig. 8. Principal coordinates analysis (PCO) of individual AFLP phenotypes of *E. arborea* from 38 populations based on the Dice similarity coefficient. 35

Fig. 9. Neighbour-joining tree of the AFLP phenotypes of 230 individual plants of *E. arborea* from 38 populations based on Nei and Li (1979) genetic distance. 37

Fig. 10. The intra-population gene diversity of *E. arborea*. 40

Fig. 11. Structure analysis of the AFLP data for *E. arborea*. 42

Fig. 12 Geographic origin of the 38 *E. arborea* populations analysed and their grouping according to Bayesian clustering analysis (Structure) based on AFLP markers. 43

Fig. 13. Principal coordinates analysis (PCO) of individual AFLP phenotypes of *H. revolutum* from 33 populations based on the Dice similarity coefficient. 48

Fig. 14. Neighbour-joining tree of the AFLP phenotypes of 227 individual plants of *H. revolutum* from 33 populations based on Nei and Li (1979) genetic distance. 50

Fig. 15. Map showing intra-population gene diversity of *H. revolutum*. 53

Fig. 16. Structure analysis of the AFLP data for *H. revolutum*. 56

Fig. 17. Map showing the population structure of *H. revolutum*..57

Fig. 18. Principal coordinate analysis (PCO) of individual AFLP phenotypes of *L. giberroa* from 25 populations based on the Dice similarity coefficient 63

Fig. 19. Neighbour-joining tree of the AFLP phenotypes of 191 individual plants of *L. giberroa* from 25 populations based on Nei and Li genetic distance. 65

Fig. 20. The Intra-population gene diversity of *L. giberroa*..67

Fig. 21. Structure analysis of the AFLP data for *L. giberroa*. 71

Fig. 22. Map showing the population structure of *L. giberroa*...72

List of Appendices

Appendix 1a. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *E. arborea* …………...……………..93

Appendix 1b. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *H. revolutum* …………...……94

Appendix 1c. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *L. giberroa* ………………………….95

Abstract

The afro-alpine region s.l. refers to the high mountains of Ethiopian and Topical East Africa. The flora of this region is unique in composition compared to the surrounding lowlands and subdivided into three altitudinal vegetation belts: the afro-montane, ericaceous and afro-alpine belts. The origin and extant distribution of the flora had been influenced strongly by the Pleistocene climatic oscillations. During glacial (cold) periods, the afro-alpine and ericaceous belts were broad while the afro-montane belt was reduced and fragmented. The montane forests had probably persisted in refugia located at mountain foothills or in river basins. Afro-montane forest was extensive during interglacial (warm) periods whereas the afro-alpine and ericaceous belts were compact. Based on amplified fragment length polymorphisms (AFLPs) data, we provide insights into the origin, fragmentation and evolution of some key afro-montane species in relation to Pleistocene climatic oscillations. In view of this, we focus on the genetic diversity and population genetic structure of these afro-montane plant taxa without obvious long distance dispersal morphology and that are widely distributed in the East African mountain systems (i.e., *Erica arborea*, *Hypericum revolutum* and *Lobelia giberroa*). Regardless of some general observed phylogeographic trends such as divergent lineages, contact zones, altitudinal shift and complex refugia, our analyses highlight that each species had a unique phylogeographic pattern and the geographical consistency of the genetic patterns differ considerably. Our observation further suggest that *L. giberroa* and *H. revolutum* genetic groups occupied geographically well defined areas while the geographic pattern of *E. arboreia* is rather complex. Furthermore, we have attempted to identify important areas for conservation of the current study species based on concepts of hotspots of intraspecific diversity and evolutionary significant units (ESU). This approach could be followed to assess or design appropriate genetic conservation priorities as well as a robust sampling strategies

for further studies. This study is the first of its kind concerning the impacts of Pleistocene glaciations on the genetic structures of afro-montane/-alpine plants and it will certainly guide to scope future phylogeographic studies in Africa. In prospect, it is important to cover the whole distribution range of the study species to have more complete phylogeographic picture of the region. In addition, it is appropriate to investigate many wide-spread plant species with well-documented fossil data and use appropriate genetic criteria to deduce the impact of Pleistocene climatic fluctuations on the genetic structure of the present day afro-montane/-alpine flora.

Key words: AFLP, Afro-alpine, Afro-montane, Conservation, *Erica arborea, Hypericum revolutum, Lobelia giberroa*, Phylogeography

1. Introduction

1.1. The afro-montane/-alpine ecosystem

The Great Rift Valley of East Africa (Fig. 1), which begun to form during the upper Cretaceous (a period of warm climate) run across highlands of Ethiopia and tropical East Africa from north to south with two main branches, i.e. Eastern and Western Rift (Hedberg 1961). Along the faults of the Rift Valley there appeared an intense volcanic activity, which in a due course has formed a number of high volcanic peaks (Hedberg 1951). Ruwenzori and the Virunga volcanoes are along the Western rift, while Mt. Elgon, Cherangani Hills, Aberdare, Mt. Kenya, Mt. Kilimanjaro, and Mt. Meru and all mountains of Ethiopia (e.g. Bale Mountains, Mt. Kaka, Mt. Chilallo, Gara Muleta, Mt. Choke and Simen Mountains) are located along the Eastern rift (Hedberg 1961). These scattered and isolated high mountains constitute afro-alpine s.l. environment of East Africa. Most of these mountains are of volcanic origin and have unequal ages ranging from the Miocene to Late Pleistocene (Hedberg 1970). They form biological 'islands in the sky' and have altitudes between 3500 and 6000 m (Hedberg 1969).

Thus, the afro-alpine s.l. environment is comprised of scattered high mountains of Ethiopia and tropical East Africa (Kenya, Tanzania and Uganda) and has a remarkable climate which has been characterized as 'summer every day and winter every night' (Hedberg 1964). High altitude and proximity of these mountains to the equator are apparent geographical circumstances governing the climate of the afro-alpine environment. The latter is also endowed with unique flora. Commonly, three altitudinal differentiated vegetation belts are recognized on these African high mountains, i.e., the montane forest belt with dominant formation of broad-leaved species and some conifers, the ericaceous belt where

sclerophyllous trees and shrubs are predominant, and the afro-alpine belt which varies greatly in terms of its species composition but with its landmarks such as giant ground sels and lobelias (Hedberg 1951; Hamilton 1982). Generally, the afro-alpine environment is known for its large number of endemic giant senecios and giant lobelias which exhibit distinct adaptations to high altitude climate. Some species of the giant lobelias and giant senecios have restricted geographic range while other species are widespread through out the afro-alpine region (Hedberg 1969; Knox 1993). The adaptations of African giant senecios and lobelias are paralleled only by *Espeletia* of tropical America.

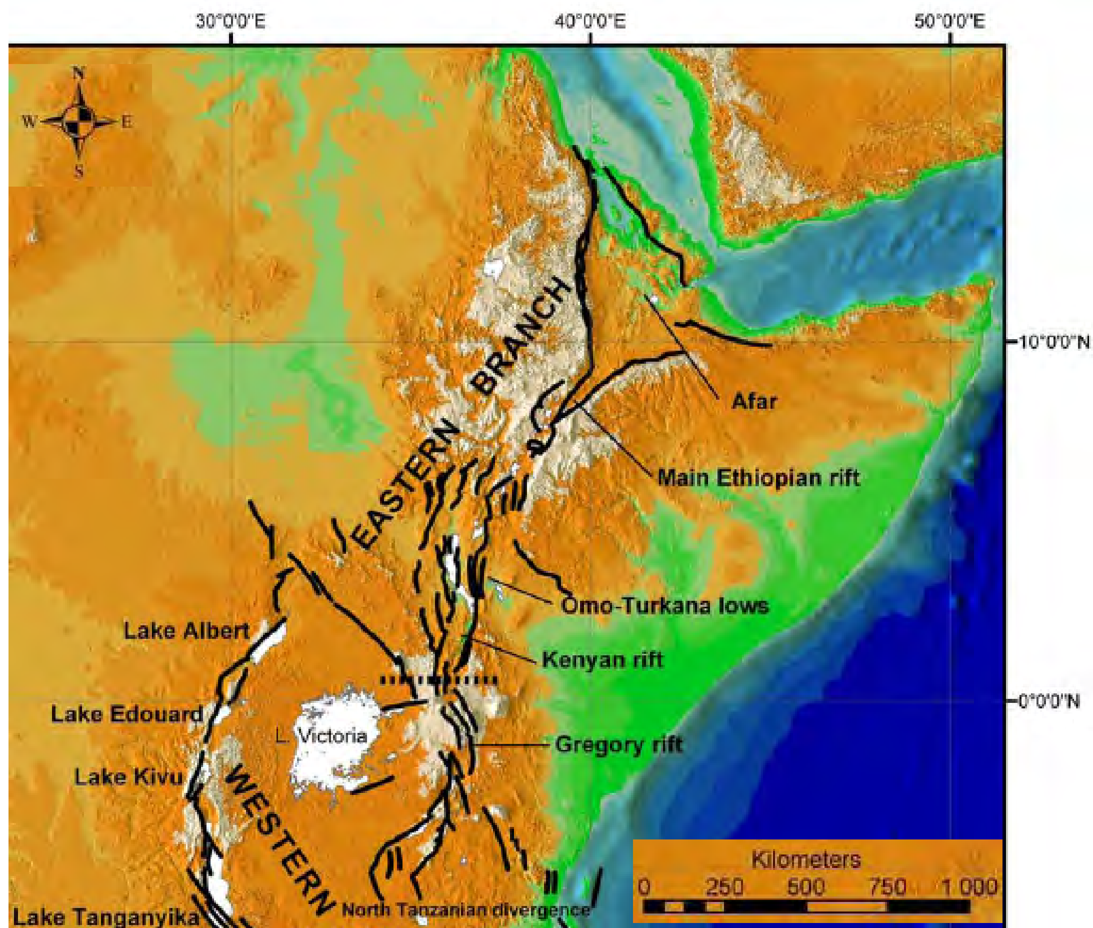


Fig. 1. Map of East Africa showing the Eastern and Western branches of the East African Rift System. Black lines represent the main faults (Modified from Chorowicz 2005).

Outside Ethiopia and tropical East Africa, afro-alpine flora type occurs on the high mountains of Yemen, Cameroon, South tropical Africa and South Africa but with a few taxa. Thus, the origin of the afro-alpine flora is predominantly African-tropical or Palearctic with smaller contributions from South Africa and other more distant sources (Harmsen *et al*., 1991). Immigration from Paleartictic region was suggested due to the presence of some linking species in the afro-alpine (e.g. *Arabis alpina*, *Cardamine hirsuta* and most mosses) which have their cradle of origin in the Northern Hemisphere populations. It furthermore appears that the flora is of mixed derivation since it contains important Boreal, Mediterranean, Himalayan, South African, Cape and South-hemispheric temperate elements (Hedberg 1965).

Once the ecosystem was established, dispersal from one mountain to others became a main source of increasing complexity. More than 80% of afro-alpine vascular taxa are endemic to the high mountains of tropical East Africa and Ethiopia, indicating that it has long been isolated from other high mountain and temperate floras (Hedberg 1970). For instance, the outlier of afro-montane flora present outside the afro-alpine region (e.g. in Cameroon) has been attributed to long distance dispersal, but this is open to a debate. Fragmentation due to a vicariant event cannot be excluded as explanation for the present day disjunction of afro-alpine flora (Burgoyne *et al*., 2005). For most of the species, dispersal is difficult among the mountains due to the scattered nature of the mountains which is like an island in the sea of low lands. Hedberg (1969) suggested cyclones as massive long-distance dispersal agents in connection to afro-alpine flora. Dispersal among adjacent mountains, especially for montane plant elements, was more likely when montane forest was more wide-spread during both the previous interglacials and in earlier period of the present interglacial, i.e., prior to the expansion of agriculture (Hedberg 1969). In such a case, populations on adjacent mountains or on mountains connected by highlands would be expected to be more closely related to each

other than to populations on more isolated mountains. If, on the contrary, most dispersal events were random such as cyclones or independent rare long-distance dispersal, more random or unstructured genetic patterns would be expected.

1.2. Pleistocene climatic fluctuations and the afro-montane/-alpine flora

Quaternary (the last two million years) is the most recent period which includes two epochs, namely, the Pleistocene (most recent) and Holocene (started 10,000 years BP), which is wholly recent and the present warm interval within which we live (Lowe & Walker 1997). Quaternary is characterized by climatic oscillations between glacial and interglacial periods (Fig 2). Glacial (cold) period is known for the expansion of ice sheets and glaciers at the high latitude which has resulted in a reduced sea level and formation of land bridges whereas interglacial (warm) periods mark the melting of ice and rise of sea levels. The major glaciation has occurred at about 20,000 years (20 kyr) ago BP and it is known as the last glacial maxima (LGM).

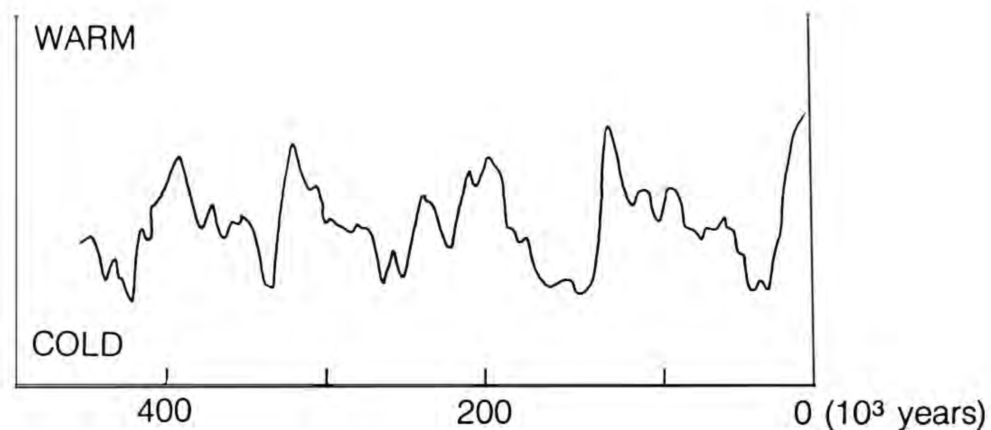


Fig. 2. Heavy oxygen isotope (^{18}O) record in deep sea sediments showing climatic oscillations during Quaternary (Hays *et al*., 1976).

The cause of climatic fluctuations, i.e., the onset of glaciations and subsequent interglacial periods, is explained by Milankovitch theory, named after a Serbian Engineer and Mathematician. According to this theory, the Quaternary glacial–interglacial cycles are due to changes in the parameters of Earth's orbit around the sun. These long-term parameters are orbital eccentricity, obliquity and precession with a cycle spanning 100,000, 41,000 and 19,000 to 23,000 years, respectively (Webb & Bartlein 1992; Lowe & Walker 1997; Taberlet & Cheddadi 2002). Evidences for global climatic changes and their causes (data of palaeo-climates) comes from various physical and biological sources (animal and plant remains, e.g. fossil records of pollen and beetles) obtained from cores of sea bed, lake bottoms and ice sheets (Bennett 1997; Williams *et al*., 1998). The sources are analyzed for carbon and oxygen isotope level, CO_2, magnetic and mineral signatures (Hays *et al*., 1976; Hewitt 1999).

In general, climate fluctuations of the past few million years have caused major biological events such as extinction and repeated range shifts of taxa. The Quaternary climate oscillations have induced extensive extinction and re-colonization from refugia in higher latitudes and altitudinal shift and manifestation of complex refugia near the tropics (Hewitt 2004). The Quaternary refugia represent areas of special value for the long-term persistence of biodiversity (Taberlet & Cheddadi 2002). Expansion of species from such refugia depends on sharpness of the climate change, the latitude and the topography of the region, dispersal and the reproductive capabilities of the organism. Thus, it is apparent that the major shifts in climate were felt differently across the globe owing to regional differences in landform, ocean currents and latitude. Furthermore, species responded individually, and their range changes were meticulous to local geography and climate (Hewitt, 2000).

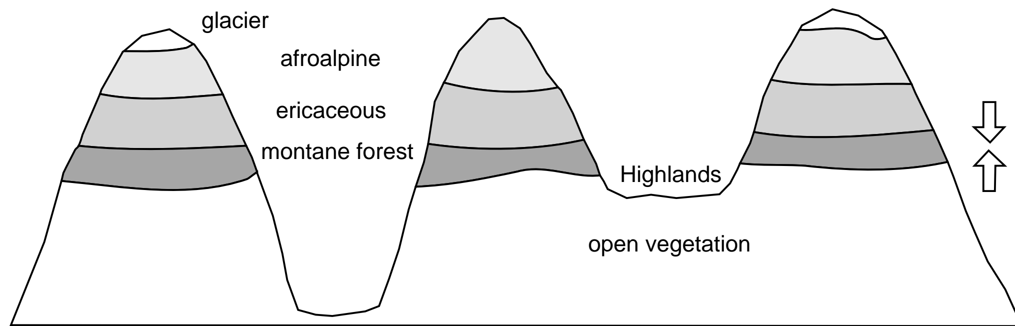
Obviously, Pleistocene climatic fluctuations have shaped the distribution of the present day African flora (Livingstone 1962; Hamilton 1982; Harmsen *et al.*, 1991; Mohammed & Bonnefille 1998; Gottelli *et al.*, 2004). However, many studies have been dedicated to the studies of the impact of the Pleistocene climate fluctuations on the phylogeographic history of northern hemisphere species (e.g. Taberlet *et al.*, 1998; Hewitt 2000; Brochmann *et al*., 2003 for reviews). Only very few such studies have been recently undertaken for afro-montane bird species in Africa (e.g. Bowie *et al*., 2006), insects (Brühl 1997) and for plants (Kebede *et al*., 2005, 2007 a & b). Climate changes and vegetation history of eastern Africa have been documented based on studies on sediment cores (e.g. Bakker 1962; Mohammed & Bonnefille 1998; Trauth *et al.*, 2005) and have revealed cycles of varying temperature and humidity. Traces of extensive earlier glaciations on some of the mountains, such as Kilimanjaro and Mt Kenya, provide information about past climates in the region as well. During the last glacial maxima, temperature decrease was estimated at 4-6°C below the present mean temperature on most of the East African Mountains (Hedberg 1969). Despite changing climates, the mountains provided relatively stable habitats where older species survived by altitudinal range shifts and new lineages were generated (Fjeldså & Lovett 1997; Hewitt 2000; Tzedakis *et al.,* 2002).

It is to be noted that most of Africa and Madagascar were not glaciated during the LGM and they have, therefore, largely escaped the drastic vegetation extinctions that affected so much of the terrestrial habitats in the temperate parts of the northern hemisphere. This may account for the current relatively large proportion of palaeo-endemics for African and Madagascan floras (Burgoyne *et al*., 2005). Furthermore, a high level of endemism could be that the afro-alpine environment is isolated from the neighbouring lowland African ecosystem and the long distance between the African mountains and similar environments elsewhere in the world

throughout the Pleistocene (Harmsen *et al*., 1991). Thus, the present genetic diversity, species richness and patterns of endemism in the afro-alpine region are of complex origin, associated with different factors such as age of isolation, historical climate oscillations and some other outstanding factors. In this study we will address one particular aspect, i.e. the impacts of glacial-interglacial cycles on the geographical distribution and genetic structure of some afro-montane species of East Africa.

During humid periods (interglacials at high latitudes) the afro-montane forest has expanded to cover low-lying ridges and highlands. Aridification in response to glaciation at higher latitudes and altitudes, on the contrary, has favoured the expansion of open vegetation and reduced the extent of the afro-montane forest, i.e., the latter is spatially fragmented (Fig. 3). Three major periods of aridity (peaked near 2.8, 1.7, and 1.0 Ma) were inferred from a study of deep sea cores (deMenocal 1995 & 2004). These periods of aridity roughly corresponded to high latitude ice ages. As a result, the African tropics were colder and drier during the last glaciation (Bonnefille *et al*., 1990) and the present vegetation belts of the afro-alpine region were pushed down by more than 1000 m (Mareau 1963; Flenley 1979). However, the afro-alpine and ericaceaous vegetation belts were larger than at present (e.g. Gottelli *et al*., 2004) while the afro-montane forest belt was contracted (Fig. 3). The upper limit of the afro-montane forest was pushed down due to expansion of glaciers and afro-alpine and ericaceous vegetations at the top of the mountains and its lower limit were pushed up by the expansion of open vegetation in the lowlands due to aridification. Despite this general trend, local climatic variability resulted in cool and relatively humid periods in some areas creating favourable patches for afro-montane forest during glacial periods (Osmaston & Harrison 2005).

1. Glacial: cool and arid



2. Interglacial: warm and humid

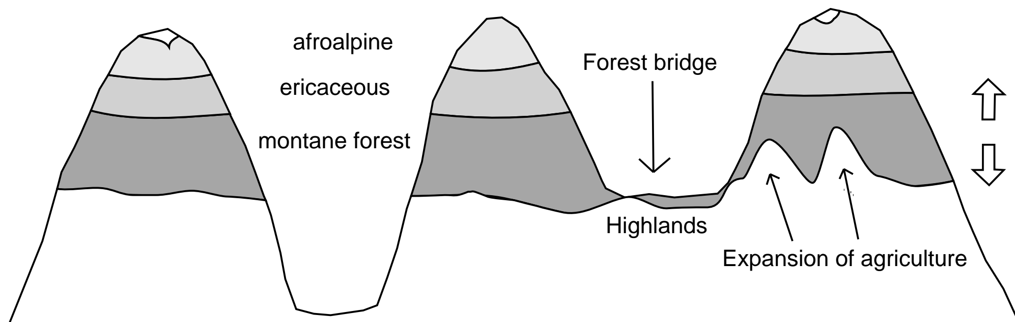


Fig. 3. Schematic representation of range shifts of the three vegetation belts of the afro-alpine region in response to climate fluctuations during the Pleistocene. 1. During glaciations, glaciers formed on some mountain tops and the afro-alpine and ericaceous zones expanded. The lower limit of the montane forest was in many places pushed up by aridification of the lowlands. 2. During interglacials, the montane forest expanded. At present, it is however fragmented by the expansion of agriculture (Kebede *et al.*, 2007b).

The afro-alpine environment has in all probability existed on at least some mountains for 2-3 million years (Harmsen *et al.*, 1991). Most of the regions now occupied by the afro-alpine ecosystem have been extensively glaciated several times during the Pleistocene shifting the entire biosphere, including most suitable soils. During the interglacial and Holocene, on the other hand, most of the areas occupied by the afro-alpine ecosystems in glacial times where

covered with afro-montane forest. Unglaciated ridges and nunataks at high altitude may be the only areas which remained continually part of the afro-alpine ecosystem, but climatic changes would have had major effects on the biological communities (Harmsen *et al*., 1991).

In the recent past, climate has not been the only factor that governed the extent of afro-montane forest in Africa. Its lower limits on most mountains have been pushed up in historical time by the expansion of agriculture. It appears that without human impact the afro-montane forests of Mt Elgon and Cherangani Hills, Aberdare and Mt. Kenya could have been in direct contact (Hedberg 1969). Similarly, up to 35% of the Ethiopian highlands were covered by afro-montane forest until a few hundred years ago (EFAP 1994). At present, many mountain massifs are used for extensive agriculture on almost all sides. As a result, the afro-montane forest belt has become highly fragmented and reduced to small patches due to habitat degradation (Burgoyne *et al*., 2005). Therefore, we have used the phylogeographic history of three ecologically key species (*Erica arborea*, *Hypericum revolutum* and *Lobelia giberroa*), without obvious long distance dispersal to provide insights into the interconnections of fragmentation patterns and evolution of the afro-montane flora and Pleistocene climatic oscillations on one hand and recent anthropogenic impacts on the other.

1.3. Characterization and Distribution of the Study Species

Phylogeographic studies of a **s**ingle species provide an interesting story about the individual species but say very little about the biogeographic history of the region. Comparative studies of multiple, unrelated species with different life history strategies are the key to gain a complete picture of regional history (Morris *et al.*, 2005). Thus, we identified three widespread afro-montane species for this investigation. The nomenclature of the species

follows Edwards *et al*., (1995), Hedberg *et al*., (2003 & 2006), Polhill (1984) and Agnew (1974).

1.3.1. Erica arborea (Ericaceae)

Erica arborea is a richly branched shrub or small tree up to 8 m high with a grey-brown stem and young twigs which have densely branched hairs. It grows between 2200-3900 m on steep and rocky slopes, in wooded, often burnt and grazed grassland and sometimes on exposed lava flows. It is also often kept down by burning but sprouts shortly after the rainy season begins. *Erica arborea* is the only species in the genus that is widespread in Europe, Arabian Pensula and Africa (Fig. 4). The species exhibit remarkable geographical disjunction. It occurs along the Mediterranian coast from Morocco to Tunisia, an isolated population on Emi Koussi in the Tibesti Mountains of the Sahara, the Ethiopian highlands, and the mountains of East central Africa from southern Uganda to northern Malawi (McGuire & Kron 2005). In Europe, *E. arobra* occurs on the islands and the northern coastline of the Mediterranean Sea in addition to populations along the Atlantic cost of Spain and in the Pyrenees (Quezel 1978). *Erica arborea* displays considerable variation in the amount of pubescence, size of flowers, size and shape of bracteoles, etc., but the variation range in each feature is so continuous that morphological taxonomic subdivision is impossible.

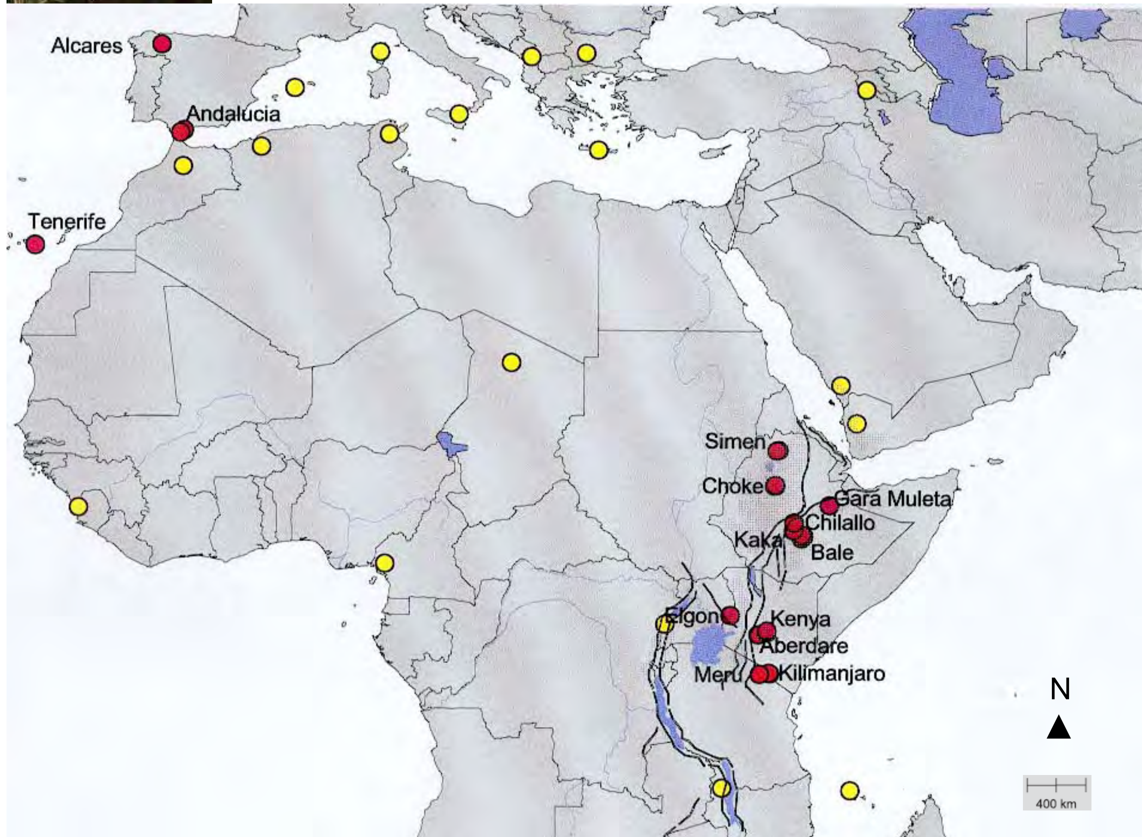
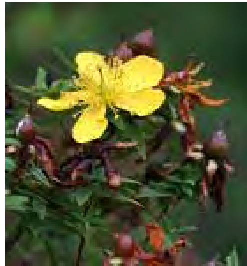


Fig. 4. Distribution map of *E. arborea*. Red dots show sampled areas where as yellow dots show areas which were not sampled.

1.3.2. Hypericum revolutum (Hypericaceae)

Hypericum revolutum (St. Johns wort) is a much branched evergreen shrub or tree with white scaly bark and about 1-12 m high. Flowers are solitary at the ends of the branches. It grows around upland dry evergreen forest, upland evergreen bush land and stream sides in upland grass land between 1800-3360m. It occurs on Mountains of eastern Africa, from Ethiopia to

Cape Province, Cameroon, Bioko (Fernando Po), Madagascar and the Comoro Islands, Yemen and Saudi Arabia (Fig. 5).



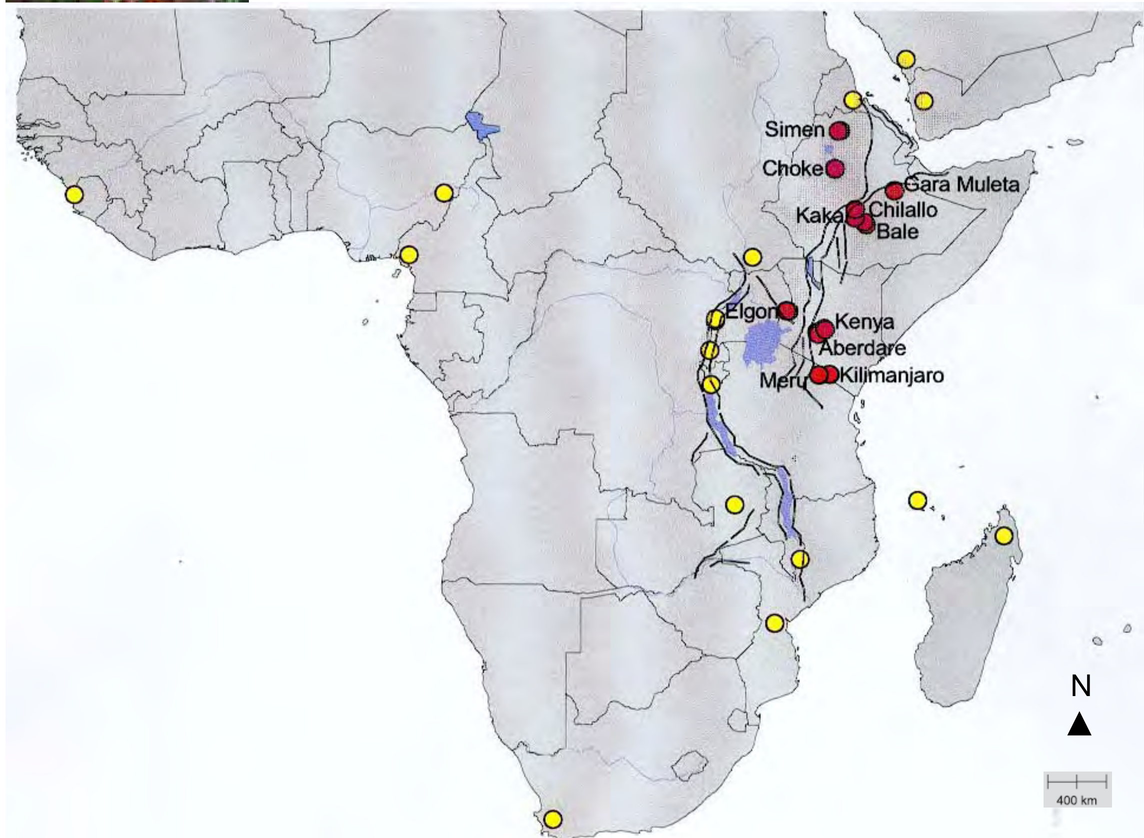


Fig. 5. Distribution map of *H. revolutum*. Red dots show sampled areas where as yellow dots show areas which were not sampled.

1.3.3. Lobelia giberroa (Lobeliaceae)

Lobelia giberroa is a widespread species and is the sole East African giant lobelia restricted to the afro-montane forest. Other species of giant lobelias grow in the afro-alpine and ericaceous vegetation belts (Knox 1993; Knox & Palmer 1998). *L. giberroa* grows in small patches along streams, forest edges or in moist depressions from 1550 to 3000 m, occasionally as low as 1260 m and as high as 3350 m (Knox 1993). The plants have a giant–rosette growth form and can reach 9 m in flower. They have very small, numerous and narrowly winged seeds, probably dispersed by wind. *L. giberroa* is distributed from Eritrea to northern Malawi and inland to eastern Congo (Fig. 6, Knox 1993).

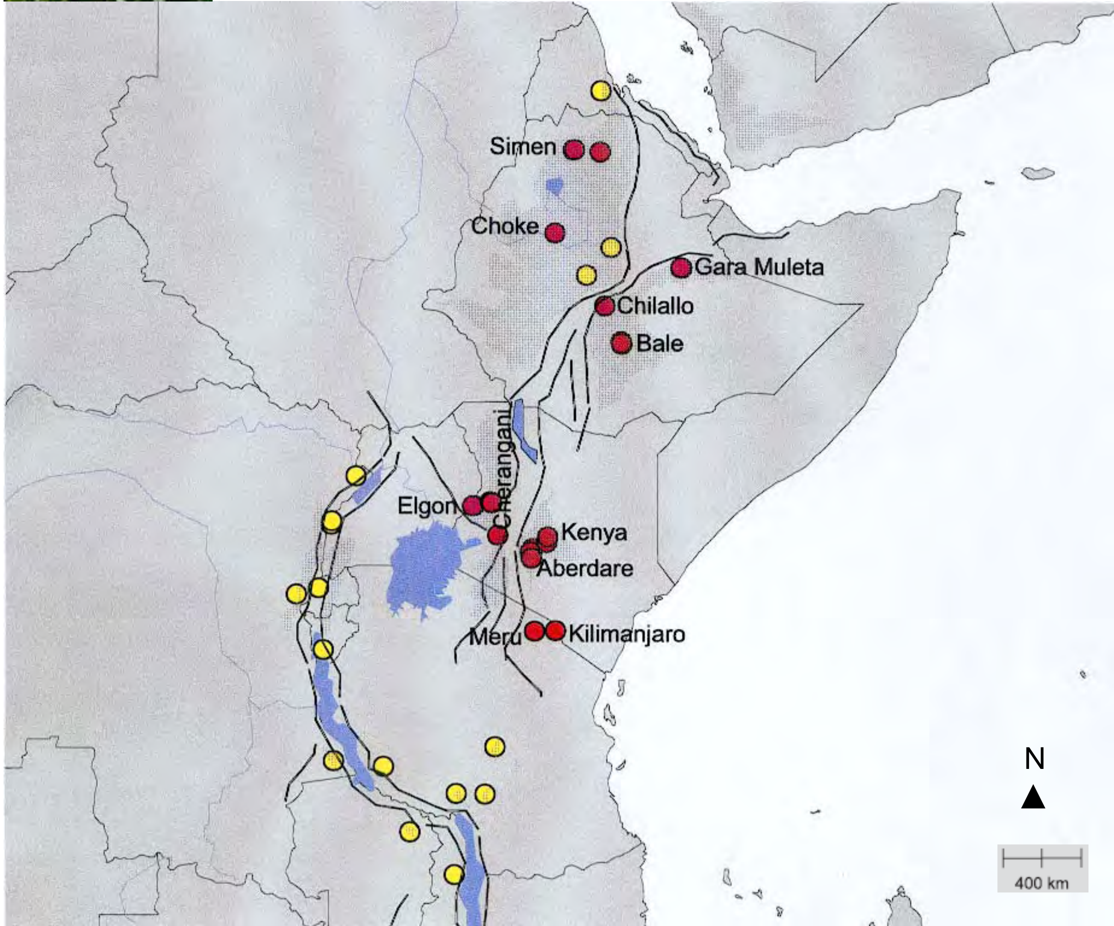


Fig.6. Distribution map of *L. giberroa*. Red dots show sampled areas where as yellow dots show areas which were not sampled.

1.4. Amplified fragment length polymorphism (AFLP) and its power to address Phylogeographic histories of species

1.4.1. Basic principles of AFLP

AFLP technology was introduced by Vos *et al.*, (1995) and it represents an ingenious combination of restriction fragment length polymorphism (RFLP) analysis and polymerase chain reaction (PCR). AFLP technology is applicable to all organisms without previous sequence information and generally results in highly informative fingerprints. It is an ideal genotyping method that produces results that are invariable from laboratory to laboratory and allows unambiguous comparative analysis and the establishment of database. Thus, it rapidly become one of the most popular and powerful approaches to detect DNA polymorphisms (Savelkoul *et al.*, 1999).

The AFLP reaction is comprised of three successive principal steps (Fig. 7). First, genomic DNA is digested with two different restriction enzymes producing sticky ends. One of the enzymes is a frequent cutter (the four base restriction enzyme, eg. *Mse*I) and the other a rare cutter (the six base restriction enzyme, e.g. *Eco*RI). Various enzyme/primer combinations can be used. *Mse*I and *Eco*RI are best used in AT-rich genomes as they give fewer fragments in GC-rich genomes. Following restriction digestion, specific double-stranded synthetic oligonucleotide adapters for each restriction site are ligated to the digested DNA. Both the restriction and ligation steps can be preformed in a single reaction. Adapter and restriction site sequences then provide universal primer binding sites for subsequent PCR reactions. Double-stranded oligonucleotide adapters are designed in such a way that the initial restriction site is not restored after ligation, which allows simultaneous restriction and ligation, while relegated

fragments are cleaved again, i.e. restriction and ligation can be preformed in a single step (Janssen *et al.*, 1996).

Second, specific subsets of DNA digestion products are then subjected to two subsequent PCR amplifications, using combination of selective primers. A first PCR (pre-selective) is preformed by using oligonucleotide primers complementary to the adapter and restriction sites. A nucleotide is added to the primers to select only a subset of fragments. To achieve this, the 5' portions of the primers are made complementary to the adapters whereas the 3' ends extended by a few, arbitrarily chosen nucleotides (often termed as selective bases or selective nucleotides) into the restriction site. Exact matching of the 3' end of a primer is essential for amplification. For example, an extra nucleotide A is added to pre-selective primers. As a result, only a subset of the fragments of the mixture is amplified (i.e. those in which the restriction site sequence is followed directly by an A). Statistically, each selective base added to one of the primers reduces the complexity of banding patterns fourfold. An extension of one selective nucleotide on each side amplifies 1/16 of the ligated fragments whereas 1/256 in case of two, and 1/4096 in case of three selective nucleotides in both primers (Janssen *et al*., 1996; Weising *et al*., 2005). Only one or two selective nucleotides are required for templates with low complexity such as bacterial genomes (Lin *et al*., 1996), whereas up to eight selective nucleotides may be required for species with very large genomes (Han *et al*., 1999). Amplification primers are usually 17 to 21 nucleotides in length, and anneal perfectly to the target sequences.

Pre-selective amplification products undergo another PCR run (selective) and again a subset of those fragments is selected. Usually a second selective amplification is then carried out using similar oligonucleotide primers but with two extra nucleotides (e.g. AC). Therefore,

only a subset of the first amplification reaction will undergo subsequent amplification during the second round of PCR (i.e. those in which the AC sequence follows the restriction site sequence).

Third, the final step is fragment separation and detection. Polymorphism detection is possible with radioisotopes, fluorescent dyes or sliver staining. In addition to the advantage of not requiring radioisotopes, fluorescent primers can be loaded as set of three, each labelled with a different coloured dye (blue, green and yellow) into the same gel line, thus maximising the number of data points gathered. At last, the subset of fragments are separated by denaturing polyacrylamide gel electrophoresis or capillary gel electrophoresis to generate a fingerprint and DNA bands which may be detected using different methods (Weising *et al*., 2005).

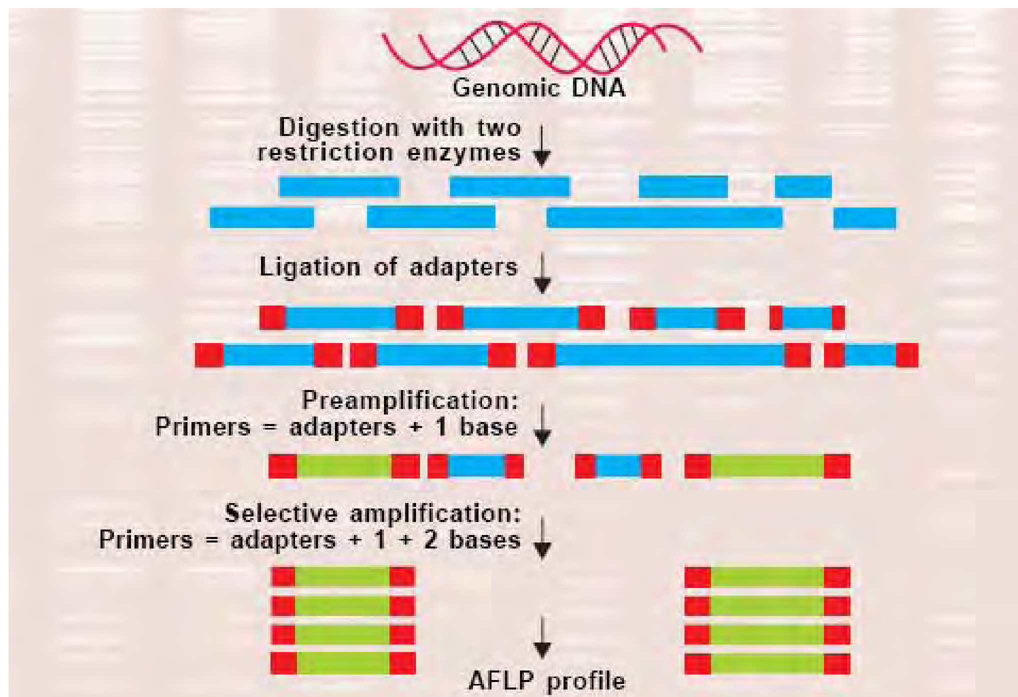


Fig. 7. Schematic representation of the basic principles of AFLP analysis. Genomic DNA is digested with two restriction enzymes (*Eco*RI and *Mse*I) and specific adapters are ligated to

both ends of all resulting fragments. Two successive PCRs are then preformed using specific primer pairs, of which the 5' portions are complementary to the adaptors and the restriction site, and the 3' ends extend by one or a few selective bases into the interior of restriction fragment (http://irc.igd.cornell.edu/MolecularMarkers/AFLPs).

1.4.2. Interpreting AFLP bands

The molecular basis of AFLP polymorphisms will usually be caused at the nucleotide level. Polymorphisms between two or more genotypes may arise due to (1) mutations in the actual restriction sites, (2) mutations in the sequences adjacent to the restriction sites (not assayed in the analysis) and complementary to the selective primer extensions, which cause the primers to mis-pair at the 3' end and prevent amplification, and (3) insertions or deletions within the amplified fragments (Weising *et al.*, 2005). Different restriction enzymes can be used and different combinations of pre- and selective nucleotides will increase the probability of finding useful polymorphisms. The more selective bases, the less detectable polymorphism will be. Bands are usually scored as either present or absent. Heterozygous versus homozygous bands may be detected based on the thickness of the signal, although this can be tricky. Most AFLP markers will be mono-allelic, meaning that only one allele can be scored and the corresponding allele is not detected.

1.4.3. Advantages and limitations of AFLP

No any single technique is universally ideal because each available technique exhibits both strength and weaknesses (Mueller & Wolfenbarger 1999). The advantage of AFLP is that it allows a quick scan of the whole genome for polymorphisms. It can be applied to any DNA sample of human, animal, plant and microbial DNA, giving it the potential to become a

universal DNA fingerprinting system. Because of the large number of bands generated, each marker gives a highly informative fingerprint. No prior sequence information or probe generation is needed. Because of the nature of AFLP primers, the markers obtained are highly reliable and robust (Janssen *et al*., 1996), unaffected by small variations in the amplification process (i.e. they are highly reproducible, Weising *et al*., 2005).

Like any type of molecular marker used for analysis of genetic polymorphism, AFLP technique has also limitations. AFLP generate huge quantities of information, which may need automated analysis and therefore computer technology. AFLP markers display dominance. They are technically demanding in the laboratory and, especially, in data analysis. A further drawback of AFLP technology is perhaps the lack of guarantee of homology between bands of similar molecular weight (MW), thus creating difficulties for sum type of studies such as phylogenetic analysis.

1.4.4. AFLP Applications

The AFLP technique is used to address several problems in various disciplines including Phylogeography (Schönswetter *et al*., 2002; Wang *et al*., 2003; Skrede *et al*., 2006; Kebede *et al*., 2007 a & b), Population Genetics (Gaudeul *et al*., 2000; Campbell *et al*., 2003), Molecular Taxonomy (Kardouls *et al*., 1998; Koopman *et al*., 2001; Despres *et al*., 2003; Brout *et al*., 2004), Conservation Genetics (Besse *et al*., 1998; McGregor *et al*., 2002; Rottenberg & Parker 2003), and etc. In such areas AFLP is used for genetic diversity assessment, genetic distance analysis, genetic fingerprinting, and analysis of germplasim collections, genome mapping, and monitoring diagnostic markers.

1.5. Phylogeographic study

Current environmental factors and the historical events such as the last glacial period are relevant to the contemporary distribution of species and their genes (Hewitt 1999). Understanding how historical events have helped to shape the current geographical dispersion of genes, populations and species is the major goal of Phylogeography, a term introduced by Avise *et al*., (1987). Phylogeography can be defined as a field of study concerned with the principles and processes governing the geographical distribution of genealogical lineages, especially those within and among closely related species (Avise 2000). It has deep roots in historical biogeography and population genetics. By comparing the evolutionary relationships of genetic lineages with their geographical locations, we may gain a better understanding of which factors have most influenced the distribution of genetic variation. Phylogeography, therefore, embraces aspects of both time (evolutionary relationships) and space (geographical distributions). The analysis and interpretation of lineage distributions usually requires input from molecular genetics, population genetics, phylogenetics, demography, and historical geography. Thus, phylogeography is an integrative discipline (Avise1998).

In general, molecular phylogeography can be used, bearing the differences in geography and climatic history in mind, to seek possible generalities of the spatial and genetic distribution of species (Hewitt 2000). Recent developments seek to use genetic data to estimate the demographic history of a population, the dates of historical bottlenecks or expansions, the size of ancestral populations, the location of refugia, the dates of divergence, the extent of migration and gene flow, the extent of fragmentation, and the sequence of such events to produce the present geographic distribution of genotypes (Beaumont 2004; Templeton 2004).

Since the birth of the discipline by Avise *et al*., (1987), most phylogeographic studies were conducted at Northern hemisphere flora. As a result, it has been variously reported that the level of intraspecific polymorphism was low at higher latitude, particularly in areas glaciated during Pleistocene cold periods and most of the variation lies in areas that correspond to refugia during range contractions (Taberlet 1998). At the moment, there is no such a sound explanation on the phylogeographic history of the most diverse, unique but little explored afro-montane/-alpine flora. The current study will generate at least suggestive idea about the flora of the region.

1.6. Conservation Genetics and Biodiversity

Every conservation project needs to answer different questions about the organisms under investigation. Their systematics, phylogeography, phylogenetic relationships and the relatedness among individuals are only some of the different aspects of fundamental importance in planning the conservation of the biodiversity of a particular area. Therefore, conservation genetics has become one of the most important aspects in the conservation of the biodiversity and its management (Lucchini 2003). It takes an understanding of genetics and evolution and applies it to conservation problems. In a broader sense, it is a mixture of ecology, molecular biology, population genetics, mathematical modelling and evolutionary systematics (the construction of family relationships). Although the scope of conservation genetics is not limited, the study of gene diversity in natural populations remains central to the field (Petit *et al*., 1998). One goal of conservation genetics is to use an information of range wide surveys of genetic diversity, based on genetic markers to implement conservation policies for given species, and in particular to identify areas for on-site conservation (Riggs 1990; Petit *et al*., 1998).

Accordingly, molecular techniques are used in the field to determine the amount of inter-breeding between different populations of the same species (e.g. do populations intermingle, or are they cut off from each other by some kind of barrier?). Geographical barriers lead a population to diverge and form lineages which are treated as distinct evolutionarily significant units (ESUs). These evolutionarily significant units are important components in the evolutionary history of a species (Moritz 1994; Taberlet & Cheddadi 2002; Petit *et al.*, 2003) and they are often of critical importance to conservation efforts. On one hand, it may be desirable to keep certain populations separate to minimize genetic mixing and thereby maintain maximum diversity. Conversely, small, isolated populations from other populations of the same species are more likely to become extinct. The effect of fragmentation is currently an important topic in evolutionary and conservation biology (Heino & Hanski 2001). Fragmentation affects both the exchange of individuals and gene flow (Young *et al.*, 1996; Aldrich & Hamrick 1998; Dayanandan *et al.*, 1999). Finally, fragmentation contributes to the evolution of dispersal-related traits (Heino & Hanski 2001). Consequently, the implementation of conservation strategies requires the monitoring of gene flow in relation to habitat structure (Imbert & Lefèvre 2003).

1.7. Objectives of the study

General Objective

- To investigate the historical biogeography of some key afro-montane species using amplified fragment length polymorphism (AFLP) and identify important areas for conservation of the species based on hotspots of intraspecific diversity and ESUs.

Specific objectives

- To investigate the population genetic structure of *Erica arborea, Hypericum revolutum* and *Lobelia giberroa* using amplified fragment length polymorphism (AFLP).
- To describe the extent of dispersal and fragmentation patterns of these species and the effect of such events on their current geographic distribution.
- To identify potential glacial refugia for these plant species.
- To describe the phylogeographic structure of each species.
- To analyze comparative phylogeographic patterns in the afro-montane/-alpine environment as a whole.
- To identify important areas for biodiversity conservation based on hotspots of intraspecific diversity and evolutionarily significant unit (ESU).

2. Material and Methods

2.1. Sampling

Thirty eight, thirty three and twenty five populations of *E. arborea*, *H. revolutum* and *L. giberroa* respectively were collected from mountain systems in Ethiopia and tropical East Africa in 2003 and 2004 (Appendix 1a, b, & c; Fig. 4, 5 & 6). Reference populations for *Erica arborea* were collected from Canary Islands and Spain. One to three distant populations and two to eleven individuals per population were sampled per mountain system. From each population, leaf material was sampled in tubes containing silica gel, from individuals at 25 m intervals along 250 m straight line transects. A duplicate (from a randomly chosen plant sampled twice) marked "X" was collected for each population. Voucher specimens were collected for each population and deposited at the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia.

2.2. DNA isolation and AFLPs

2.2.1. DNA isolation

Total genomic DNA was extracted from silica gel dried leaf material following the CTAB protocol (Doyle & Doyle 1987) with some modifications. A dried leaf material was placed in an Eppendorf tube together with carbamide ball and crushed with grinding machine at frequency of 22 for 5 minutes. 700 µL of CTAB buffer (4.0 g CTAB, 56 mL of 5M NaCl, 20 mL of 1M Tris-HCl [pH=8], 4g PVP, 8 mL of 0.5 M EDTA [pH=8], 115 mL of ddH_2O and 0.4 mL of β-mercaptoethanol), was added to the powder, mixed with vortex and incubated on a heating block at 60°C for 30 minutes while shaking every 15 minutes. This step lyses the cells. Then it was mixed with 500 µL of Chloroform/Isoamyl alcohol with 24:1 ratio and

allowed to stand for 5 minutes to precipitate proteins. Afterwards the tube was centrifuged at 13,000 revolutions per minute (rpm) for 2 minutes. Gently, 500 µL of the upper clear phase was removed to another Eppendorf tube with 350 µL Isopropanol and mixed well. The tube was kept in Refrigerator at 4°C for 5 minutes to precipitate the DNA. Then the tube was centrifuged at 13,000 rpm, the upper supernatant was removed and the DNA pellet remained at the bottom of the tube. 1 mL of 70% Ethanol was added and centrifuged at 13,000 rpm to wash the DNA pallet. This step improves the quality of DNA to ensure complete digestion by the restriction enzymes during digestion. After ethanol was discarded and drained, the DNA pallet was allowed to dry in vacuum centrifuge for 10 minutes. Then, the DNA pallet was re-suspended in 100 µL TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8) together with 2 µL RNAse (to remove RNA), vortexed and allowed to stand at room temperature for 30 minutes. The quality of extracted DNA was checked on 1% TBE-Agarose gel. Finally, the isolated total genomic DNA can be stored in Freezer at -20°C until needed for the consecutive AFLP procedures.

2.2.2. AFLPs

The AFLP procedure includes consecutive steps. First, Restriction–ligation: 2 µL extracted DNA (Genomic template DNA, 200 ng on average) was added to a 9 µL master mix composed of 1.1 µL of 10X T4 ligase buffer, 1.1 µL of 0.5 M NaCl, 0.55 µL of 1 mg/mL bovine serum albumin (BSA), 0.02 µL of 50 U/µL *Mse*I, 0.125 µL of 40 U/µL *Eco*RI, 0.2 µL of 5 U/µL T4- DNA ligase, 1 µL of 10 µM *Mse*I-adapter, 1 µL of 10 µM *Eco*RI-adapter and 3.91 µL of double distilled sterile water (ddH_2O). The adapters were denatured at 95°C for 5 minutes, warped with Aluminium foil and kept on table at room temperature for few minutes to cool before added to the master mix. The final 11 µL total reaction volume (9 µL master mix + 2 µL DNA template) of the mixture was then incubated at 37 °C for 3 h on the

PCR machine. The final restriction ligation reaction product was diluted 10 times with ddH_2O before the next step proceeded.

The double-stranded adapters used for ligation, in the master mix were prepared as follows. To prepare 10 µM solutions of the adapters (Eco-adap. and Mse-adap.), 10 µL adapter A§T (100 µM), 10 µL adapter A§B (100 µM) and 80 µL ddH_2O were mixed to have 100 µL of the final volume. The corresponding oligonucleotide sequences for the adapter A§T and A§B for both Eco and Mse are as shown below.

Eco-adap. A§T: 5'-CTCGTAGACTGCGTACC-3'

A§B: 5'-AATTGGTACGCAGTCTAC-3'

Mse- adap**.** A§T: 5'-GACGATGAGTCCTGAG-3'

A§B: 5'-TACTCAGGACTCAT-3'

The complementary oligonucleotides were mixed and annealed to form the double-stranded adaptors with the conformation shown in Table 1.

Table 1. Restriction sites for the restriction enzymes (symbols ↓↑include the actual cutting sites), adapters (double stranded) and pre-selective prime sequences (only single-stranded primers are shown) used in the AFLP analysis of *E. arborea*, *H. revolutum* and *L. giberroa*. Selective bases are in bold.

Restriction Enzyme (RE)	***Eco*RI**	***Mse*I**
Restriction sites for RE	*Eco*RI 5'- G↓AATT C-3' 3'- C TTAA↑G-5'	*Mse*I 5'- T↓TA A-3' 3'- A AT↑T-5'
Adapters	Eco-adap. 5'- CTCGTAGACTGCGTACC-3' 3'- CATCTGACGCATGGTTAA-5'	Mse-adap 5'- GACGATGAGTCCTGAG-3' 3'- TACTCAGGACTCAT-5'
Pre-selective PCR primers	Eco-A 5'- GACTGCGTACCAATTC**A**-3'	Mse-C 5'- GATGAGTCCTGAGTAA**C**-3'

Second, pre-selective PCR was performed using 1.5 µL of the diluted restriction ligation product and 11µL of pre-selective master mix composed of 1.25 µL of 10X Taq buffer (100 mM Tris-HCl [pH= 8.3], 500 mM KCl & 0.01% gelatine), 0.75 µL of 25 mM $MgCl_2$, 1 µL of 10 mM dNTP (containing all four types), 0.25 µL of 10 µM *Eco*RI primer (Eco-A), 0.25 µL of 10 µM *Mse*I primer (Mse-C), 0.05 µL of 5 U/µL Ampli*Taq* DNA Polymerase (Perkin-Elmer), and 7.45 µL of ddH_2O. The final total reaction volume was 12.5 µL. PCR reactions

were performed with the following profile: 2 min at 72 °C, 30 cycles of 30 sec denaturing at 94 °C, 30 s annealing at 56 °C, and 1 min extension at 72 °C, ending with 10 min at 72 °C to complete extension. Successive amplification was checked with 1% agarose gel electrophoresis by loading 5 µL of pre-selective product, 2.5 µL of loading buffer and 1 µL of ethidium bromide. After checking for the presence of a smear of fragments (300-600 bp in length) under UV- light, the amplification product was diluted 20 times in double distilled water and stored at -20°C or used immediately.

Third, a selective PCR was performed in which 2.5 µL of the diluted pre-selective product was added to a 10 µL selective master mix composed of 1.25 µL of 10X Gold Taq buffer, 1.25 µL of 25 mM $MgCl_2$, 0.1 µL of 1 mg/mL bovine serum albumin (BSA), 1 µL of 10 mM dNTP, 0.1 µL of 10 µM fluorescent *Eco*RI primer (E.AXX), 0.25 µL of 10 µM *Mse*I primer (M.CXX), 0.1 µL of 5 U/µL Ampli*Taq* Gold DNA Polymerase (Perkin-Elmer) and 5.95 µL of ddH_2O to make the final reaction volume 12.5 µL. PCR reactions were performed with the following profile: 10 min at 95 °C, 13 cycles of 30 sec denaturing at 94 °C, 1 min annealing at 65°C and 1 min elongation at 72 °C, 23 cycles of 30 sec denaturing at 94 °C, 1 min annealing at 56°C and 1 min elongation at 72 °C , and ending with 10 min at 72 °C to complete extension. Annealing was initiated at a temperature of 65 °C, which was then reduced by 0.7 °C for the next 12 cycles and maintained at 56 °C for the subsequent 23 cycles.

For each species twelve primer pair combinations with base sequence 5'-GACTGCGTACCAATTC**AXX-**3' for *Eco*RI and 5'-GATGAGTCCTGAGTAA**CXX-**3' for *Mse*I were tested on four individuals from two different mountain systems. AFLP profiles with many polymorphic markers and well separated fragments were selected. A second primer test was carried out using six primer pair combinations chosen from the first primer test on 16 individuals sampled from three geographic regions (Ethiopia, Kenya and Tanzania).

Finally, three of the primer pair combinations were chosen and reproducibility was confirmed. The final AFLP analysis was carried out with three primer pair combinations for each species (Table 2).

Table 2. Selective primer sequences and the primer pair combinations (*Eco*RI with the corresponding *Mse*I) used for AFLP analysis for each species. Only single-stranded primers are shown. Selective bases are in bold.

Species	*Eco*RI	*Mse*I
Erica arborea	5'-GACTGCGTACCAATTC**AGA-**3' (6FAM) 5'-GACTGCGTACCAATTC**ACA-**3' (VIC) 5'-GACTGCGTACCAATTC**AAC-**3' (NED)	5'-GATGAGTCC TGAGTAA**CTC-**3' 5'-GATGAGTCCTGAGTAA**CAT-**3' 5'-GATGAGTCCTGAGTAA **CAG-**3'
Hypericum revolutum	5'-GACTGCGTACCAATTC**ATG-**3' (6FAM) 5'-GACTGCGTACCAATTC**AAG-**3' (VIC) 5'-GACTGCGTACCAATTC**AGC-**3' (NED)	5'-GATGAGTCCTGAGTAA**CTA-**3' 5'-GATGAGTCCTGAGTAA**CTG-**3' 5'-GATGAGTCC TGAGTAA**CTG-**3'
Lobelia giberroa	5'-GACTGCGTACCAATTC**AGA-**3' (6FAM) 5'-GACTGCGTACCAATTC**ACA-**3' (VIC) 5'-GACTGCGTACCAATTC**AGC-**3' (NED)	5'-GATGAGTCCTGAGTAA**CAC-**3' 5'-GATGAGTCCTGAGTAA**CAT-**3' 5'-GATGAGTCCTGAGTAA**CTG-**3'

Selective primer pair combinations were chosen for each species because they produced a manageable number of fragments that were well separated.

Forth, for each individual, 2.0 μL 6-FAM (Blue), 2.0 μL VIC (Green) and 4.0 μL NED (Yellow) labeled selective PCR products were mixed with 11.7 μL formamide and 0.3 μL GeneScan ROX 500 size standard. The mixture was denatured at 95°C for 5 minutes and cooled on ice water for 5 minutes and run on an automated sequencer ABI 3100 (Applied Biosystems) with the following parameters: Project, 3100; dye, filter set C; run module, Gene Scan 36_pop4_35ing, and analysis module, GS500 Analysis. gsp.

2.3. Visualizing and scoring AFLP bands

AFLP patterns were then visualized with ABI prism GeneScan version 3.7 analysis software (Applied Biosystems). A fluorescence peak corresponds to the presence of an amplified restriction fragment. Subsequently, the GeneScan files were imported into Genographer (version 1.6. available at http://hordeum.msu.montana.edu/genographer/) for scoring the fragments. Each AFLP fragment was scored using the 'thumbnail' option of the program, which allows comparison of the signal per locus over all samples. Peaks of low intensity were included into the analysis when unambiguous scoring was possible. Fragments in the size range of 50-500 bp were scored as present (1) or absent (0). AFLP fragments that exhibited ambiguous peaks (not amplified well) were excluded from the analysis. The duplicates were used to test the reproducibility of the markers. The average reproducibility was calculated for each species as the average proportion of correctly reproduced bands over all replicates (Bonin *et al*., 2004).

2.4. Data Analyses

Principal coordinate analysis (PCO) was used to visualize pair-wise similarities among the AFLP multilocus phenotypes. Analyses were executed in NTSYS-pc (Rohlf 1990), using the

Dice similarity coefficient. The data sets were also subjected to a neighbour-joining analysis based on Nei & Li (1979) genetic distances using the software TREECON 1.3b (Van de Peer & De Wachter 1994). The Nei & Li (1979) and Dice coefficients are equivalent, and take into account only similarity in presence of fragments. They are thus more conservative than the simple matching coefficient, which takes into account both presence and absence of fragments. The trees were midpoint rooted and branch supports were estimated with 1000 bootstrap replicates.

For each population, genetic diversity was estimated using Nei's unbiased diversity estimator for each marker, $H_e = [1- (P_0^2 + P_1^2)]\ n/(n-1)$, where n is the sample size, P the frequency of the band, absence (0) and presence (1) respectively, and computing the average over all markers (Nei 1978). The average gene diversity within the mountain systems and major groups were calculated from the intra-population estimates. Total genetic diversity was estimated for groups by pooling all samples from the populations concerned. Analyses of molecular variance (AMOVA) were computed with the software Arlequin 2.0 (Schneider *et al.*, 1997) to estimate genetic differentiation at different hierarchical levels.

As an alternative approach, the population structure was examined by genetic mixture analysis using the software BAPS ver. 3.2 (Corander *et al.*, 2006) and Structure ver. 2.1 (Pritchard *et al.*, 2000). BAPS is a program for Bayesian inference of population structure, which infers the optimal number of clusters as well as the cluster each individual belongs to. The analysis was carried out using a maximum possible number of groups of 25 (K). The program Structure implements a model based clustering method using Markov Chain Monte Carlo estimation. By comparing the likelihood of the data estimated in different runs for different numbers of groups (K) it is possible to identify the optimal K. Individuals are

assigned (probabilistically) to one of the clusters defined by allele frequencies at each locus. Our data were analyzed with Structure program at the Bioportal, University of Oslo (http://www.bioportal.uio.no), with number of clusters, *K*, 1 – 10 and 10 replicates; burn-in period of 2 x 10^{5} and 10^{6} iterations. The no admixture model (each individual purely comes from one of the *K* populations) and uncorrelated allele frequencies were assumed for the analysis. The AFLP data were coded as recommended in the user manual. Similarity coefficients among pairs of Structure runs were calculated according to Rosenberg *et al.*, (2002) using the R-script AFLPdat (Ehrich 2006).

3. Results and Discussions

3.1. Erica arborea

3.1.1. Results

The AFLP analysis of 230 individuals from 38 populations of *E. arborea* provided a total of 106 markers of which 103 (98.6%) were polymorphic. The calculated average reproducibility of the markers was found to be 96%. The PCO plot based on the Dice similarity coefficient (Fig. 8a), where the first and second axis explained 37.7% and 10.7% of the variation respectively, grouped the populations into three main gene pools. The first group which is the most divergent and a cluster composed of Spain and Canary Island populations (reference populations) together with some populations from Ethiopia (Simen Mountains, Gara Muleta, Mt. Chilallo and Mt. Kaka), and from tropical East Africa (Mt. Elgon and Aberdare, almost one population from each). The second group is a cluster of Ethiopian populations (Mt. Choke, Mt. Chilallo, Mt. Kaka and Bale Mountains) and individuals from the entire tropical East African Mountains. The third and last group is entirely from Tropical East African populations (Mt. Elgon, Aberdare and Mt. Kilimanjaro). All the three populations originated from a mountain system belong to the same cluster group except those which originate from Mt. Elgon, Aberdare, Mt. Kilimanjaro, Mt. Chilallo and Mt. Kaka. Particularly, representatives (almost one entire population) from Mt. Elgon and Aberdare were present in each of the three main groups. The third axis explained 5.8% of the variation (Fig. 8b) and groups the reference populations distinctively and almost the entire population of Tropical East Africa and part of Ethiopian population within one gene pool. However, a population from Mt. Elgon and Aberdare were grouped together with some Ethiopian populations (Simen Mountains, Gara Muleta, Mt. Chilallo and Mt. Kaka). Thus, some individuals from Mt. Chilallo, Mt. Kaka, Mt. Elgon and Aberdare were present in more than one cluster. Therefore,

the PCO plots did not show a clear geographical structuring of the species. The PCO analyses based on simple matching similarity coefficient (not shown) showed exactly the same structure to the analysis based on Dice similarity coefficient.

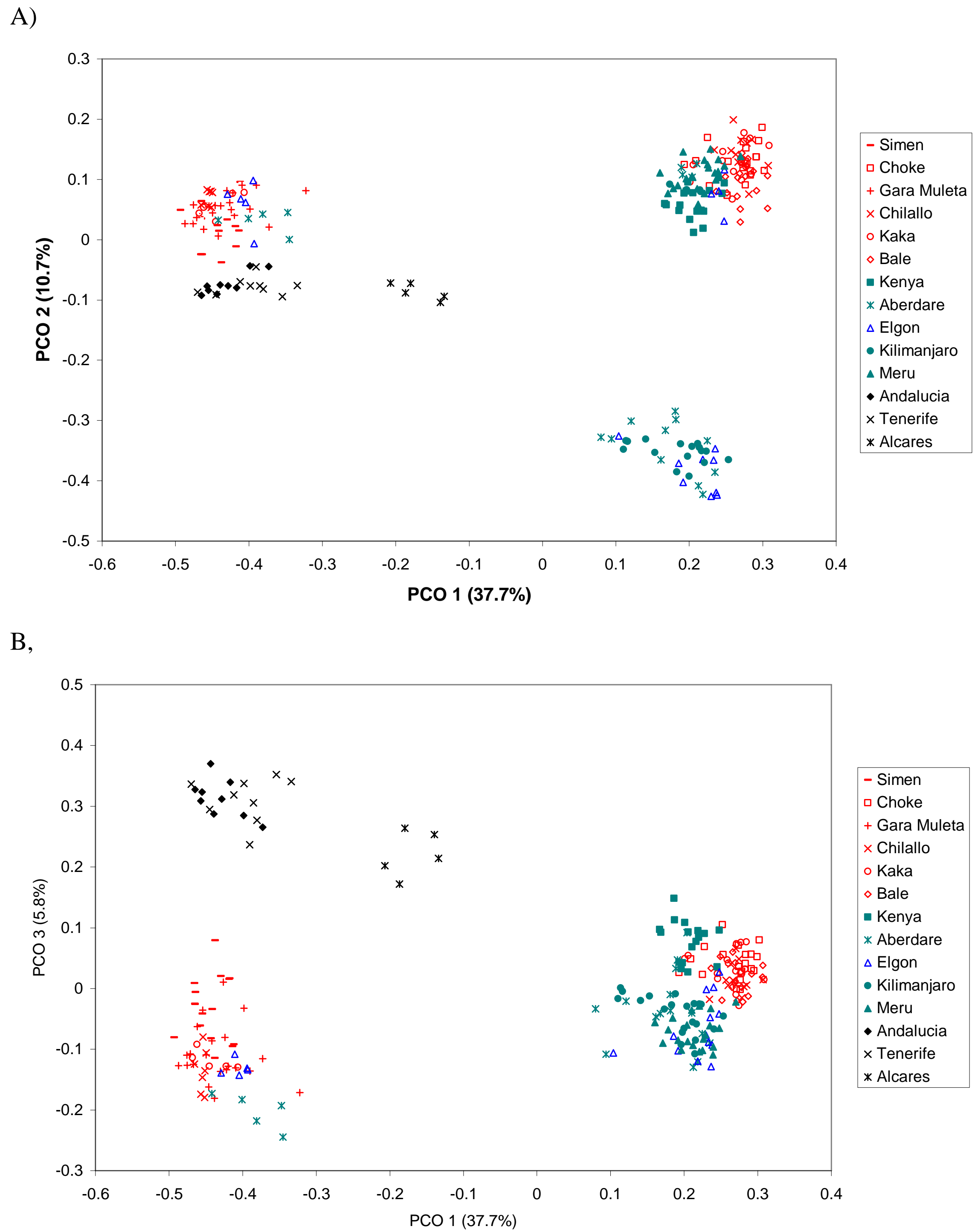


Fig. 8 Principal coordinates analysis (PCO) of individual AFLP phenotypes of *E. arborea* from 38 populations based on the Dice similarity coefficient. (a) Axes 1 & 2 (b) Axes 1 & 3.

The neighbour-joining tree based on Nei and Li genetic distance (Fig. 9) has revealed that each of the three groups shown on PCO plot (Fig. 8a) were clustered separately with strong bootstrap values. However, this analysis strongly discerned two main groups with a high bootstrap value (98%), i.e., the Ethiopian – tropical East African group and Ethiopian – Aberdare and Elgon-Spain and Canary Islands group. It is to be noted that some of the Ethiopian and Tropical East African populations shared gene pools between the two groups. The topology and the support values were nearly identical when the analysis was based on the Nei and Li's and simple matching genetic distances.

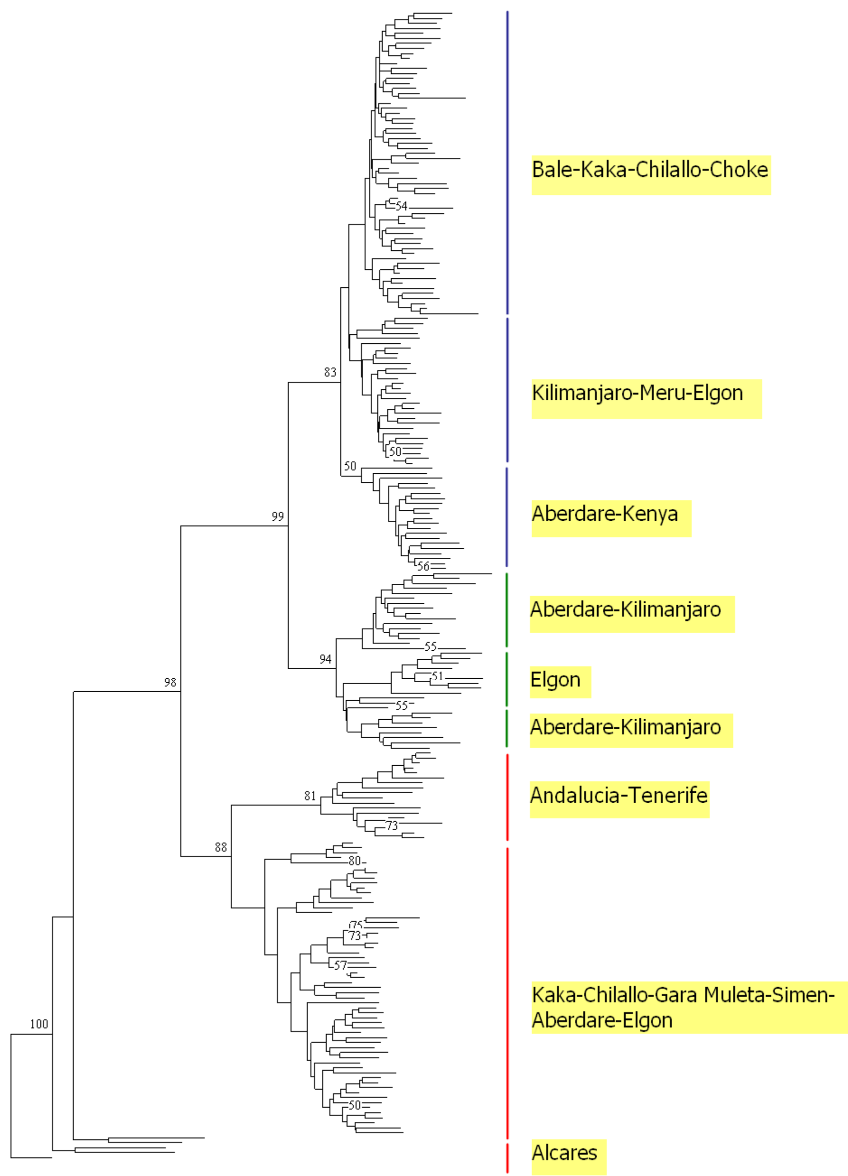


Fig. 9 Neighbour-joining tree of the AFLP phenotypes of 230 individual plants of *E. arborea* from 38 populations based on Nei and Li (1979) genetic distance. The three major genetic groups identified in *E. arborea* are indicated by different colours on the tree. The numbers above the branches are bootstrap values of 50% and higher (1000 replicates).

The average gene diversity among populations was 0.111. The highest values were observed in population from Mt. Kaka, Mt. Chilallo and Mt. Elgon (D = 0.225 to 0.270; Table 3). The lowest intra-population gene diversity (D = 0.058) was observed in the populations from Ethiopia (E004) and Spain (E3226). Relatively high intra-population gene diversity was observed in populations of Ethiopia compared to the tropical East African and Reference populations from Spain and Canary Islands (Fig. 10).

Table 3. Locality, number of individuals (n) and gene diversity (D) of the investigated populations of *E. arborea.*

Pop.	Country	Mountain	Lat/Long	Altitude (m)	n	D
E061	Ethiopia	Simen	N13.26066/E038.13575	3713	4	0.097
E065	Ethiopia	Simen	N13.23181/E038.03970	3251	5	0.100
E066	Ethiopia	Simen	N13.21222/E038.00089	3187	7	0.079
E068	Ethiopia	Choke	N10.63408/E037.84431	3772	10	0.085
E069	Ethiopia	Choke	N10.63755/E037.82961	3714	5	0.060
E074	Ethiopia	Choke	N10.68319/E037.83739	3794	5	0.085
E108	Ethiopia	Gara Muleta	N09.22841/E041.79417	2962	11	0.089
E114	Ethiopia	Gara Muleta	N09.20987/E041.79477	2768	5	0.079
E116	Ethiopia	Gara Muleta	N09.20955/E041.79327	2860	5	0.091
E046	Ethiopia	Chilallo	N07.93842/E039.22580	3708	9	0.142
E048	Ethiopia	Chilallo	N07.93724/E039.21493	3497	5	0.236
E051	Ethiopia	Chilallo	N07.91853/E039.18356	3089	4	0.241
E028	Ethiopia	Kaka	N07.36887/E039.18170	3817	5	0.092
E030	Ethiopia	Kaka	N07.36773/E039.18508	3764	3	0.270
E032	Ethiopia	Kaka	N07.36624/E039.18676	3690	11	0.230
E004	Ethiopia	Bale	N06.77974/E039.75373	3376	4	0.058
E007	Ethiopia	Bale	N06.91311/E039.92304	3627	10	0.077
E015	Ethiopia	Bale	N07.04575/E039.75152	3332	2	0.085
E166	Kenya	Kenya	S00.16791/E037.25395	3931	5	0.079
E170	Kenya	Kenya	S00.16765/E037.24820	3847	10	0.083
E181	Kenya	Kenya	S00.05820/E037.29133	3528	2	0.085
E137	Kenya	Aberdare	S00.33997/E036.65958	3560	5	0.153
E139	Kenya	Aberdare	S00.33389/E036.65130	3669	5	0.072
E142	Kenya	Aberdare	S00.34058/E036.66967	3484	10	0.141
E206	Kenya	Elgon	N01.08708/E034.63153	3383	5	0.225
E207	Kenya	Elgon	N01.10912/E034.60957	3786	4	0.068
E208	Kenya	Elgon	N01.09678/E034.62244	3615	9	0.171
E075	Tanzania	Kilimanjaro	S03.17803/E037.50838	2851	11	0.107
E077	Tanzania	Kilimanjaro	S03.18684/E037.51462	2641	5	0.085
E080	Tanzania	Kilimanjaro	S03.13998/E037.44009	3706	5	0.072
E094	Tanzania	Meru	S03.21762/E036.77106	3611	5	0.072
E098	Tanzania	Meru	S03.21697/E036.76657	3606	11	0.073
E099	Tanzania	Meru	S03.21817/E036.76739	3629	5	0.081
E3231	Canary Islands	Tenerife	N28.05833/W16.08333	875	5	0.087
E3241	Canary Islands	Tenerife	N28.03333/W16.06014	760	4	0.096
E3226	Spain	Andalucia	N36.50301/W 05.21667	1220	4	0.058
E3228	Spain	Andalucia	N36.31667/W05.46667	950	5	0.085
E3250	Spain	Alcares	N42.81667/W06.85012	1550	5	0.143

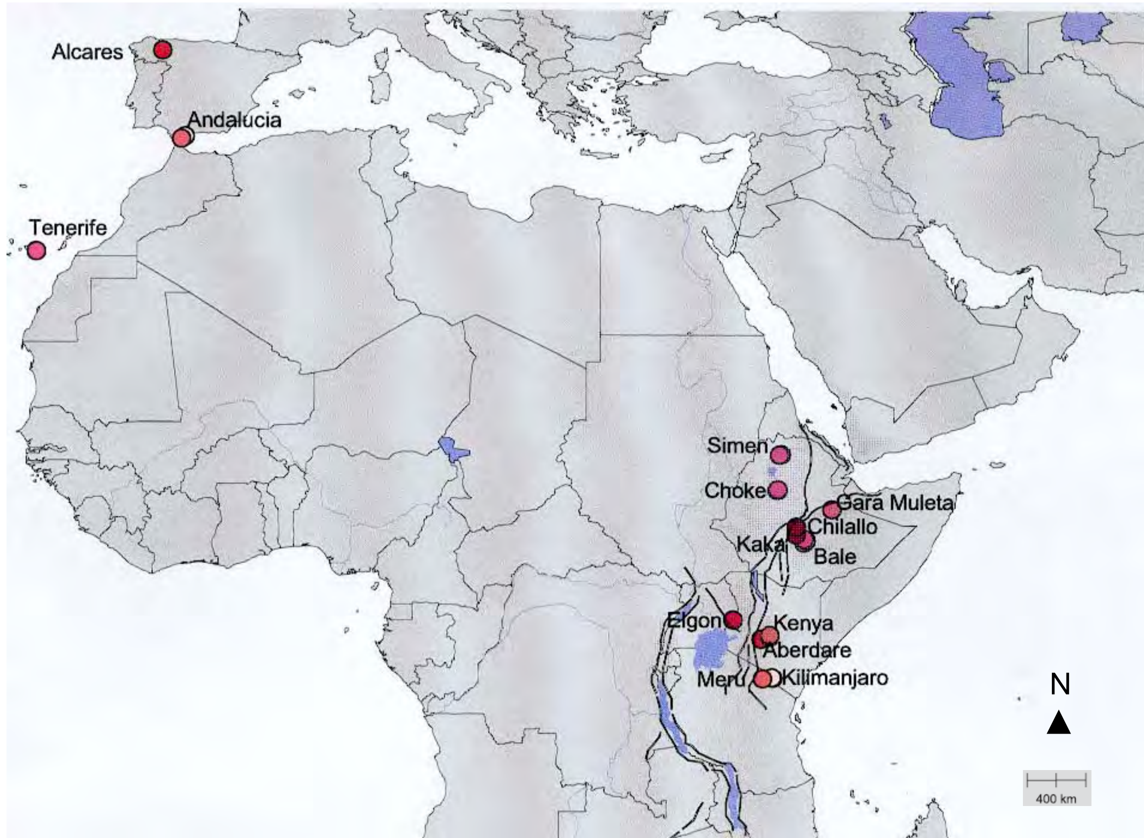
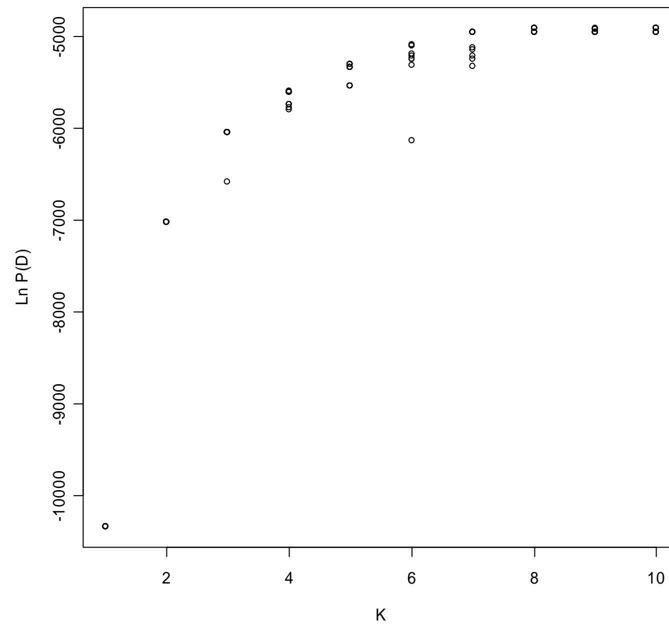


Fig. 10. The intra-population gene diversity of *E. arborea*. Colour differences show variation in gene diversity. The darker the dot the higher is the gene diversity whereas the paler the lower the gene diversity.

Hierarchical AMOVA analysis was not performed for this species because the data set was not clearly divided into phylogeographic groups which originate from distinct geographical regions. Analysis of the data with BAPS (not shown) revealed grouping of populations into eight clusters: 1) Andalucia-Tenerife, 2) Kaka-Chilallo-Simen-Gara Muleta-Aberdare-Elgon, 3) Bale-Kaka-Chilallo-Choke, 4) Kilimanjaro-Meru-Elgon, 5) Alcares, 6) Kilimanjaro-Aberdare, 7) Aberdare-Kenya and 8) Elgon. In some of the clusters, individuals from geographically related mountain systems were grouped together, whereas some clusters were formed from individuals entirely originated from one mountain system. Also populations

from Mt. Elgon and Aberdare were grouped in to three separate clusters in accordance with the PCO and neighbour-joining analyses. However, in the BAPS result, the genetic distance between cluster 3 and 4, and 6 and 8 (estimated in a Külbach-Leibler distance matrix by BAPS, not shown), was 0.28 which is relatively much smaller than the values among the other clusters. The result of the Structure analysis was also congruent with the results from neighbour-joining analysis (the two main clusters, Fig. 11 & 12), but with the exception that no tropical East African populations shared gene pool with those from Spain and Canary Islands. In this Analysis, populations were grouped into two main gene pools (Fig. 12). The graph of the likelihood of the AFLP data estimated from the different runs showed a clear point of inflection for K = 2 (Fig. 11a). Up to K = 2 outputs from the program were also absolutely identical, as shown on the plot of similarity coefficients against K (Fig. 11b). For larger values of K, the likelihood values were slightly higher, but there was no convergence for a particular configuration of clusters.

A)



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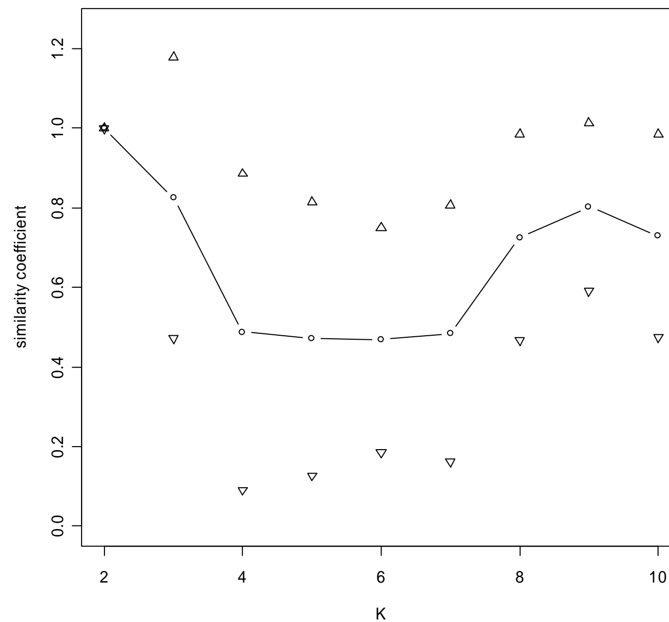


Fig. 11 Structure analysis of the AFLP data for *E. arborea*. (a) Estimated likelihood for values of K ranging from one to 10. (b) Similarity coefficients of the results from different runs of Structure calculated according to Rosenberg *et al.*, (2002). Dots represent the average similarity coefficient for the pairwise comparisions among 10 runs and triangles show the standard deviation.

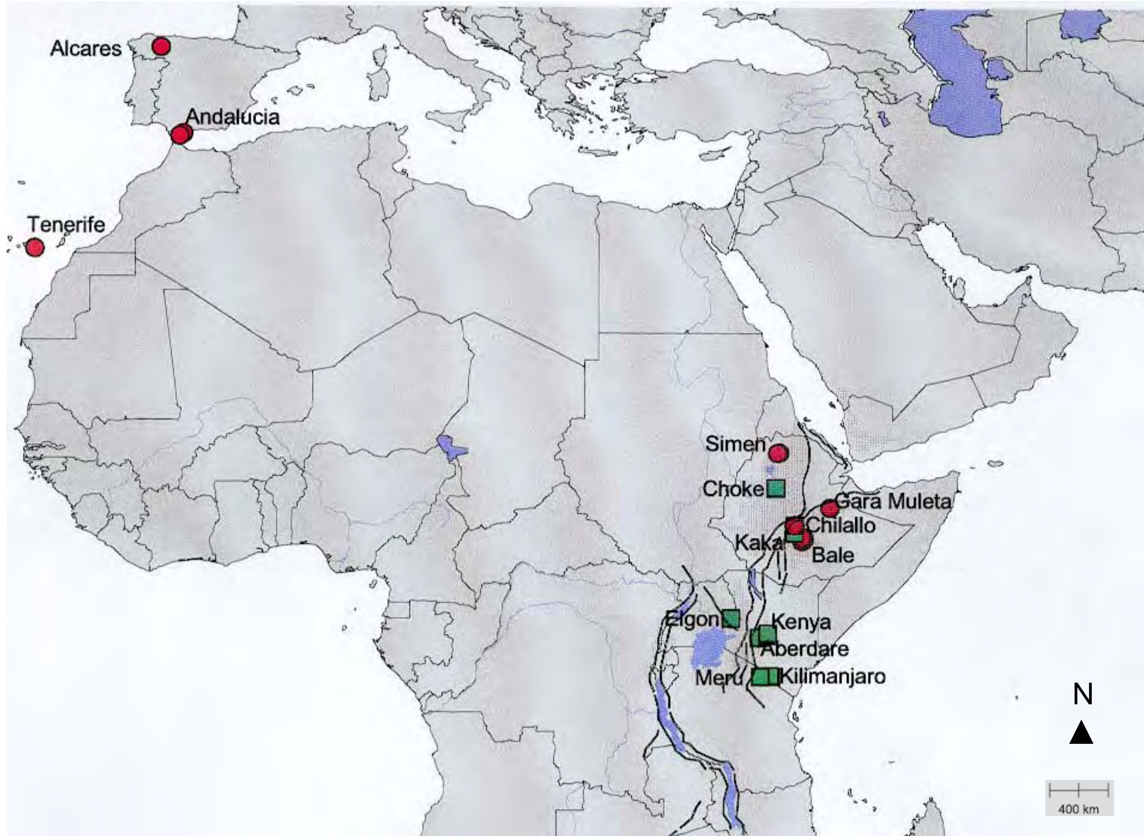


Fig. 12 Geographic origin of the 38 *E. arborea* populations analysed and their grouping according to Bayesian clustering analysis (Structure) based on AFLP markers.

3.1.2. Discussion

Erica arborea shows a pattern of mixed gene pool clustering among populations originating from different mountain systems. As revealed by the first PCO (37. 7% of variation), three main gene pools were identified (Fig. 8a). Of these clusters, only one cluster (Mt. Elgon, Aberdare and Mt. Kilimanjaro) is composed of entirely population from a distinct geographical region. However, some other populations or individuals from this particular region have also shared gene pool with the other two clusters. These observations show that geographically close populations of *E. arborea* were genetically well differentiated.

Conversely, populations at distant locations are genetically related making the pattern of historical gene flow more complicated. Furthermore, this observed pattern may be accounted for by considering the proportion of polymorphic loci of the data set (98.6% were polymorphic). There appears to be no straight forward explanation for the strong differentiation observed among the populations of *E. arborea*.

Our finding is in accordance with the gradual dispersal model of species, but the direction of the dispersal events has remained unclear for this species. Long distance dispersal (e.g. by cyclones) is a less probable mechanism to explain the observed pattern. The contractions, expansions and distant colonizations involved in the late Quaternary range shift have influenced the genetic diversity and should have left some signs. Low genetic diversity and shallow clades are expected when populations have been severely contracted. But, this is in disagreement with the observed data set of *E. arborea*.

The reference populations have revealed distinct genetic structure following different analyses which may be attributed to their geographical location. However, some populations from Ethiopia (populations of Gara Muleta and Simen Mountains) share gene pool with the reference populations. This genetic similarity may show that the populations from the two mountain massives mentioned above are historically linked with reference populations at some time in the past, probably at the beginning of the recent interglacial period. They may be originated from the same ancestral population during postglacial colonization. Alternatively, afro-alpine environment encompasses several scattered mountain ranges which had been connected to Yemen, Saudi Arabia, Turkey and then to the West, the Mediterranean region in the recent past. Thus, this corridor which extends to Eritrean highlands linking to past land bridges across the Red Sea (Gottelli *et al*., 2004) may have provided a link between the

reference and Ethiopian Populations. However, our data does not suggest the direction and actual time of dispersal for the species in these regions. The manifestation of more genetic similarity between reference populations and some of the Ethiopian populations rather than tropical East African populations may suggest that the Ethiopian highland have perhaps served as a centre of diversity or contact zone between the two populations or a corridor for gene flow in the migration history of this species.

Noteworthy is that few populations of *E. arborea* have shown the lowest intra-population gene diversity probably due to population bottlenecks. On the other hand, the genetic diversity analysis revealed that the Ethiopian populations have relatively the highest intra-population gene diversity within population. The two divergent subgroups in Ethiopian populations (Fig. 9) clearly show the persistence of a strong genetic diversity within this group. The principal coordinate analysis also suggests that populations from Mt. Kaka and Mt. Chilallo share gene pool with the reference populations as well as those of tropical East African (Fig. 8a) in view of their highest intra-population gene diversity. Populations from Gara Muleta and Simen Mountains have grouped together with Reference populations while those of Mt. Choke and Bale Mountains have clustered with those of tropical East Africa. Thus, all these patterns may show that the Ethiopian populations exhibit a strong genetic diversity and which may suggest Ethiopia as a source of radiation for this species.

Tropical East African populations did not show sub-division into smaller phylogeographic groups in the region. Except for the second PCO axis which revealed two-subgroups, the first and the third PCO axes have not separated the tropical East African populations into distinct sub-groups. This may indicate that population sub-structuring within this group is less pronounced and the populations were exercising some sort of local gene flow. Nevertheless,

some of these populations share a gene pool with the Ethiopian ones. Moreover, the grouping of one population from Aberdare with the reference populations may show some degree of genetic link in the past although it is difficult to a full account of it based on the present data.

Although three major groups were observed with the PCO analysis and the neighbour-joing (Fig. 9), the population genetic mixture analyses (Fig. 12) have clearly revealed two main lineages in *E. arborea*. There is no sharp boundary between the two main lineages but with little overlap of gene pools from different geographical regions. Thus, the observed patterns of *E. arborea* could be explained by three alternative scenarios. First, this species has perhaps radiated from the Ethiopian highlands to the north (Mediterranean region) and to the south to the tropical East African Mountains (i.e. Ethiopia as centre of diversity or simply 'out of Ethiopia'). Second, Ethiopia could be a contact zone for the Mediterranean and tropical East African populations and the genetic diversity of this species remained relatively very high. Third, as suggested by McGuire & Kron (2005) but less likely according to our result is that the species could have expanded to Africa from the Mediterranean region. However, we are not ruling out other alternative hypothesis but further studies covering the whole distribution range of the species may shade light on these complex patterns of genetic structure of *E. arborea*. Although the current data set exhibits no clear geographic patterns of the gene pools the identified divergent lineages of *E. arborea* have conservation implications.

Generally, no clear phylogeographic pattern has been found for this species may be due to our population samples are restricted to part of the whole distribution range of the species. However, the absence of a clear phylogeographic structure in *E. arborea* may be the interplay of several factors: i) presence of refugia with large population sizes during the last glacial

maximum, ii) a high speed of re-colonization and dispersal ability and iii) a high mutation rate. Further studies are required to test the contributions of each of these factors.

3.2. *Hypericum revolutum*

3.2.1. Results

The AFLP analysis of 227 individuals from 33 populations of *H. revolutum* provided a total of 152 markers, of which 146 (96%) were polymorphic. The calculated average reproducibility of the markers was found to be 96%. The PCO plot based on the Dice similarity coefficient (Fig. 13a), where the first and second axis extracted 15.8% and 7.5% of the variation, grouped the populations into two main clusters: Ethiopian mountains and tropical East African mountains. Remarkably, populations from Mt. Elgon were not completely aligned with the tropical East African group. Two of them were clustered with Ethiopian populations. The third axis explained 5.9% of the variation and has not clearly separated the Ethiopian mountain into subgroup (Fig. 13b). However, the tropical East African group was subdivided into two (Kenya-Aberdare and Kilimanjaro-Meru). All populations from Mt. Elgon have formed a separate cluster in this plot. The distinct Ethiopian mountain clusters revealed by the third axis of the PCO plot were also supported by the neighbour-joining analysis (Fig. 14).

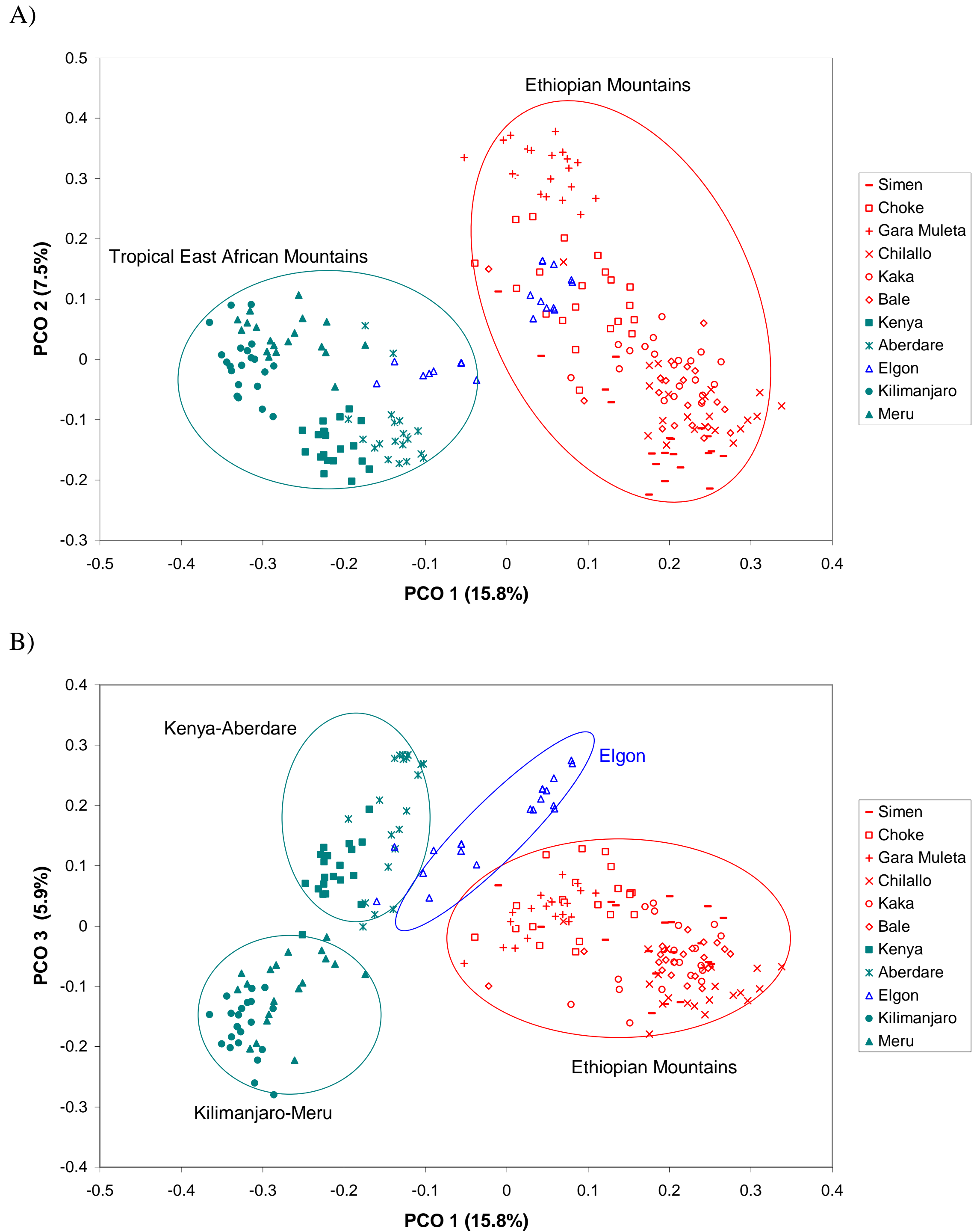


Fig. 13. Principal coordinates analysis (PCO) of individual AFLP phenotypes of *H. revolutum* from 33 populations based on the Dice similarity coefficient. (A) Axes 1 and 2 (B) Axes 1 and 3.

In the neighbour-joining analysis (Fig. 14), almost all the recognized populations in the third PCO plot form a separate cluster with some of which supported with bootstrap values. Kenya-Aberdare and Kilimanjaro-Meru were recognized as separate clusters on the tree.

Fig. 14. Neighbour-joining tree of the AFLP phenotypes of 227 individual plants of *H. revolutum* from 33 populations based on Nei and Li (1979) genetic distance. The two major genetic groups identified in *H. revolutum* are indicated by different colours on the tree. The numbers above the branches are bootstrap values higher than 50% (1000 replicates).

The calculated average gene diversity among populations was 0.15. The highest values were observed in some population from Mt. Choke, Simen Mountains, Mt. Chilallo and Bale Mountains (Table 4, Fig. 15). Relatively the lowest values were recorded for two populations from Mt. Elgon (H205, D = 0.011) and Aberdare (H152, D = 0.038). From the two major groups (Ethiopian Mountains and tropical East African Mountains, Fig. 13a), a relatively high average gene diversity among populations was observed in Ethiopian mountains and two populations from Elgon (D = 0.17, Table 5).

Table 4. Locality, number of individuals (n) and gene diversity (D) of the investigated populations of *H. revolutum.*

Pop.	Country	Mountain	Lat/Long	Altitude (m)	n	D
H059	Ethiopia	Simen	N13.26133/E038.19872	3679	5	0.176
H063	Ethiopia	Simen	N13.26879/E038.10975	3609	10	0.214
H070	Ethiopia	Simen	N13.23236/E038.04013	3253	5	0.195
H067	Ethiopia	Choke	N10.72652/E037.80306	3553	5	0.211
H072	Ethiopia	Choke	N10.63755/E037.82961	3714	5	0.214
H073	Ethiopia	Choke	N10.68458/E037.83550	3797	11	0.218
H111	Ethiopia	Gara Muleta	N09.22622/E041.79065	3151	11	0.127
H112	Ethiopia	Gara Muleta	N09.22172/E041.78778	3149	5	0.121
H117	Ethiopia	Gara Muleta	N09.20955/E041.79327	2860	5	0.132
H049	Ethiopia	Chilallo	N07.93456/E039.21193	3447	11	0.204
H050	Ethiopia	Chilallo	N07.93536/E039.20113	3239	5	0.179
H055	Ethiopia	Chilallo	N07.92032/E039.18398	3047	5	0.182
H034	Ethiopia	Kaka	N07.36367/E039.18821	3641	10	0.162
H039	Ethiopia	Kaka	N07.37116/E039.19295	3528	5	0.176
H042	Ethiopia	Kaka	N07.36780/E039.19612	3506	5	0.167
H009	Ethiopia	Bale	N06.92182/E039.93216	3585	11	0.174
H022	Ethiopia	Bale	N07.10455/E039.79150	3122	5	0.226
H027	Ethiopia	Bale	N07.05563/E039. 61990	3526	5	0.180
H160	Kenya	Kenya	S00.17046/E037.21450	3051	10	0.135
H161	Kenya	Kenya	S00.10302/E037.13880	3224	5	0.125
H173	Kenya	Kenya	S00.17101/E037.20844	2983	5	0.142
H138	Kenya	Aberdare	S00.33923/E036.65603	3573	5	0.149
H146	Kenya	Aberdare	S00.34058/E036.66968	3484	5	0.178
H152	Kenya	Aberdare	S00.47638/E036.72564	2885	11	0.038
H185	Kenya	Elgon	N01.05820/E034.76339	2610	11	0.112
H204	Kenya	Elgon	N01.10151/E034.61759	3675	5	0.138
H205	Kenya	Elgon	N01.08708/E034.63153	3383	5	0.011
H076	Tanzania	Kilimanjaro	S03.17648/E037.51234	2823	11	0.095
H078	Tanzania	Kilimanjaro	S03.18889/E037.51605	2583	5	0.122
H087	Tanzania	Kilimanjaro	S03.18232/E037.51229	2721	5	0.079
H089	Tanzania	Meru	S03.23212/E036.81414	2142	10	0.121
H090	Tanzania	Meru	S03.23158/Eo36.80417	2375	5	0.117
H093	Tanzania	Meru	S03.21686/E036.77339	3591	5	0.136

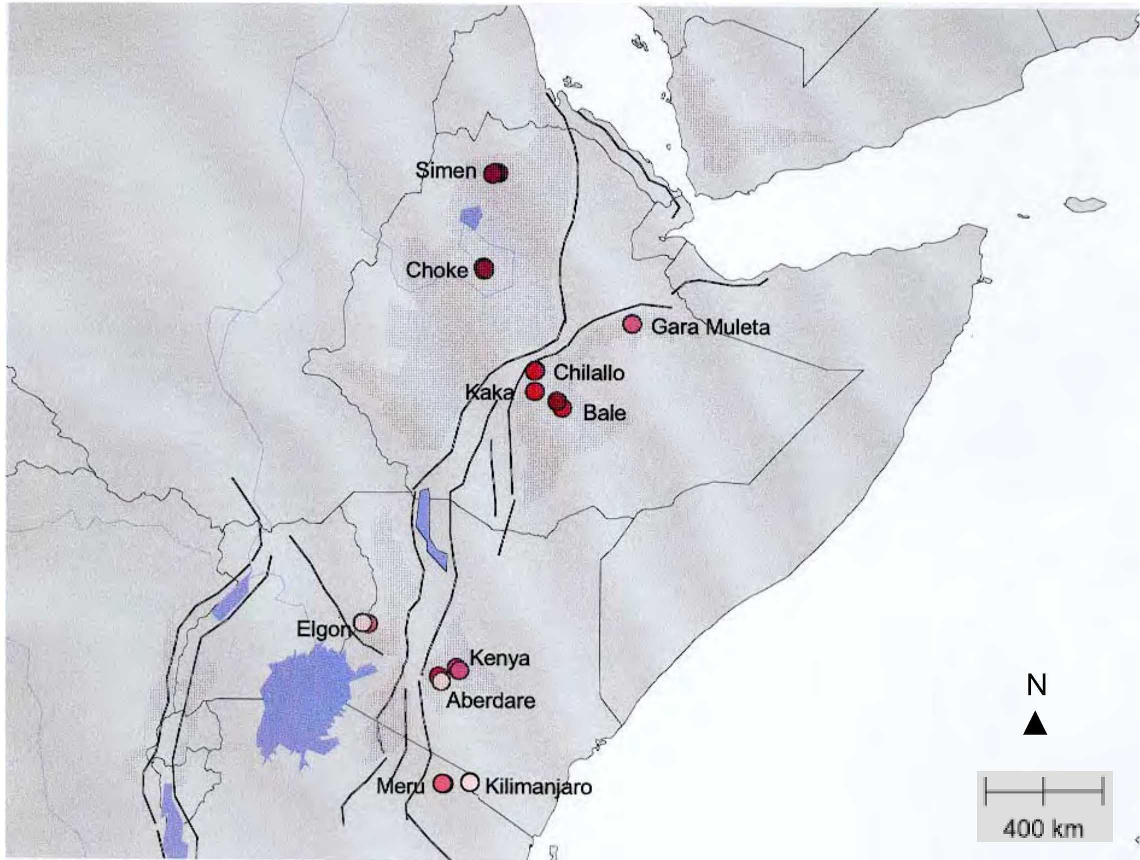


Fig. 15 Map showing intra-population gene diversity of *H. revolutum*. Colour intensity of the dots corresponds to gene diversity of populations: deep darker dots represent relatively higher gene diversity while pales (lighter) dots characterize lower gene diversity and the intermediate colours are in between.

Table 5. Average gene diversity among populations for the two main groups of *H. revolutum*.

Group	Number of mountains	Number of populations	Average gene diversity ± SD
Ethiopian mountains and two populations from Elgon	7	20	0.1690 ± 0.0501
Tropical East African Mountains and one population from Elgon	5	13	0.1211 ± 0.0347

We detected 8 private AFLP markers in Ethiopian mountains (excluding two populations of Mt. Elgon which clustered with Ethiopian populations). No private markers were recorded from the three Mt. Elgon populations as well as for those from tropical East African Mountains.

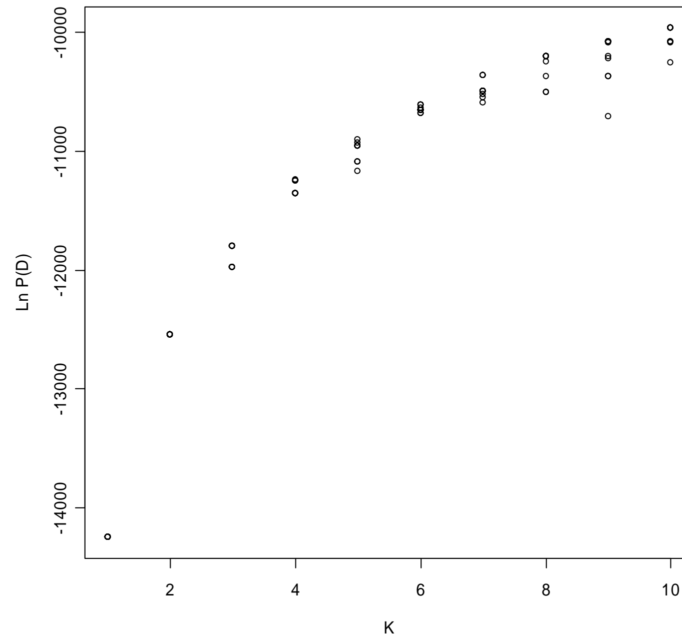
In a hierarchical AMOVA, 19.7% of genetic variation was found among the two main groups identified by PCO (Table 6). There was also some degree of genetic differentiation among the subgroups. In separate AMOVA analyses for each main group, 13.2% of the variation was found among Ethiopian mountains, and 17.6% among mountains of tropical East Africa. These differentiations were not strong as observed from the groups. However, high differentiation was observed during separate analysis of both groups within populations (Table 6).

Table 6. Analysis of molecular variance (AMOVA) of the AFLP data for *H. revolutum*. p-values were estimated in a permutation test (10000 permutations).

Source of variation	% of total variance	
Among the two major groups	19.7	p = 0.001
Among mountains within groups	24.23	p < 0.0001
Within mountains	56.07	
Ethiopian Mountains and Elgon-Eth		
Among mountains	13.2	p = 0.03
Among populations within mountains	18.36	p < 0.0001
Within populations	68.44	
TEA Mountains and Elgon-Tea		
Among mountains	17.6	p = 0.1
Among populations within mountains	22.12	p < 0.0001
Within populations	60.28	

Analysis of BAPS of the populations of this species has formed ten main groups. Most of the clusters (e.g. Elgon, Simen, Choke, Gara Muleta and Aberdare) were formed from populations originated from a mountain system. Other clusters were groups of populations from two or more mountain systems (e.g. Aberdare-Kenya, Kilimanjaro-Meru, Bale-Kaka-Chilallo, and Bale-Choke-Kaka-Chilallo). This shows that representatives of a mountain system were distributed into more than one cluster groups. Like the PCO analysis, Structure analysis has resulted into two main gene pools of *H. revolutum* (Fig. 17). The graph of the likelihood of the AFLP data estimated from the different structure runs showed a clear point of inflection for K = 2 (Fig. 16a). Up to K = 2, outputs from the program were also absolutely identical, as shown on the plot of similarity coefficients against K (Fig. 16b). For large values of K, the likelihood values were slightly high but there was no convergence for a particular configuration of clusters.

A)



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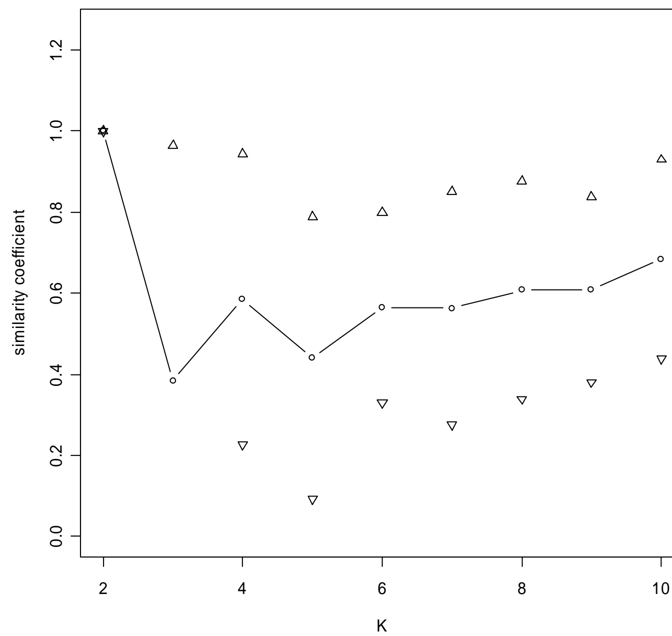


Fig. 16. Structure analysis of the AFLP data for *H. revolutum*. (a) Estimated likelihood for values of K ranging from one to 10. (b) Similarity coefficients of the results from different runs of Structure calculated according to Rosenberg *et al.*, (2002). Dots represent the average similarity coefficient for the pairwise comparisons among 10 runs and triangles show the standard deviation.

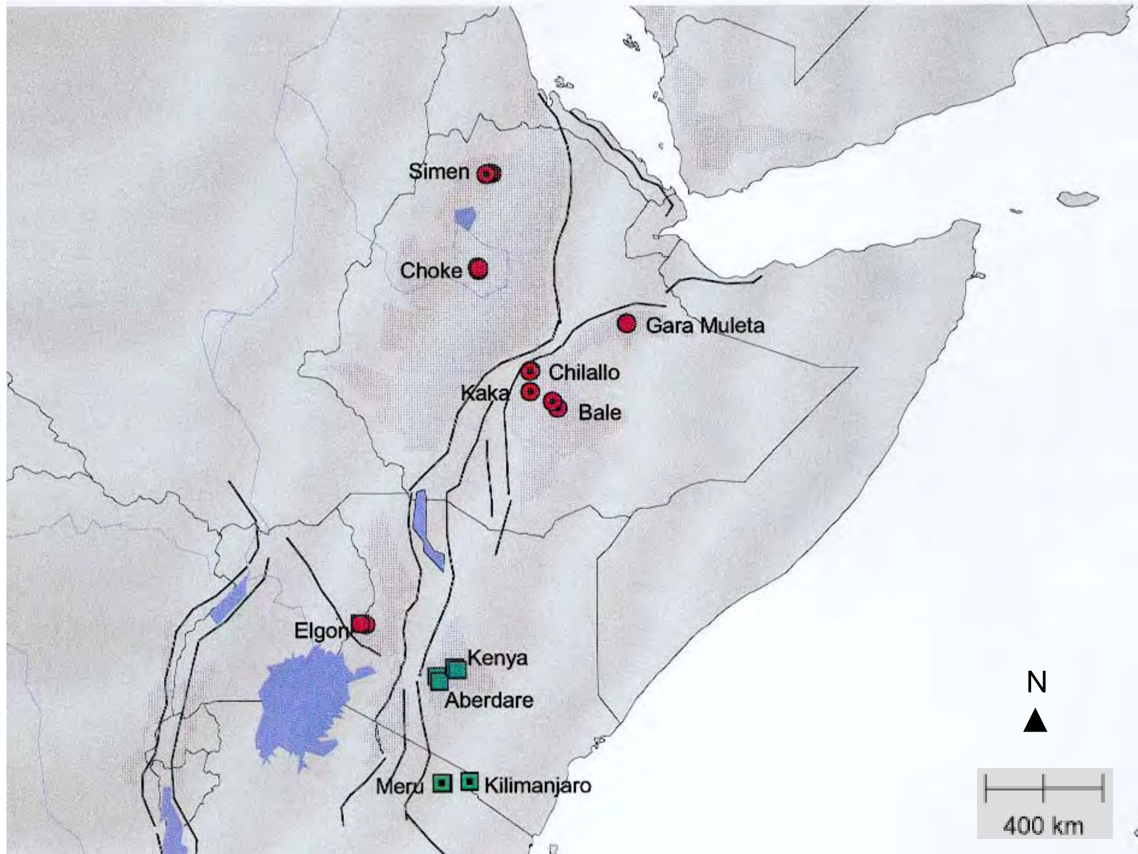


Fig. 17. Map showing the population structure of *H. revolutum*. Two clusters were identified. Red and green colours represent the two main groups and the marks inside the squares and circles correspond to the sub-groups.

Population sub-structuring was observed within the two main groups (Fig. 17). However, this sub-structuring was not in accordance with the geographical locations of the mountains in the Ethiopian lineage. On the other hand, sub-grouping of populations of tropical East African lineage is inline with the geographical proximity.

3.2.2. Discussion

Two major lineages (Ethiopian and tropical East African) were revealed from PCO analysis (Fig. 13a) for this species. Although there is concordance between geographical location and genetic structure of the populations at a large scale, the pattern is not as distinct as observed in *Lobelia giberroa*. The two major divergent lineages were also revealed by the genetic mixture analyses (Fig. 17), which more or less correspond to geographically distinct regions. Generally, all populations originated from different mountain systems have grouped into their respective geographical areas. However, two populations from Mt. Elgon have grouped with those from Ethiopia and while one population has clustered with those from tropical East Africa. These observed patterns may suggest that Mt. Elgon could be a contact zone for the populations of Ethiopia and tropical East Africa. In the nested analysis of molecular variance, 19.7% ($P = 0.001$) of total variance was described among the two major groups (Table 6). This low level of variance might be an indication of recent divergence between the two major lineages. However, the level of differentiation within mountains was found to be very high, 56.07% ($P < 0.0001$). In the neighbour-joining tree (Fig. 14), the two populations of Mt. Elgon which were grouped with Ethiopian ones were nested within the tropical East African group but with a little bootstrap support. Given the observed patterns and high degree of polymorphic markers (96%) among the populations, we suggest that populations of *H. revolutum* are undergoing strong genetic differentiations within a mountain system. We also rule out long-distance dispersal as mediated by cyclones for *H. revolutum.* Furthermore, the absence of mass dispersal is apparent owing to the observed heterogeneity of intra-population gene diversity (Table 4 & Fig. 17) and the number of private AFLP markers observed in the Ethiopian populations. Therefore, the general pattern observed between the two main lineages

of *H. revolutum* corresponds well to a gradual dispersal model of species mediated by montane forest bridges (Kebede *et al.*, 2007b).

In Ethiopia, the mountains are connected by continuous plateaux of highlands and must have supported extensive afro-montane forests. Although Simen Mountains and Mt. Choke (north of the Ethiopian Rift Valley) are connected through highlands, they harbour populations with different genetic structures. Similarly, Gara Muleta which is connected to Chilallo-Kaka-Bale through mountain ridges has populations which have exhibited close genetic similarities to those from a remote geographic area, i.e., Mt. Choke (Fig. 13a, 14). Similarly, populations from Chilallo-Kaka-Bale had strong genetic similarity with those from Simen Mountains. This observed genetic discontinuity between geographically close mountains could be explained by considering genetic drift as the habitat of the species fragmented due to anthropogenic activities. The environments of Choke and Gara Muleta are subject to more human influences compared to the other mountain systems of Ethiopia. The populations of *H. revolutum* are highly fragmented on Mt. Choke and Gara Muleta such that genetic drift leads to loss of diversity and induces divergence of isolated (fragmented) populations.

The Tropical East African populations formed a distinct cluster as revealed by different analytical methods such as neighbour-joining tree, PCO and genetic mixture analysis. This group is also unique compared to Ethiopian group due to its lowest gene diversity. Low level of genetic diversity may be a manifestation of a founder effect or could result from leading edge colonization as well where diversity is gradually lost due to recurring colonization of new area by only a few individuals (Hewitt 1996; Petit *et al.*, 2002). In these mountains, the populations of *H. revolutum* are probably facing a founder event due to one of the above factors or other possible causes.

Further sub-structuring was observed in the Tropical East African Populations. The third PCO (Fig. 13b) results into Kenya-Aberdare and Kilimanjaro-Meru sub-groups where the mountains have clustered according to their geographical proximity. In this case, montane forest bridges must have played a significant role in the exchange of genes among populations of these mountain pairs. Although no subgrouping was observed for the populations of the Ethiopian mountain systems, the same axis has grouped all populations from Mt. Elgon as a distinct cluster. This may suggest that Mt. Elgon could be a contact zone for the two major lineages of *H. revolutum*. A geogrpahical contact among forest patches was as recent as the present interglacial and there is considerable evidence that the climate was more humid in large parts of eastern Africa during the beginning of Holocene (Kuper & Kröpelin 2006). In some regions where habitat was not fragmented by agriculture, it is also more likely that forest patches were in direct contact (Hedberg 1969). Our data do not provide a strong evidence for the age for the last montane forest bridge in East Africa since estimation of a time frame for divergence from AFLP data is difficult.

Although the tropical East African populations exhibit lower gene diversity and lack private AFLP markers compared to the Ethiopian populations, the identification of further divergent geographically structured subgroups of *H. revolutum* indicates that these lineages should be treated as distinct evolutionarily significant units (ESUs). Any conservation effort should take these independent ESUs of this species into consideration. ESUs are important components in the evolutionary history of a species (Moritz 1994; Taberlet & Cheddadi 2002; Petit *et al.*, 2003). Therefore, each independent unit should be conserved in all mountain systems.

In conclusion, a phylogeographic pattern of the populations of a widespread afro-montane *H. revolutum* is congruent to expectations of a hypothesis of montane forest bridges between mountain systems of Ethiopia and tropical East Africa during interglacial periods. Since our investigation did not include the Western Rift (Mt Ruwenzori and Virunga volcanoes), we could not resolve the migration history of this species in its entire distribution range. Further phylogeographic study covering the entire distribution area of the species is necessary to deduce a concrete migration history of *H. revolutum*.

3.3. Lobelia giberroa

3.3.1. Results

The AFLP analysis of 191 individuals from 25 populations of *L. giberroa* using 3 primer pair combinations provided a total of 173 markers, of which 132 (76.3%) were polymorphic. The PCO plot based on Dice similarity coefficient (Fig. 18a), where the first and second axis explained 33.4% and 17.1% of the variation, respectively, grouped the populations into three main geographically distinct gene pools, i.e., (1) the Ethiopian group, (2) the Elgon-Cherangani group and (3) the Mt. Kenya-Aberdare-Kilimanjaro-Meru group. The Elgon-Cherangani group had the most divergent position and the main division in the dataset was thus observed across the eastern Rift Valley in tropical East Africa. The third axis explained 4% of variation (Fig. 18b) and further splitted two of the major groups into subgroups. The Ethiopian group was divided into three subgroups: Simen-Choke (north of the Ethiopian Rift Valley), and Chilallo-Bale and Gara Muleta (south of the Ethiopian Rift Valley). In the latter case, the subdivision was in accordance with geography as Mt Chilallo and Bale Mountains are quite close to each other and Gara Muleta is situated at the other end of a long mountain ridge. Two divergent subgroups were also observed in the third main group: the Kilimanjaro-

Meru subgroup and the Kenya-Aberdare subgroup, each of which were found on two geographically close mountain pairs. The PCO based on simple matching similarity coefficient showed exactly the same structure (not shown).

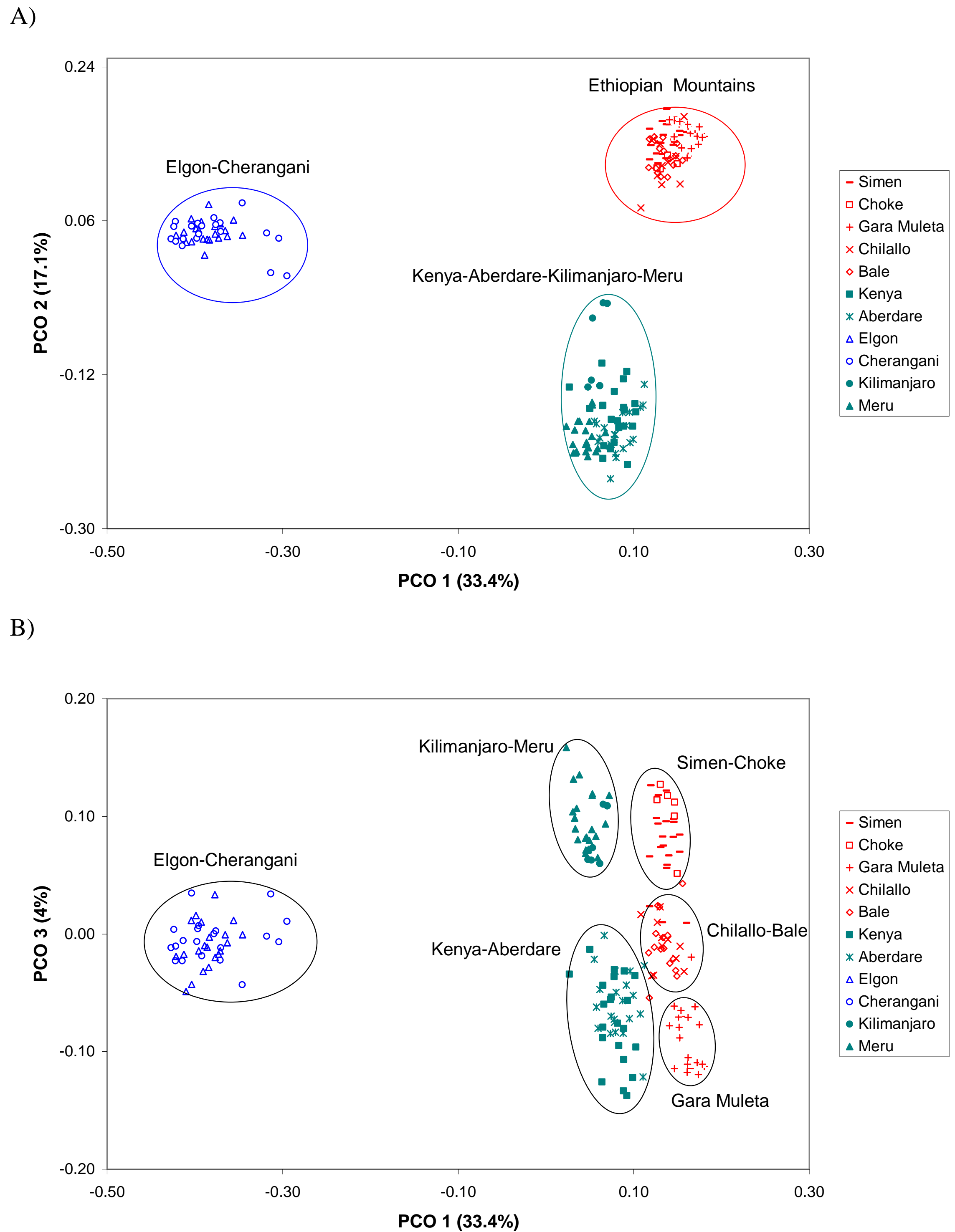


Fig. 18. Principal coordinate analysis (PCO) of individual AFLP phenotypes of *L. giberroa* from 25 populations based on the Dice similarity coefficient. (a) Axes 1 and 2 (b) Axes 1 & 3.

In the neighbour-joining analysis (Fig. 19), the Ethiopian group and the Elgon-Cherangani group had high bootstrap supports and were identified as the most divergent group. On the other hand, the Kenya-Aberdare-Kilimanjaro-Meru group was not supported and did not form a distinct cluster. It is to be noted that Nei and Li and simple matching coefficients has also yielded the same topology and bootstrap support values.

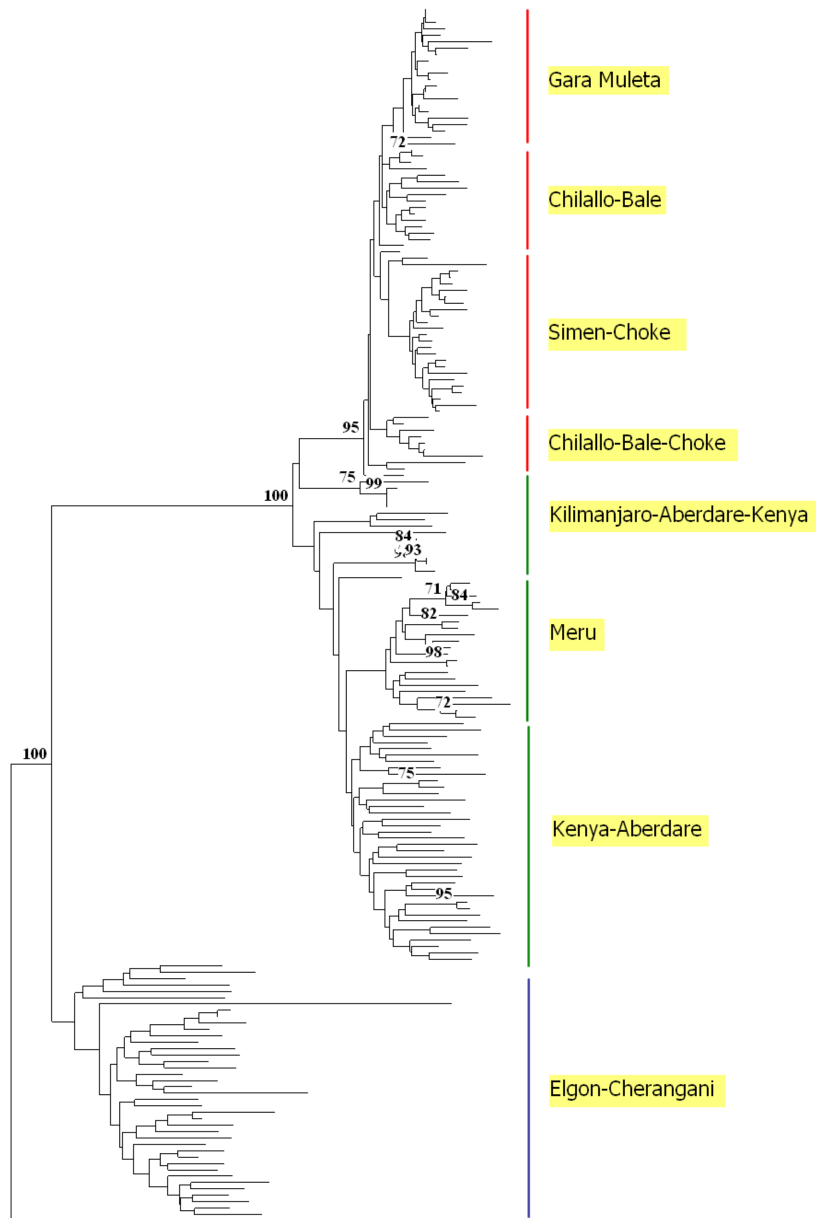


Fig. 19. Neighbour-joining tree of the AFLP phenotypes of 191 individual plants of *L. giberroa* from 25 populations based on Nei and Li genetic distance. The three major genetic groups identified in *L. giberroa* are indicated by different colours on the tree. The numbers above the branches are bootstrap values higher than 50% (1000 replicates).

The average gene diversity among populations was 0.0605. The highest values were observed in population L224 from Cherangani Hills (D = 0.1017; Table 7). The average gene diversity among populations in the major groups (Table 8, Fig. 20) was relatively high in the Elgon-Cherangani (0.085) and Kenya-Aberdare-Kilimanjaro-Meru (0.077) groups and lowest in the Ethiopian mountains (0.036).

Table 7. Locality data, number of individuals (n) and gene diversity (D) of the investigated populations of *L. giberroa.*

Pop.	Country	Mountain	Lat/Long	Altitude (m)	n	D
L056	Ethiopia	Simen	N13.22166 / E038.99902	2370	5	0.028
L057	Ethiopia	Simen	N13.29127 / E038.09282	2470	11	0.034
L058	Ethiopia	Simen	N13.29853 / E038.12611	2590	5	0.043
L500	Ethiopia	Choke	N10.42490 / E037.47320	2850	6	0.035
L120	Ethiopia	Gara Muleta	N09.22051 / E041.78170	2520	5	0.036
L122	Ethiopia	Gara Muleta	N09.23278 / E041.74273	2590	11	0.027
L124	Ethiopia	Gara Muleta	N09.23316 / E041.74327	2600	5	0.022
L312	Ethiopia	Chilallo	N07.92914 / E039.16980	2850	11	0.040
L125	Ethiopia	Bale	N06.70370 / E039.72004	2310	6	0.052
L126	Ethiopia	Bale	N06.61483 / E039.73801	1840	4	0.032
L127	Ethiopia	Bale	N06.65274 / E039.73223	1920	5	0.044
L104	Tanzania	Meru	S03.22517 / E036.79781	2630	11	0.065
L105	Tanzania	Meru	S03.22582 / E036.79834	2470	11	0.064
L088	Tanzania	Kilimanjaro	S03.20025 / E037.51874	2400	11	0.052
L134	Kenya	Aberdare	S00.39531 / E036.73089	2960	11	0.075
L154	Kenya	Aberdare	S00.50920 / E036.64869	2950	5	0.087
L155	Kenya	Aberdare	S00.72276 / E036.67835	2820	5	0.090
L158	Kenya	Kenya	S00.17617 / E037.19933	2820	11	0.095
L182	Kenya	Kenya	N00.00469 / E037.24118	2610	11	0.085
L186	Kenya	Elgon	N01.07194 / E034.72719	2540	5	0.080
L200	Kenya	Elgon	N01.06383 / E034.70394	2920	5	0.086
L201	Kenya	Elgon	N01.06134 / E034.68774	3000	11	0.091
L213	Kenya	Cherangani	N01.20582 / E035.28007	2580	11	0.079
L214	Kenya	Cherangani	N01.16556 / E035.33190	2710	4	0.071
L224	Kenya	Cherangani	N00.05275 / E035.53882	2730	5	0.102

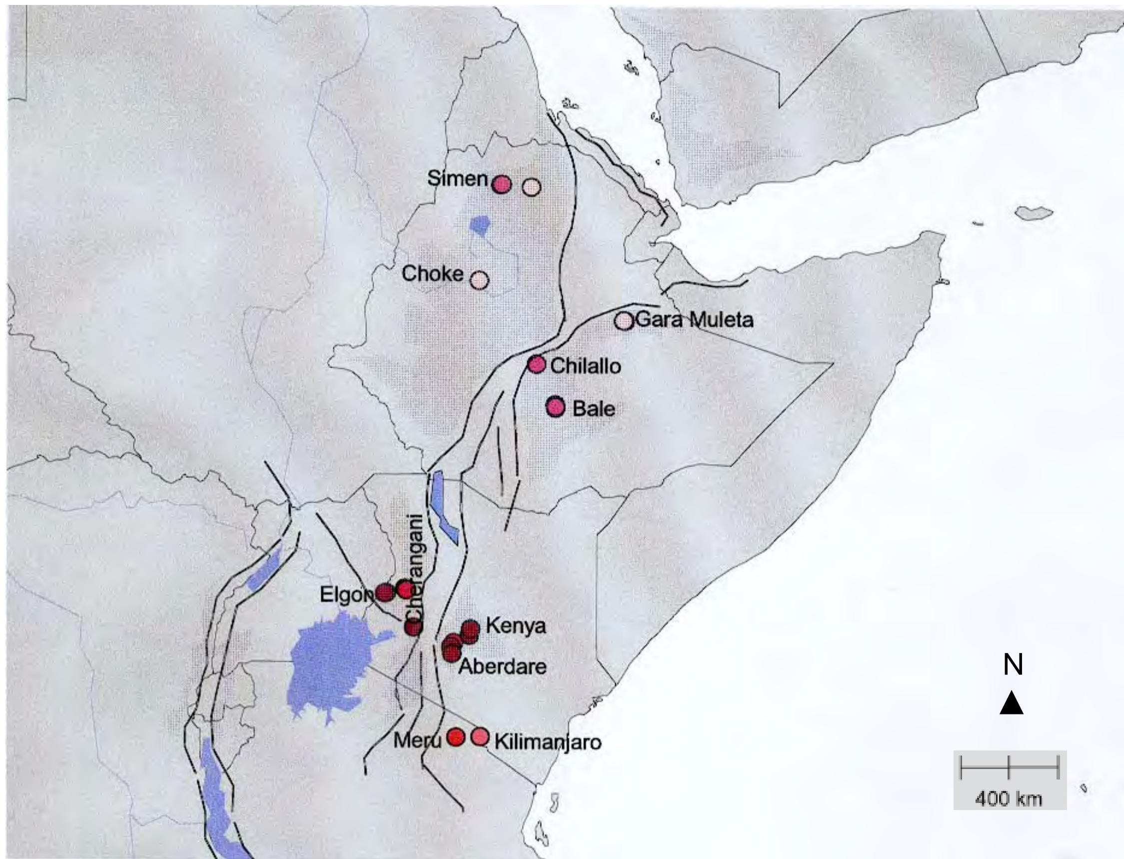


Fig. 20. The Intra-population gene diversity of *L. giberroa*. Colour differences show variation in gene diversity. The darker the dot the higher is the gene diversity whereas the paler the lower the gene diversity.

No pronounced differences were observed in the number of private AFLP markers among the three main groups. We detected four private AFLP markers in the Ethiopian mountains, eight in Elgon-Cherangani and six in Kenya-Aberdare-Kilimanjaro-Meru (Table 8).

Table 8. Average gene diversity among populations and the number of private AFLP markers for the three major groups of *L. giberroa*.

Group	Number of mountains	Number of populations	Average gene diversity ± SD	Private AFLP markers
Ethiopian mountains	5	11	0.036 ± 0.009	4
Kenya-Aberdare-Kilimanjaro-Meru	4	8	0.077 ± 0.015	6
Elgon-Cherangani	2	6	0.085 ± 0.011	8

In a hierarchical AMOVA, the largest proportion of the genetic variation was found among the three main groups identified by PCO with an estimate of 55.71% (Table 9). There was also considerable differentiation among the subgroups. In separate AMOVA analyses for each main group, 36.7% of the variation was found among the subgroups in Ethiopia, and 16.8% between Kenya-Aberdare and Kilimanjaro-Meru. Differentiation between Elgon-Cherangani groups was not significant (Table 9). In the Ethiopian mountains, the genetic differentiation across the Rift Valley corresponded to 31.5% of the variation ($p = 0.003$), whereas 27.9% of the variation was found between Gara Muleta and Bale-Chilallo ($p = 0.029$) and 30.0% ($p = 0.029$) between Simen-Choke and Bale-Chilallo. Genetic differentiation was even high (50.6%, $p = 0.027$) between the two divergent subgroups, i.e., Simen-Choke and Gara Muleta. On the other hand, differentiation of the gene pools was found to be low and not significant (Simen-Choke: 14%, $p = 0.25$; Bale-Chilallo: 18.4%, $p = 0.25$) among mountain massifs within subgroups.

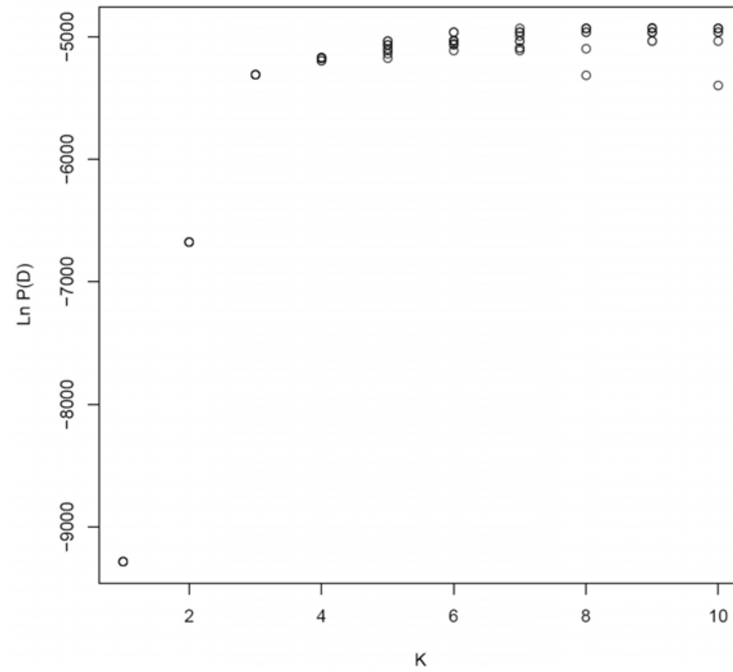
Table 9. Analyses of molecular variance (AMOVA) of the AFLP data for *L. giberroa*. p-values were estimated in a permutation test (10000 permutations).

Source of variation	% of total variance	
Among the three major groups	55.71	p < 0.0001
Among mountains within groups	12.31	p < 0.0001
Within mountains	31.98	
Ethiopian Mountains		
Among subgroups	36.69	p = 0.0001
Among populations within subgroups	10.39	p < 0.0001
Within populations	52.92	
Elgon-Cherangani		
Among mountains	2.64	p = 0.1
Among populations within mountains	14.58	p < 0.0001
Within populations	82.78	
Kenya-Aberdare-Kilimanjaro-Meru		
Among subgroups	16.79	p = 0.017
Among populations within subgroups	18.96	p < 0.0001
Within populations	64.25	

The result from BAPS analysis revealed clustering of the populations into three main groups. In addition, the populations from Kenya-Aberdare were separated from Kilimanjaro-Meru. However, the genetic distance between these two groups (estimated in a Külbach-Leibler distance matrix by BAPS, not shown), was much smaller than the values among the other clusters. The result of the Structure analysis was congruent with the results from other analyses. The populations were grouped into the same three main gene pools (Fig. 21 & 22). The graph of the likelihood of the AFLP data estimated from the different runs showed a clear

point of inflection for K = 3 (Fig. 21a). Up to K = 3 outputs from the program were also absolutely identical, as shown on the plot of similarity coefficients against K (Fig. 21b). For larger values of K, the likelihood values were slightly higher, but there was no convergence for a particular configuration of clusters.

A)



B)

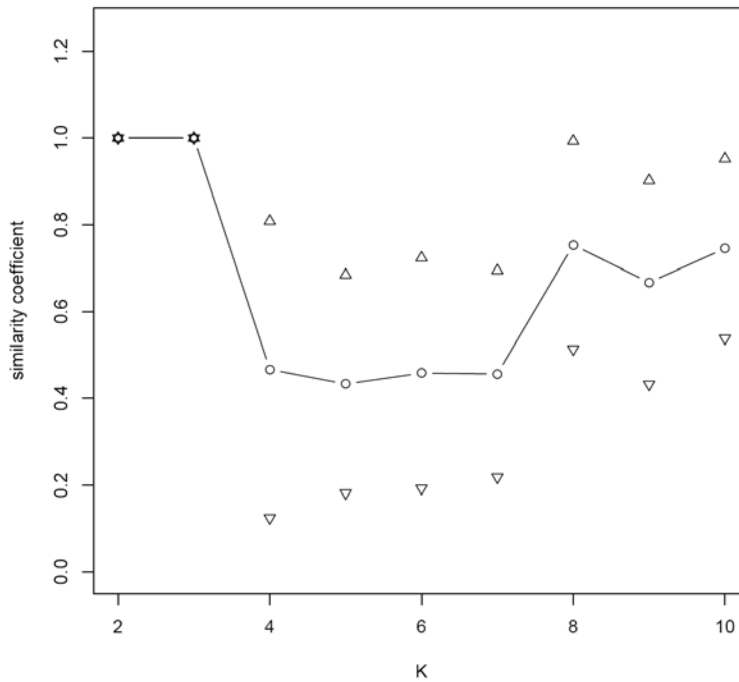


Fig. 21. Structure analysis of the AFLP data for *L. giberroa*. (a) Estimated likelihood for values of K ranging from one to 10. (b) Similarity coefficients of the results from different runs of Structure calculated according to Rosenberg *et al.*, (2002). Dots represent the average similarity coefficient for the pairwise comparisions among 10 runs and triangles show the standard deviation.

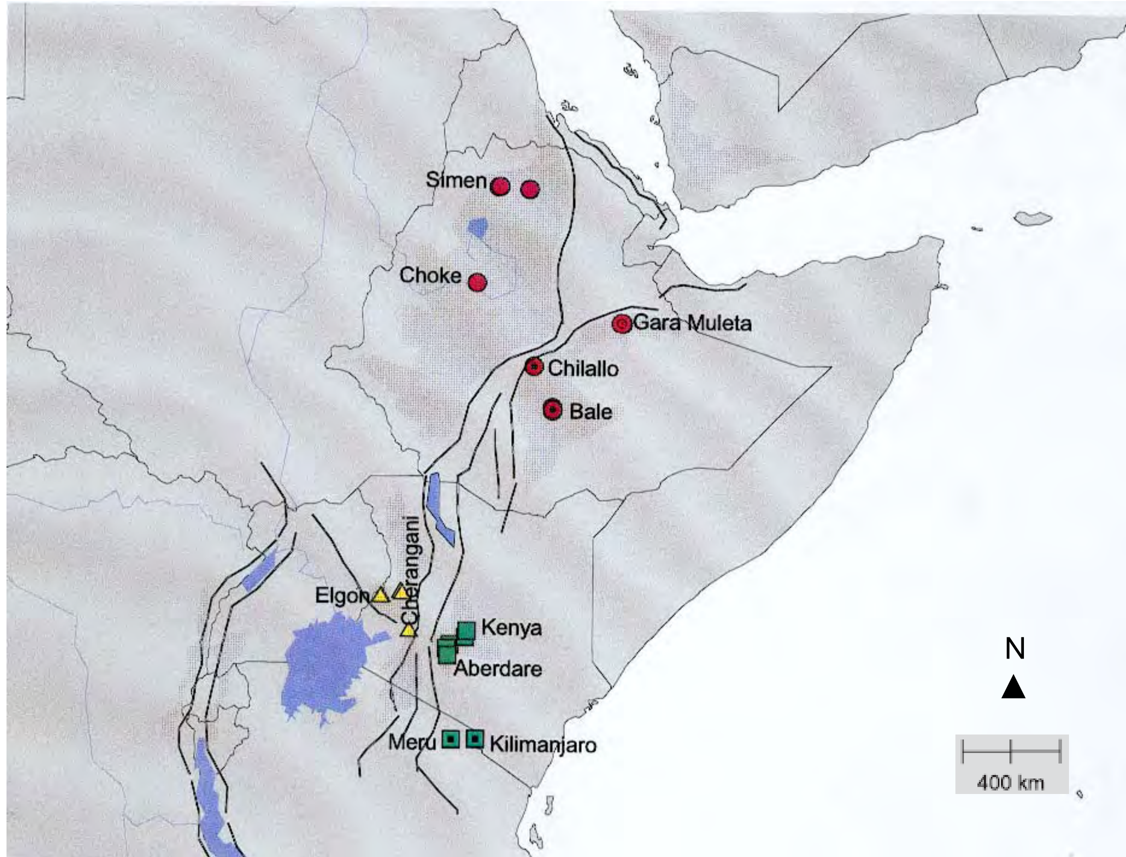


Fig. 22. Map showing the population structure of *L. giberroa*. Three clusters were identified. Red, green and yellow colours represent the main groups where as the shapes correspond to the sub groups.

3.3.2. Discussion

The PCO and genetic mixture analyses clearly revealed three main groups, which corresponded to three geographically distinct regions: the Ethiopian mountains, Kenya-Aberdares-Kilimanjaro-Meru on the eastern side of the Rift Valley, and Elgon-Cherangani on the western side of the Rift in the southern part of the study area. In the neighbour-joining tree, the Ethiopian group and Elgon-Cherangani were identified with substantial bootstrap support, whereas the samples from Kenya-Aberdares-Kilimanjaro-Meru did not form a

supported group. Based on the PCO and the AMOVA results, two of the groups were divided into geographically separated subgroups. Altogether this phylogeographic pattern corresponds to expectations from a gradual dispersal model and indicates that *L. giberroa* did not colonize its large range by random long-distance dispersal mediated for example by cyclones.

In Ethiopia, one genetic subgroup (Simen-Choke) was identified north of the Rift Valley and two spatially separated groups (Chilallo-Bale and Gara Muleta) south of it. In an Ethiopian context, all mountain systems are connected by extensive highlands, which have once in the recent past supported almost continuous montane forests. Although Gara Muleta is connected to Mt. Chilallo and Bale Mountains by a narrow mountain ridge, its populations formed a distinct subgroup. Simen Mountains and Mt. Choke are geographically far apart but they are connected by a continuous mass of highlands. These two mountain systems harbour genetically very similar populations. Generally, the geographic patterns of the gene pools of *Lobelia giberroa* in Ethiopia agree well to the expectations of the montane forest bridge hypothesis.

In the mountains of tropical East Africa, our data did not clearly support a montane forest bridge across the Rift Valley between Cherangani and Aberdares, as postulated by Hedberg (1969). On the contrary, we observed the largest genetic discontinuity in our dataset between these two mountain regions and this agrees with the findings of Knox & Palmer (1998). Although montane forests occur as an altitudinal vegetation belt in the afro-alpine environment, its distribution is strongly influenced not only by altitude but also by precipitation (Hedberg 1951). Notably, although *L. giberroa* has quite large altitudinal range, it requires high humidity (Knox 1993, Lüttge *et al.*, 2001). Therefore, altitude alone may not provide all inferences concerning past distribution of this taxon and/or of the montane forests.

The bottom of the Rift Valley between Cherangani and Aberdares is quite high in some places (up to 1600 m, Chorowicz 2005), but this region is much drier than the bordering mountain ranges, which attract most of the rain clouds. A phylogeographic barrier has been reported in this region also for a montane forest bird, the olive sunbird (*Nectarinia olivacea/Nectarinia obscura*; Bowie *et al.*, 2004) suggesting the absence of a historical forest bridge.

On each side of the Rift Valley in tropical East Africa, populations from adjacent mountain massifs were genetically closely related (Elgon and Cherangani; Aberdares and Kenya) indicating the importance of montane forest bridges for dispersal of *L. giberroa.* This argument was also further supported by a noticeable differentiation between the two isolated volcanoes, i.e., Mt. Meru and Mt. Kilimanjaro, which are separated only by about 50 km of arid lowland. Altogether we conclude that our data, both in Ethiopia and tropical East Africa, are best explained by the montane forest bridge hypothesis.

Our genetic data seem consistent with the hypothesis that this contact among forest patches was as recent as the present interglacial. There is considerable evidence that the climate was more humid in large parts of eastern Africa during the first part of the Holocene (e.g. Kuper & Kröpelin 2006) which may suggest that montane forest bridges were extensive during this period. Furthermore, in areas where habitats were not fragmented by agriculture, forest patches were in contact (Hedberg 1969). However, because of the difficulties associated with the estimation of time frame for divergence from AFLP data, we will not provide a firm evidence for an older age for the last montane forest bridge in our study area.

The divergence among the major groups is likely to be old for at least two reasons. First, all three groups have private AFLP markers, i.e., there is no indication of recent colonization of

one region from another. Second, AMOVA revealed high differentiation among them (55.71%; Table 9). It is thus likely that the three lineages identified here survived several glacial periods when the montane forest was contracted by aridification, in separate refugia. Furthermore, we suggest that the subgroups found both in Ethiopia and in Kenya-Aberdares-Kilimanjaro-Meru may descend from distinct refugial populations during the last ice age and then expanded to occupy their respective current ranges over montane forest bridges at the beginning of the present interglacial.

The relative genetic distances among the three main groups, of which the Elgon-Cherangani group is the most divergent (Fig. 19), is consistent with the cpDNA restriction analysis data of Knox and Palmer (1998). Their study showed that *L. giberroa* from Mt Elgon and the Cherangani Hills had cpDNA haplotypes belonging to the clade of the Western Rift, whereas other mountains of the Eastern Rift (Kenya-Aberdare-Kilimanjaro-Meru group in our case) and the two Ethiopian individuals included in their study belonged to the other cpDNA clade. As we lack samples from the Western Rift (Mt. Ruwenzori and Virunga volcanoes), we can not confirm that the populations from Elgon-Cherangani are more closely related to the Western Rift populations than to the rest of the populations.

From the point of view of conservation, the identification of three highly divergent gene pools in this part of the range of *L. giberroa* indicates that these should be treated as distinct evolutionarily significant units (ESUs). Thus, conservation of this unique afro-montane giant *Lobelia* and indeed of the afro-montane forest ecosystem of Ethiopia and tropical East Africa should consider these distinct ESUs. Furthermore, despite the higher genetic diversity observed in tropical East Africa, the distinctness of the Ethiopian populations (including four

private markers) clearly support their conservational value and their status as an independent ESU.

Knox & Palmer (1998) suggested that the geographical origin of *L. giberroa* was situated either along the Western Rift or in the southern Highlands. According to their interpretation, Ethiopia has been colonized from the south. Our results show that the Ethiopian populations are more closely related to the plants from Kenya-Aberdare-Kilimanjaro-Meru than to those from Elgon-Cherangani indicating that the former region may have been the source for the ancient northward expansion of *L. giberroa*. This is congruent with the result from the cpDNA analysis of Knox & Palmer (1998), which placed the two available Ethiopian samples into the Eastern Rift clade.

Ethiopian mountains have lower average gene diversity within populations than tropical East Africa ones and have the fewest private AFLP markers. This low level of genetic diversity may reflect a founder effect or a manifestation of a leading edge colonization which leads to gradual loss of diversity due to repeated colonizations of new land by only a few individuals (Hewitt 1996; Petit *et al.*, 2002). In this case, fewer private AFLP markers would be the rule than exception. Genetic diversity may, however, also be reduced by habitat fragmentation. Today, *L. giberroa* typically occurs only in isolated patches due to the strong impact of agriculture on the slopes of most mountains. In a highly fragmented habitat, diversity will be lost from each patch, but at the same time the isolated populations will diverge from each other due to genetic drift. Therefore, gene diversity can still be quite large due to a high level of differentiation over the whole area .In the Ethiopian populations of *L. giberroa* where average intra-population genetic diversity is lower than in tropical East African ones (Table 8), we argue that the relatively low diversity is most likely caused by their colonization

history. A support for this assertion has emerged from the distribution of genetic diversity of this species within Ethiopia. Higher genetic variability was recorded for the southern Ethiopia populations (Bale Mountains and Mt. Chilallo) than elsewhere in the country perhaps suggesting that this part of Ethiopia was colonized first by *L. giberroa* coming from the south and which has subsequently served as the source for further northwards colonization (Simen Mountains and Mt. Choke).

Gillett (1955) suggested that the high mountain flora of southern Ethiopia shows a stronger resemblance to that of the East African Mountains than to the mountains of northern Ethiopia. This is clearly not the case for intra-specific differentiation in *L. giberroa*. The closest relatives of the southern Ethiopian populations of *L. giberroa* were found in northern Ethiopia and all Ethiopian populations together formed a distinct genetic cluster. Nevertheless, the strongest subdivision among Ethiopian populations was found on both sides of the Ethiopian Rift Valley which may suggest this physical feature as a barrier to dispersal of afro-montane plant species.

In summary, our results have demonstrated that there is a strong phylogeographic structure among the populations of afro-montane giant lobelias, *L. giberroa*, in the afro-alpine environment of Ethiopia and tropical East Africa. This observed geographical structure of its gene pools corresponds to expectations of hypothesis of montane forest bridges between mountains during interglacial periods. Early divergence among the three main lineages was inferred. Since our samples were all from the Eastern Rift, we could not resolve the history of this ecologically important species in its entire range. However, this first phylogeographic study of an afro-montane plant has considerably contributed to our understanding of the history of the afro-montane flora.

4. Conclusion

The contemporary distribution of biological diversity can not be understood without knowledge of how organisms responded to the geological and climatic history of Earth. In particular, Quaternary expansions and contractions of glacial ice sheets are thought to have played an important role in shaping the distribution of biodiversity among current populations of the afro-alpine s.l. region. Thus, the history of a species or a population can have a great impact on its current genetic structure and the latter can also be used to deduce species past history. Phylogeographic patterns from contemporary genetic samples have proven highly informative in recovering the demographic histories of species.

Previously, very few studies have described the phylogeograpic pattern of some representatives of African biota. Particularly, this study is the first to describe the phylogeographic pattern of the afro-alpine flora with emphasis on the afro-montane plant species. The present study revealed some pattern of phylogeographic structure in this region. Although the study indicated no evidence of common phylogeographic structure among the three investigated species, it is possible to draw some general trends.

I) For some of the mountains which are geographical close, the montane forest bridge hypothesis provides plausible explanations for the genetic data sets of the study species. This assertion is supported by evidences of historical gene flow among populations.

II) Divergent lineages were identified within each of the investigated species through different analytical methods (PCO, neighbour-joining, BAPS and population

structure analysis). Although there are distinct genetic groups within each of the studied species shaped by Pleistocene climate oscillations, differentiations into a species level was not noticed.

III) Quaternary climatic oscillations of the last 2 My caused repeated range shifts by the surviving taxa which has been manifested through altitudinal shifts and formation of complex refugia in tropical mountains. The afro-montane forests probably persisted in refugia located at mountain foothills or in river basins.

IV) The populations of Ethiopia are exercising genetic drift due to habitat fragmentation caused by human activity compared to the tropical East African populations.

V) Even though the overall genetic diversity of investigated species is high in the afro-alpine region, the pattern observed in populations of Elgon should be given priority.

VI) The probable contact zones suggested for *E. arborea* and *H. revolutum* are also the phylogeographic patterns observed in these species. Actually, the contact zone (probably a hybrid zone) is not localized exactly in the same place for different organisms as we have seen from our analyses. In general, such observed common patterns have implications to design a management plan for conservation of the afro-alpine flora.

Generally, tropical mountains provide stable habitat for the species during the Pleistocene period. This long term stability of the habitat through out the last glaciation is thought to be a function of continued local moisture availability and varied topography (Hewitt 2000). Thus, the local conditions appear to have buffered the extreme effects of Quaternary climate variability in the afro-alpine region. However, the pattern of postglacial colonization depends

on many factors such as habitat preferences, propagation and dispersal strategies, cold tolerance, abundance and competition. Thus, the observed phylogeographic pattern of a species might be complicated. For example, when gene pools from two or more refugia come together, as seen in the contact zones, genetic diversity will be increased by the presence of diverged lineages. Two populations in the same region living in similar habitats, but from different colonizing refugia will possess very different alleles and genomes, while two very distant populations in distinct habitats may have the same refugial genome. These events are probably related to the pattern observed in *E. arborea* and points out the importance of population history in the process of post glacial adaptation.

In fact, the analysis of fossil pollen and the study of genetic patterns of populations are the two complementary ways of deducing the past migration patterns of plant species. Although fossil and pollen data of our study species are missing, our genetic data set demonstrates that the specific history of *E. arborea, H. revolutum* and *L. giberroa* was different. A lack of perfectly congruent phylogeographic pattern may suggest that the three species have not had the same history of dispersal pattern. Each species displayed a unique phylogeographic pattern and different distributions of genetic diversity. This may suggest that different species have responded individually to the Pleistocene climatic fluctuations owing to their different ecological requirements and morphological traits. As described elsewhere in the text, the phylogeographic patterns of *E. arborea* were more complex than those of *L. giberroa* and *H. revolutum*. *L. giberroa* and *H. revolutum* are relatively cold sensitive compared to *E. arborea* where the latter has morphological features for adaptation to cold environment. Thus, refugia of *L. giberroa* and *H. revolutum* were probably of limited size and isolated from each other, allowing for population differentiation to develop among refugia. Whereas *E. arborea* is

relatively cold tolerant and was perhaps apparently able to survive the Last Glacial Maxima at higher altitudes with large population size.

Fragmentation due to local climatic instability was also identified as having the most likely influence on the differentiation of the lineages. Within species several lineages and in most cases geographically localized gene pools were identified, indicating that populations in one or a few mountain systems should be considered as independent ESUs. Thus, each species and indeed the whole afro-alpine habitat, needs to be protected. Interestingly, species survived in the tropical mountains via altitudinal shifts induced by climate change and locally in a varied topography. These factors may have contributed to the survival of various lineages during Pleistocene climate changes and resulting in gnome divergence. Thus, the current findings have significant implications to any conservation effort and highlighting areas where conservation priorities should be concentrated in the afro-alpine flora.

The current study, being the first of its kind in tropical Africa, has meaningful contributions to any future phylogeographic studies in the afro-alpine region. In prospect, it is appropriate to undertake extensive paleoenvironmental studies of the afro-alpine environment based on pollen fossils and other proxies to provide a platform for a robust phylogeographic studies. Well documented pollen records provide basic background information on which many hypotheses could be formulated and tested.

In conclusion, molecular methods are now widely accepted tools for approaching phylogeographical questions. In addressing such questions, it is important to highlight the importance of incorporating several plant species in the study and use some appropriate genetic criteria as much as possible. These might demonstrate the power of a comparative

phylogeographical approach and provide sharp picture of how the glaciations of the Pleistocene shaped the genetic constitution of plants in the afro-alpine region.

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Appendices

Appendix 1a. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *E. arborea.*

Pop.	Country	Mountain	Lat/Long	Altitude (m)	n
E061	Ethiopia	Simen	N13.26066/E038.13575	3713	4
E065	Ethiopia	Simen	N13.23181/E038.03970	3251	5
E066	Ethiopia	Simen	N13.21222/E038.00089	3187	7
E068	Ethiopia	Choke	N10.63408/E037.84431	3772	10
E069	Ethiopia	Choke	N10.63755/E037.82961	3714	5
E074	Ethiopia	Choke	N10.68319/E037.83739	3794	5
E108	Ethiopia	Gara Muleta	N09.22841/E041.79417	2962	11
E114	Ethiopia	Gara Muleta	N09.20987/E041.79477	2768	5
E116	Ethiopia	Gara Muleta	N09.20955/E041.79327	2860	5
E046	Ethiopia	Chilallo	N07.93842/E039.22580	3708	9
E048	Ethiopia	Chilallo	N07.93724/E039.21493	3497	5
E051	Ethiopia	Chilallo	N07.91853/E039.18356	3089	4
E028	Ethiopia	Kaka	N07.36887/E039.18170	3817	5
E030	Ethiopia	Kaka	N07.36773/E039.18508	3764	3
E032	Ethiopia	Kaka	N07.36624/E039.18676	3690	11
E004	Ethiopia	Bale	N06.77974/E039.75373	3376	4
E007	Ethiopia	Bale	N06.91311/E039.92304	3627	10
E015	Ethiopia	Bale	N07.04575/E039.75152	3332	2
E166	Kenya	Kenya	S00.16791/E037.25395	3931	5
E170	Kenya	Kenya	S00.16765/E037.24820	3847	10
E181	Kenya	Kenya	S00.05820/E037.29133	3528	2
E137	Kenya	Aberdare	S00.33997/E036.65958	3560	5
E139	Kenya	Aberdare	S00.33389/E036.65130	3669	5
E142	Kenya	Aberdare	S00.34058/E036.66967	3484	10
E206	Kenya	Elgon	N01.08708/E034.63153	3383	5
E207	Kenya	Elgon	N01.10912/E034.60957	3786	4
E208	Kenya	Elgon	N01.09678/E034.62244	3615	9
E075	Tanzania	Kilimanjaro	S03.17803/E037.50838	2851	11
E077	Tanzania	Kilimanjaro	S03.18684/E037.51462	2641	5
E080	Tanzania	Kilimanjaro	S03.13998/E037.44009	3706	5
E094	Tanzania	Meru	S03.21762/E036.77106	3611	5
E098	Tanzania	Meru	S03.21697/E036.76657	3606	11
E099	Tanzania	Meru	S03.21817/E036.76739	3629	5
E3231	Canary Islands	Tenerife	N28.05833/W16.08333	875	5
E3241	Canary Islands	Tenerife	N28.03333/W16.06014	760	4
E3226	Spain	Andalucia	N36.50301/W 05.21667	1220	4
E3228	Spain	Andalucia	N36.31667/W05.46667	950	5
E3250	Spain	Alcares	N42.81667/W06.85012	1550	5

Appendix 1b. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *H. revolutum.*

Pop.	Country	Mountain	Lat/Long	Altitude (m)	n
H059	Ethiopia	Simen	N13.26133/E038.19872	3679	5
H063	Ethiopia	Simen	N13.26879/E038.10975	3609	10
H070	Ethiopia	Simen	N13.23236/E038.04013	3253	5
H067	Ethiopia	Choke	N10.72652/E037.80306	3553	5
H072	Ethiopia	Choke	N10.63755/E037.82961	3714	5
H073	Ethiopia	Choke	N10.68458/E037.83550	3797	11
H111	Ethiopia	Gara Muleta	N09.22622/E041.79065	3151	11
H112	Ethiopia	Gara Muleta	N09.22172/E041.78778	3149	5
H117	Ethiopia	Gara Muleta	N09.20955/E041.79327	2860	5
H049	Ethiopia	Chilallo	N07.93456/E039.21193	3447	11
H050	Ethiopia	Chilallo	N07.93536/E039.20113	3239	5
H055	Ethiopia	Chilallo	N07.92032/E039.18398	3047	5
H034	Ethiopia	Kaka	N07.36367/E039.18821	3641	10
H039	Ethiopia	Kaka	N07.37116/E039.19295	3528	5
H042	Ethiopia	Kaka	N07.36780/E039.19612	3506	5
H009	Ethiopia	Bale	N06.92182/E039.93216	3585	11
H022	Ethiopia	Bale	N07.10455/E039.79150	3122	5
H027	Ethiopia	Bale	N07.05563/E039. 61990	3526	5
H160	Kenya	Kenya	S00.17046/E037.21450	3051	10
H161	Kenya	Kenya	S00.10302/E037.13880	3224	5
H173	Kenya	Kenya	S00.17101/E037.20844	2983	5
H138	Kenya	Aberdare	S00.33923/E036.65603	3573	5
H146	Kenya	Aberdare	S00.34058/E036.66968	3484	5
H152	Kenya	Aberdare	S00.47638/E036.72564	2885	11
H185	Kenya	Elgon	N01.05820/E034.76339	2610	11
H204	Kenya	Elgon	N01.10151/E034.61759	3675	5
H205	Kenya	Elgon	N01.08708/E034.63153	3383	5
H076	Tanzania	Kilimanjaro	S03.17648/E037.51234	2823	11
H078	Tanzania	Kilimanjaro	S03.18889/E037.51605	2583	5
H087	Tanzania	Kilimanjaro	S03.18232/E037.51229	2721	5
H089	Tanzania	Meru	S03.23212/E036.81414	2142	10
H090	Tanzania	Meru	S03.23158/Eo36.80417	2375	5
H093	Tanzania	Meru	S03.21686/E036.77339	3591	5

Appendix 1c. Population numbers, country, locality, geographical coordinates, altitudes above sea level, and number of individuals investigated in *L. giberroa.*

Pop.	**Country**	**Mountain**	**Lat/Long**	**Altitude (m)**	**n**
L056	Ethiopia	Simen	N13.22166 / E038.99902	2370	5
L057	Ethiopia	Simen	N13.29127 / E038.09282	2470	11
L058	Ethiopia	Simen	N13.29853 / E038.12611	2590	5
L500	Ethiopia	Choke	N10.42490 / E037.47320	2850	6
L120	Ethiopia	Gara Muleta	N09.22051 / E041.78170	2520	5
L122	Ethiopia	Gara Muleta	N09.23278 / E041.74273	2590	11
L124	Ethiopia	Gara Muleta	N09.23316 / E041.74327	2600	5
L312	Ethiopia	Chilallo	N07.92914 / E039.16980	2850	11
L125	Ethiopia	Bale	N06.70370 / E039.72004	2310	6
L126	Ethiopia	Bale	N06.61483 / E039.73801	1840	4
L127	Ethiopia	Bale	N06.65274 / E039.73223	1920	5
L104	Tanzania	Meru	S03.22517 / E036.79781	2630	11
L105	Tanzania	Meru	S03.22582 / E036.79834	2470	11
L088	Tanzania	Kilimanjaro	S03.20025 / E037.51874	2400	11
L134	Kenya	Aberdare	S00.39531 / E036.73089	2960	11
L154	Kenya	Aberdare	S00.50920 / E036.64869	2950	5
L155	Kenya	Aberdare	S00.72276 / E036.67835	2820	5
L158	Kenya	Kenya	S00.17617 / E037.19933	2820	11
L182	Kenya	Kenya	N00.00469 / E037.24118	2610	11
L186	Kenya	Elgon	N01.07194 / E034.72719	2540	5
L200	Kenya	Elgon	N01.06383 / E034.70394	2920	5
L201	Kenya	Elgon	N01.06134 / E034.68774	3000	11
L213	Kenya	Cherangani	N01.20582 / E035.28007	2580	11
L214	Kenya	Cherangani	N01.16556 / E035.33190	2710	4
L224	Kenya	Cherangani	N00.05275 / E035.53882	2730	5

Declaration

This dissertation is my original work and has not been presented for a degree in any other university, and that all sources of material used for the dissertation have been duly acknowledged.

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This dissertation has been submitted for examination with our approval as research advisors.

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