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BIOLOGICAL CONSEQUENCES OF PLEISTOCENE GLACIATIONS IN EAST AFRICAN AFRO-ALPINE ENVIRONMENT AS INFERRED FROM MOLECULAR DATA OF SOME KEY PLANT SPECIES

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DEDICTION

TO MY PARENTS

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
BAPS	Bayesian Analysis of Population Structure
cDNA	Complementary DNA
CI	Confident interval
cpDNA	Chloroplast DNA
CTAB	Hexadecyl trimethylammonium bromide
DAF	DNA amplification fingerprinting
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ExoSAP	Exonuclease I and shrimp alkaline phosphatase
IGS	Intergenic spacer
ISSR	Inter-simple sequence repeats
MCMC	Marcov Chain Monte Carlo
mtDNA	Mitochondrial DNA
NCB	National Center for Biosystematics
NCPA	Nested clade phylogeographical analysis
N-J	Neighbour-joining
PCO	Principal coordinate analysis
PCR	Polymerase Chain Reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
TBR	Tree-Bisection-Reconstruction
TNT	Tree analysis using New Technology
SD	Standard deviation
SSR	Simple sequence repeat
UNESCO	United Nations Educational, Scientific, and Cultural Organization

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ABSTRACT

The afro-montane/-alpine regions representing biological 'sky islands' comprise the high mountains of Ethiopia and tropical East Africa. The vegetation of the regions is characterized by fragmentation among the various mountain massifs with numerous local endemic species. Most plant species in these ‘sky islands’ are quite different from those occurring in their respective immediate surrounding lowlands. However, some primarily arctic-alpine plants also occur in the mountains, as *Arabis alpina*. It has been suggested that this plant elements are Tertiary relict, but a recent range-wide study of *A. alpina* suggested that the species colonized the region twice during the Pleistocene period. In addition to such immigrant species, some other key plant species, as *Salvia merjamie and Cerastium afromontanum* are very common in sub-alpine and montane forests of the ‘sky islands. In this study, two overlapping phases of investigations have been carried out on these three species using two basic molecular biological techniques: in the first phase, the detail colonization history of *A. alpina* was investigated in the afro-alpine regions based on chloroplast DNA (cpDNA) sequences, and in the second phase, the phylogeographic structures and conservation status of the three species were studied using amplified fragment length polymorphic DNA data (AFLP fingerprinting) obtained from 11 mountain systems, where present-day populations are often limited to isolated mountain tops. In the first phase, the results confirm the twice-into-Africa scenario. The Asian lineage is also confined to the mountains closest to the Arabian Peninsula, on opposite sides of the Great Rift Valley (GRV), suggesting long-distance dispersal of the *A. alpina* lineage. The African lineage is divided into two phylogeographic groups with distinct geographic distribution. The observed pattern is consistent with isolation of the African lineage in at least two interglacial refugia, located on separated highlands, followed by range expansion in cooler period(s), when the afro-alpine habitat extended further down the mountains. Several long-distance dispersal events across the GRV are also suggested by haplotypes observed outside the area occupied by the phylogeographic groups they belonged to. In the second phase of the investigation, in the Ethiopian and tropical East African mountains, *A. alpina* showed high and clearly structured genetic variability. The fragmented structure in the mountains indicated that *A. alpina* disperses little among established populations. However, occasional long-distance dispersal events were also suggested in all parts of the ranges, similar to the cpDNA data findings. In *S. merjamie* and *C. afromontanum* which may consist of the subspecies complex, the diversity is so high for the former and very low for the latter species in certain isolated mountain regions in Ethiopia (*i.e*. the Simen Mts and Gara Muleta). The present analyses did also reveal very complex geographical structuring and phylogeographic patterns among the study areas. Moreover, the AFLP data clearly indicated very distinct and genetically highly divergent individuals, which might represent possible cryptic subspecies signifying the call for future detail morphological assessments of the species in the region.

Key words: cpDNA, AFLP, *Arabis alpina*, *Salvia merjamie*, *Cerastium afromontanum*, phylogeography, genetic diversity, refugia, afro-alpine, afro-montane, East Africa, Pleistocene.

CHAPTER 1 INTRODUCTION

The Earth has experienced four major episodes of glaciation over the last billion years (Crowley and North, 1991), *i.e*., the Late Precambrian (800-600 million years ago (Ma)), Late Ordovician (440 Ma), Permo-Carboniferous (330-275 Ma) and the Late Cenozoic (40-0 Ma). During these series of key geological eras, the earth had passed through various extents of and significant climatic fluctuations. Subsequently, the extreme cooling of the Cenozoic climate of the Earth in the Tertiary period, 65 million years (Myr) has increased in amplitude and eventually led to the latter major ice ages of the Quaternary (2.4 Myr to the present). However, the background of the events and the overall historical records of the scenarios are well documented only for the last glacial history, particularly the progression from full ice conditions 20 000 years BP to the warm interglacial of the present (Coope, 1977; Huntley and Birks, 1983; Huntley and Webb, 1988; Bartlein and Prentice, 1989; Webb and Bartlein, 1992; Hewitt, 1996).

During the Ice Age, large areas of the global landmass were repeatedly buried under vast sheets of ice, causing drastic alterations on continental scales. It is estimated that up to 20 glaciation events consisting of glacial advances, stabilizations and retreats have been occurred (Martinson et al., 1987). Each glaciation had spanned 100 kilo years (KY) with interglacial period lasting 10–12 KY (Dawson, 1992). Milankovitch theory attributes orbital forcing to the onset of glaciations and subsequent interglacial periods, *i.e*., change in the Earth’s orbit around the sun (Hays et al., 1976).

The primary evidence of global climate fluctuations can be obtained directly from various sources such as carbon and oxygen isotopes, radiolarian species, and pollen types (Bennett, 1997; Williams et al., 1998), and indirectly from other biological and physical proxies. The pollen record of interglacial time, for example, exhibits marked changes in vegetation at various spatial scales suggesting extensive climate oscillations over wide ranges (Tzedakis et al., 1994). It has been also reported that periodic iceberg discharges into oceans of the past 15-7 KY were possibly caused by cycles of swelling and collapse of ice (Bond et al., 1993; Lehman, 1993). This in turn has seriously affected the ocean conveyer circulation and mainly responsible for a large scale climatic fluctuation episodes (Grimm et al., 1993).

Several geological evidence indicated that some equatorial regions had been affected by Quaternary climatic changes that produced extensive glaciers in higher latitudes. During the glacial periods, in tropical Africa, the levels of some existing lakes had been much higher and the other extensive lakes in basins were quite dry (Nilsson 1949). There had also been episodes when the glaciers occupied the tops of the highest tropical East African Mountains (Lomolino et al., 2006). Evidently, however, the temperate flora and fauna had migrated into African high mountains and colonized relatively these stable habitats (Gottelli et al., 2004, Koch et al., 2006; Assefa et al., 2007). Furthermore, these mountain systems are characterized by both ecological and evolutionary dynamism and is believed to be refugial areas of certain unique organisms and long distance migrating species of the northern hemisphere (*e.g*., *Canis simensis*, Gottelli et al., 2004; *Arabis alpina*, Koch et al., 2006). Throughout the Quaternary climatic shifts of higher latitude, therefore these extensive mountain systems are dominated by recently radiated species (deMenocal, 1995, Roy, 1997, Koch et al., 2006) and provide opportunity for newly formed species to persist (Fjeldsa and Lovett, 1997).

The biological impacts of the last ice age have been thoroughly studies for high latitude flora (Taberlet et al., 1998; Scotti et al., 2000; Kremer and Goenaga, 2002; Tribish et al., 2002; Stehlik et al., 2001 &2002; Petit et al., 2002; Coyer et al., 2003) but none has been reported for the flora of Africa in general and afro-alpine environment in particular. It is only very recently that scientists start to appreciate the impacts of Pleistocene climate oscillations on faunal diversity and genetic structures (Beven et al., 1984; Fjeldsa and Lovett, 1997; Gottelli et al., 2004; Hughes et al., 2005). A major step has recently been undertaken to understand the interfaces of Pleistocene climate changes and historical biogeography of afro-alpine flora (Assefa et al., 2007; Ehrich et al., 2007; Kebede et al., 2007).

It has been reported (Mohammed and Bonnefille, 1998; Lomonilo et al., 2006) that the afro-alpine environment has been glaciated during the ice age. While the top of many mountains was covered by glaciers, both the afro-alpine and the ericaceous zones extended about 1000-1500 m lower than today and thus occupied considerably larger areas (Messerli et al., 1977; Flenley, 1979; Bonnefille et al., 1990; deMenocal, 1995; Gottelli et al., 2004). Furthermore, during glaciations in the northern hemisphere, the African tropics were cooler and drier. There are several crucial issues concerning the biological consequences of Pleistocene glaciations on afro-alpine flora. First, it is not clear how the different 'sky islands' were colonized by the higher latitude plant elements and to what degree plant populations on these mountains have remained isolated. Long-distance dispersal by cyclones, and/or by birds from one mountain to another has been proposed as the main mechanisms for plant dispersal within the fragmented afro-alpine regions (Hedberg, 1969). However, this was found to be not the case (Kebede et al., 2007). Second, whether or not the Rift Valley has served as Pleistocene barrier to dispersal is unknown.

Third, these isolated biological ‘islands’ in the sky have their close relatives not in the surrounding lowlands but on scattered isolated mountains.

The distribution patterns of afro-alpine flora on the isolated mountain systems of Africa assume one of the following forms, *i.e.*, (a) taxa which are endemic to only one mountain system; (b) taxa that exhibit vicariance; (c) taxa which are widely distributed. Pleistocene climate oscillations have certainly contributed to the currently observed distribution patterns of afro-alpine flora. The question is how and to what an extent. Did these climate changes had similar impacts on different species or not? Are there clear geographical patterns of genetic variation for all taxa? Although some of these outstanding questions require several similar studies, the current investigation has selected three widely distributed taxa which lack special dispersal mechanism to evaluate the biological consequences of Pleistocene glaciations on their genetic structures. This study draws evidence from cpDNA sequences and AFLP data to infer Pleistocene colorizations of the East African Mountains by an arctic-alpine taxon, role of Rift Valley as a dispersal barrier and phylogeography of the study species.

CHAPTER 2 LITERATURE REVIEW

2.1 Pleistocene glaciations in tropics

The Pleistocene glaciations were the most significant historical events and marked by extraordinary oscillations in global climate (Berger, 1984). Large areas of the landmasses were covered under the ice. The impacts of glaciations were much severe in the Northern Hemisphere, where major portions of the surfaces were covered with glacial ice (Dyke and Presss, 1987; Dawson, 1992). During the Pleistocene the Earth had experienced numerous glacial-interglacial cycles with massive sheets of ice which were often two to three km thick, and with a great mass that can deform the underlying lithosphere by about 200 to 300 m (Lomolino et al., 2006). It is also estimated that up to 20 glaciations events have occurred (Martinson et al., 1987). It has been variously reported that about 80% of the glacial ice occurred in the Northern hemisphere. On the other hand, in the Southern hemisphere, glaciations were mostly confined to high elevations such as the central Plateau of Tasmania and the New Zealand Alps (Flint, 1971), few regions of South America, Africa (the highest tropical East Africa mountains) and the Atlas Mountains (Lomolino et al., 2006).

The Pleistocene period was characterized by drastic fluctuations in average global temperature accompanied by repeated glacial advance and retreat over much of the areas (Dawson, 1992; Gates, 1993). These periodic incidents have produced radical changes in the geographical distributions and diversity of organisms (Pielou, 1991; Hewitt, 2000; DeChaine and Martin, 2004). There was a phenomenon of periodic iceberg discharges into oceans in the period of 15-7 KY, possibly caused by cycles of swelling and collapse of ice (Bond et al., 1993; Lehman, 1993) that seriously affected the ocean conveyer circulation, thereby causing large climatic fluctuation episode (Grimm et al., 1993).

The climate reversals of the Pleistocene period were caused by changes in the Earth's orbit, which affected the amount of solar radiation reaching the biosphere, unlike the ancient climate fluctuations that were reported to be due to some other factors (Gates 1993; Muller and MacDonald, 1995). The primary evidence of global climate fluctuations can be obtained directly from various sources such as carbon and oxygen isotopes, radiolarian species, and pollen fossils (Bennett, 1997; Williams et al., 1998), and indirectly from other biological and physical proxies. The pollen record of interglacial time, for example, exhibits marked changes in vegetation at various spatial scale suggesting extensive climate oscillations over wide ranges (Tzedakis et al., 1994). In tropical regions, pollen analysis has also emerged as the most important tool in environmental and biogeographical reconstruction of the past (Birks and Birks, 1980).

Before three decades, attempts have been made to estimate the climate of the tropics using pollen data from East Africa (Coetzee, 1967; Livingstone, 1967; Hamilton, 1982). Latter subsequent palyonological studies in most parts of the tropical African Mountains and highlands have established detailed late Quaternary vegetation history (van Zinderen Bakker and Coetzee, 1988). Thus, in the Last glacial maximum (LGM) (18 KY BP), for example, the pollen records obtained from East and central African regions showed that cool grassland and xerophytic scrub increased at the expense of tropical montane forests (Jolly et al., 1997). However, the occurrence of trees, for example, *Podocarpus* and *Olea* in low abundances suggests that forest refuges persisted in some sites during the glacial period (Hamilton, 1982; Bonnefille and Riollet, 1988).

Apparently, modern pollen fossil assemblages also confirmed that tree lines were dropped in elevation by about 1000 m, which has been attributed to a combination of extreme cooler and drier conditions at the LGM (Bonnefille et al., 1992).

Recent measurements of ^{13}C isotope on glacial-age sediments from high-elevation sites of the tropics indicate that C_4 plants were more abundant at the LGM, and such data raises the possibility that the lowered atmospheric CO_2 concentration may have influenced the vegetation through physiological effects (Aucour, et al., 1994). Bonnefille et al. (2004) showed that the Hadar fossil of pollen record from Ethiopia, which is dominated by subaquatic emergent reeds (Typha), sedges (Cyperaceae), and grasses (Gramineae) suggests locally extensive herbaceous cover surrounding the paleo-lake to withstand periodic dryness. Moreover, the Pleistocene climate changes of East Africa are clearly evidenced in the moraines of ancient glaciers far down the slopes of the mountains, and in the sediments and contours of big lakes which have more or less filled up the basins in the Great Rift Valley of the region. But currently only very small glaciers are left on some mountains (*e.g*. Kilimanjaro, Kenya and Ruwenzori) and the lakes in the eastern branch of the Rift system are rapidly shrinking or have partially dried up owing to the increasing desiccation of East Africa from time to time (Nilsson, 1949).

During the last glaciation, the advancing ice sheets drove most of the high latitudes species towards southern refugial regions. In Europe, the Pleistocene refugial areas, for example, were typically found in Iberia, Italy, the Balkans/Greece, and the Caspian/Caucasus. These sources thus made varying contributions to the recolonization process, and their subsequent dispersal northwards shaped the genetic diversity of plants in Europe (Hewitt, 1996, 2000 & 2004;

Taberlet et al., 1998). While this is for many relatively lowland species, the glacial history of alpine species is much less certain. They may have retreated to lower altitudes and latitudes, which would represent an increase in their range as the climate cooled or they would survive within the mountain ranges in central/peripheral refugia with less harsh conditions. On the other hand, the Pleistocene refugial areas in tropical regions would be expected to correspond to areas of high species and genetic diversities, and possess a fairly small number of endemics depending on the duration of isolation. They have a number of species in common with other areas owing to the mingling of biota during phases of forest expansion. However, according to refugia hypotheses, the supposed repeated advance and retreat of vegetation in response to the high latitude climate fluctuations has not produced the diverse flora and fauna (Haffer, 1982), or the expected hybrid zones (Endler, 1982). Because, many groups of African forest biota exhibit a higher degree of endemism, and fewer range disjunctions between forest blocks than would be expected had once-continuous forest survived as fragments throughout the Pleistocene.

2.2 Historical connection of the Arabian Peninsula to northern Ethiopian regions

The Red Sea regions play a significant role in the history of species movements between Africa and Eurasia, including the early migration of terrestrial plant (Kingdon, 1990; Koch et al., 2006) and wild animal (Gottelli et al., 1994 & 2004) and migration of modern humans and earlier *Homo* species from the African continent into the rest of the world (Fernandes et al., 2006). On the basis of detailed palaeontological evidence, archaeological data, and morphometric and genetic comparisons between the human populations in northeastern Africa and southwestern Asia, the early dispersal models of the African-Eurasia routes have been suggested. Accordingly, for crossing the Red Sea, two principal routes (Fig. 2.1) have been proposed for the early human species that were radiated in three different rates from the source population of the East African region (Tchernov, 1992; Cavalli-Sforza et al., 1993; Lahr and Foley, 1994). These are: (**1**) the Sinai Peninsula in the Northern part of the Red Sea for the species coming through two directions, *i.e.*, the Levantine corridor and around the Red Sea coastline, or (**2)** the Bab-el-Mandeb Strait in the southern Red Sea for the population which radiated through the south direction.

Regarding the early historical origin of the routes, it is believed that the Bab-el-Mandeb Strait in the southern Red Sea would have been emerged during Pleistocene sea-level lowstands (Delany, 1989; Robinson and Matthee, 1999, Walter et al., 2000; Mithen and Reed, 2002; Wildman et al., 2004; Winney et al., 2004). Although the precise nature of the dispersal mechanism across the Red Sea is unspecified, molecular genetic investigations supported the case of the Out-of-Africa migration of modern humans for which models involving major dispersal event along the ‘southern route (Quintana-Murci et al., 1999; Maca-Meyer et al., 2001; Underhill et al., 2001; Forster, 2004; Lovell et al., 2005; Macaulay et al., 2005). Similarly, this land bridge hypothesis has also been discussed by Shefer et al. (2004) in relation to patterns of genetic differentiation between the Red Sea and the Indian Ocean populations of marine species.

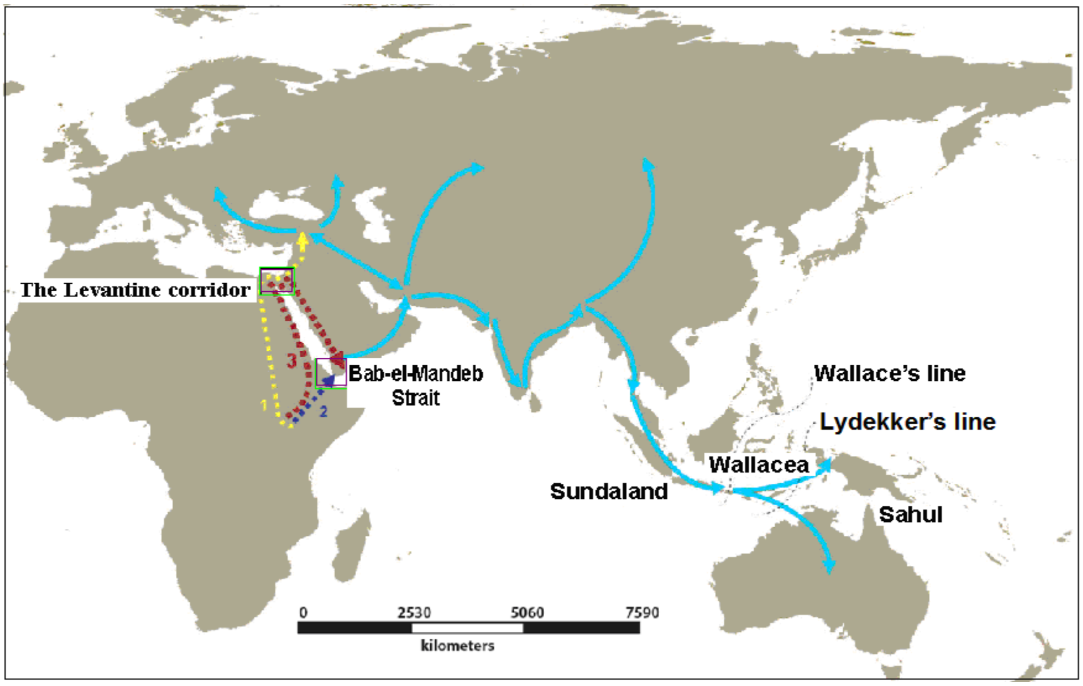

Fig. 2.1 Map showing the hypothesized past three possible dispersal routes connecting Africa and Asia that are available to terrestrial animals: (**1**) the Levantine corridor in yellow, (**2**) across the southern Red Sea in blue, and (**3**) around the Red Sea coastline in red. Source: Modified from Fernandes et al. (2006).

In another development, the existence of past continuous land-bridge between Africa, Eurasia and the Middle East across the Red Sea was confirmed. Gottelli et al. (1994) suggested that a wolf-like canid colonized the Afro-alpine ecosystems via land bridges connecting alpine habitats in a continuum from Eastern Europe through the Middle East to northeast Africa (Kingdon, 1990). Moreover, regarding the evolution history of Ethiopian wolves, the subsequent molecular phylogenetic study provided strong support for the suggestion of a recent speciation from an ancestor closely related to coyotes (*C. latrans*) and the Eurasian grey wolves (*C. lupus*) (Gottelli et al., 1994), and a latest-immigration-to-Africa highlands scenario through the land-bridges (Gottelli et al., 2004).

2.3 Range shifts and Pleistocene climate

Range shifts are the most conspicuous response of plant species to climatic fluctuations, like the Quaternary glaciations. However, regions with relatively stable climates or that were never glaciated in the past, such as most parts of the southern hemisphere, shifting the ranges is not very common and many species grow at the same latitude with periodic shifting of elevations when the size of population expands or contracts (Markgraf et al., 1995). Although individual plants cannot move to escape harsh climate, range extensions are called “migrations” for colonization of new regions through passive seed dispersal and seedlings establishment when they could not withstand the changing climate (Davis and Shaw, 2001). The rates and routes of migration were thus different among taxa (Huntley and Birks, 1983). Nevertheless, in the light of adaptation to any local environments, species migration can no longer be viewed as only an alternative to adaptation to ever changing climatic regime (Jackson and Overpeck, 2000). Instead, the range shifts often triggered by major climate changes appear to have involved adaptive evolutionary change, as changing climate altered the fitness optimum for each population throughout the species range.

The finding of adaptive differentiation in many living species indicates that species were differentiated in the past and that adaptation accompanied migration during the many climate changes of the Quaternary. Gene flow from pollen and seed dispersal is important sources of genetic variation, not only at the leading edge of a migrating species but also throughout the range of a species. Both selection against poorly adapted genotypes and genetic recombination are expected throughout a species range as it migrates to new latitudes or elevations (Davis and

Shaw, 2001). Therefore, range shifts can no longer only be viewed as an alternative to climate adaptation.

Plant taxa have persisted through a long period of variable climate of the glaciations with remarkable changes in temperature, precipitation and CO_2 concentration. The rates of these changes were being varied widely from the regional temperature changes which could be as fast as several degrees Celsius within a few decades or as slow as 1°C per millennium. Rapid or gradual changes in species distributions, and altitudinal and/or latitudinal range shifting are all seen as the expected plant response to such climate changes (Melillo et al., 1996). Moreover, compiled pollen data provided regional and continental records of abundances of some plant species as they changed through space and time (Huntley and Webb, 1988; Webb et al., 1993). For example, *Juniperus occidentalis* and *Artemisia tridentate* undergone complex changes in geographic distributions, including range shifts to new regions as well as shifts in elevation and direction. Similarly, significant changes in population size were also observed in other plant species, such as *Pinus longaeva* and *P. ponderosa* during the past 11,000 years (Thompson, 1988).

It is well known that the Pleistocene climate fluctuations caused major range shifts for many taxa (Hewitt, 2004). While temperate species contracted their ranges to southern refugia (Taberlet et al., 1998; Hewitt, 1999), the habitat of taxa adapted to cold and dry conditions was larger during cooler periods than today (Flenley, 1979; Gottelli et al., 2004). For wide-ranging species, climate fluctuations may have induced range contraction in some parts of the range and expansion in others. Range shifts in northern areas in response to climatic changes were usually latitudinal and altitudinal in mountain regions. Whereas latitudinal recolonization occurred over large areas and involved long-distance dispersal, altitudinal range shifts were more localized, and both were brought about quite different genetic consequences (Hewitt, 1999).

Presently, many taxa in temperate and northern regions are at a stage of range expansion (Ehrich et al., 2007) but this is not the case for the afro-alpine species. In the African high mountains, particularly the afro-alpine habitats occupied a considerably larger area during the last glacial period, and expanded notably over large parts of the highlands (Flenley, 1979; Gottelli et al., 2004). Nowadays, on the contrary, this habitat is highly fragmented and restricted to isolated high mountain refugia (Hedberg, 1970).

For relatively long period of time, tropical East Africa is a region of crustal instability and with major geological events including the formation of the Great Rift Valley, which extended from Ethiopia, through Kenya, Uganda and Tanzania up to Mozambique and this physical event has strongly influenced the surrounding environments and hence the climate (Denys et al., 1986). However, the temperate flora and fauna had, evidently migrated into the region and colonized relatively stable habitats of the mountain ranges and highlands (Gottelli et al., 2004; Koch et al., 2006; Assefa et al., 2007). Moreover, since the regions are characterized by unique ecological and evolutionary features, they are believed to be refugial areas of certain organisms of the northern hemisphere (Gottelli et al., 2004; Koch et al., 2006).

2.4 Impacts of Pleistocene glaciations on genetic structures

Beyond changes in geographical distribution in position or direction, it is believed that species usually undergone genetic changes in response to periodic climate oscillations. Repeated cycles of range expansions and contractions, which followed distinct patterns in different species, resulted in the present-day intraspecific genetic structures (Avise, 2000; Hewitt, 2004). Rapid latitudinal recolonization most often results in loss of genetic diversity due to repeated bottlenecks when populations are founded by few individuals, and may create large areas of reduced diversity through leading-edge colonization (Hewitt, 1996). This process is less likely in mountainous regions, where colonization usually occurs over much shorter distances (Hewitt, 1996). However, genetic diversity can be lost during recolonization in mountains as well (Tribsch et al., 2002). Once a new area has been colonized, it is more difficult for long-distance immigrants to establish and contribute to the gene pool of the new population (Hewitt, 1999), allowing the persistence of areas characterized by low genetic diversity.

Regarding the effects of Pleistocene climate fluctuations, two principal models of expansion were described. These are (1) the “pioneer expansion” due to leptokurtic dispersal with occasional long-range movements of individuals often leading to reduced genetic diversity and (2) the “phalanx-like expansion” which is the result of normal or stepping-stone models and tends to preserve most of the allelic diversity as in the southern limits of species ranges (Nichols and Hewitt, 1994; Hewitt, 1996; Ibrahim et al., 1996), which are later considered as enduring glacial refugial areas (Hewitt, 1996, 1999 & 2001).

During periods of range contractions, populations are fragmented and isolated in separate refugia. Thus, vicariance is expected, which is supposed to be the existence of habitats that acted as ecological refuges for isolated biota (Haffer, 1982), leading to genetic differentiation and formation of distinct genetic lineages (Taberlet et al., 1998). Such populations in the refuge may therefore encounter various fates (Haffer, 1982) such as extinction, surviving without changes or differentiation to the level of species or subspecies before they come into secondary contact with previously conspecific populations of other refugia during the following favorable expansive phases. Moreover, the refuge populations can disperse widely in their habitat, when isolated from the neighboring allies.

The feature of the past vegetation distribution of the tropics, particularly vegetations of the tropical Africa in response to climate oscillations of the high latitudes have been drawn from glaciological, palyonological and biogeographical data. Moreover, subsequent studies based on the distribution of plant species with propagules of high dispersal ability (White 1981), and motile organisms such as birds (Moreau, 1966; Stuart, 1981), mammals (Kingdon, 1981; Grubb, 1983; Rodgers et al., 1982; Rodgers and Homewood, 1982; Happold and Happold, 1989) and butterflies (Carcaasson, 1964) have further given similar results. The forests of tropical East Africa have thus been classified as centers of endemism that acted as refugial areas under the past climate regime (Coetzee, 1989).

In addition, the Last glacial periods of the Pleistocene that correspond to cool and dry periods in the tropics (deMenocal, 1995) have been assumed to have led to intermittent fragmentation of the rainforest biome into isolated refugia, which in turn brought about isolation of populations that led to speciation. The subsequent alternation between isolation and opportunities for dispersal was thus a significant driving mechanism responsible for the current species richness of tropical rainforests (Haffer, 1969; Diamond and Hamilton, 1980; Crowe and Crowe, 1982; Mayr and O'Hara, 1986).

DNA hybridization data were found that the evolution of the recently diversified fauna of the tropical lowland rainforests is very ancient (Fjeldså, 1994; Fjeldså and Lovett, 1997; Roy et al., 1997). According to Roy et al. (2000), the African forest robins of the genus *Sheppardia* are geographically restricted into tropical Africa, which appear to have evolved in Miocene with remarkable population expansion was in the Pleistocene. And this finding was concordant with major global climate variation and vegetation changes. Furthermore, detailed molecular phylogenetic analysis of the African green buls (genus *Andropadus*) showed that the species were highly diversified in Plio–Pleistocene period (Roy, 1997; Roy et al., 1998). The same analysis also suggested a complex relationship between early vicariance and dispersal events among the Cameroon and the Albertine mountains, and along the chain of mountains in eastern Africa, which leads to local fauna enrichment (Roy et al., 1998).

Even more accurate data were obtained from organisms with reduced mobility, such as the flightless insects of the montane habitats of tropical regions (Somme and Zachariassen, 1981; Somme, 1989). The patterns found in flightless insects of the various mountains of the tropics are only partly similar to other group of organisms, for example, features which are common in mammals, birds, butterflies and plants are very rare among the flightless insects. However, occasionally the few shared species of such insects are found on neighboring mountains, which can thus be explained by lowering of montane habitat during the past climatic oscillations (Flenley, 1979; Gottelli et al., 2004). Furthermore, passive long-distance transport and active dispersal are very improbable in many montane insects because of their size (they are often large) and lack of wings. Flightless insects are therefore an ideal group for studying the historical relationships of the different African mountains.

The evolutionary return to a flightless condition among insects may have multiple evolutionary origins in different families of Coleoptera, Dermaptera and Diptera on the mountains, and could provide with a further insight in the complex historical processes of the tropical afromontane fauna. Reduction of wings, elytra and eyes is very common with increasing altitude (Leleup 1965; Juberthine 1979; Somme and Zachariassen, 1981; Somme, 1989). These features are seen as adaptations to a cryptozoic life at high altitudes where the animals have to shelter from violent conditions, such as excessive radiation, great water saturation deficiency, as well as wide and rapid fluctuation of temperature due the early climatic episode.

2.5 Phylogeography

Comprehensive information gathered from animal, plant and mineral remains; land and ice, levels of oxygen and carbon isotopes as well as magnetic and carbon dioxide measures have contributed to great advances in the understanding of the past and the present climatic scenarios, and delivered evermore coherent descriptions and explanations of the biotic history of the Ice Age. Besides such geological evidence, the global biotic geographical distribution patterns and molecular evolutionary history are the major markers of the past climatic episodes, under a broad and well integrative geographical and biological subdiscipline known as phylogeography or historical biogeography (Avise et al., 1987). Thus under phylogeography, modern molecular ecological methods were providing powerful tools for unraveling complex historical events that played major roles in establishing present-day distribution patterns of the global biota. Phylogeography is concerned with the principles and processes governing the geographical distribution of genealogical lineages, particularly at the intraspecies levels (Avise et al., 1987). It was formulated as a method to combine phylogenies with geographical patterns to infer evolutionary processes (Avise, 2000 & 2004), and has proved successful in explaining how animal and plant distributions have been influenced by historical events extending back millions of years.

In phylogeography, the primary units of analysis are monophyletic clades that inferred from phylogenetic analyses, and eventually entail the use of any informative traits or molecular markers particularly for intraspecific populations. It grew and developed from the newly acquired technical ability, such as the novel molecular technique to obtain DNA sequence variation from individuals across a species range, which enable the reconstruction of phylogenies by plotting spatial relationships geographically and then deducing the origin and history of populations, particularly at the intraspecific level (Avise, 2000; Hewitt, 2001).

Intraspecific phylogeography deals with the evolutionary history of a species over space and time. The era of modern phylogeographical studies began with the pioneering work of Avise et al. (1979) on mitochondrial DNA (mtDNA) variation in the mouse. Subsequently, several studies of genetic variation in various molecular markers was scored and the resulting data, *e.g.*, alleles or haplotypes were used to estimate an evolutionary tree that portrays the accumulation of mutations in DNA lineages to yield the current array of, for example, sampled haplotypes (Schaal et al., 1998; Meister et al., 2005; Koch et al., 2006; Assefa et al., 2007). The resulting evolutionary trees are then overlaid upon the geographical representation of the habitat to produce phylogeographical inferences.

The most commonly used molecular marker for phylogeographic studies at populations, species or higher taxonomic levels has been animal mitochondrial DNA (mtDNA) (Avise et al., 1987). Currently, however, complementary molecular markers from nuclear and chloroplast DNA (nDNA and cpDNA, respectively) are also assisting in species delimitations and for revealing the influence of historical and demographic events in shaping the existing patterns of variation as well as determining the geographic structures of species. In phylogeography, therefore there are various nuclear and organellar markers based molecular methods, as DNA sequencing, amplified fragment length polymorphism (AFLP) and variable number of tandem repeat (VNTR), which are already identified as powerful tools to explain complex past historical scenarios and for illuminating the present day patterns of species distribution.

Phylogeography also involves the comparative analysis of the patterns in multiple co-distributed taxonomic groups and is an emerging tool in assessing the role of historical events and demographic processes that shape genetic diversity. This multispecies approach has led to recognition that phylogeographic data may be used to depict the effects of historical events on population differentiation and eventual speciation (Avise et al., 1994; Hewitt, 1996). As species distribution shifted repeatedly and frequently in response to the past climatic fluctuations, the survival of many taxa had indubitably been shaped (Pielou, 1991; Webb and Bartlein, 1992;

Green et al., 1996; Hewitt, 1996; Roy et al., 1996), and each taxon would follow its own pathway of divergence and speciation under such changing conditions (Hewitt, 1996). Analysis of DNA divergence in animals (Hewitt, 1996; Avise et al., 1998) shows that species have continued forming through the Pleistocene and that such divergence had proceeded apparently unhindered in some places (Hewitt, 2000; Willis and Whittaker, 2000), resulting in population fragmentation that would promote further speciation (Haffer, 1969) through selective differentiation and genetic drift.

There is growing evidence that species history of low latitudes including North Africa, southwestern Europe and parts of the Middle East, were influenced by the periodic ice ages (Horn et al., 2006; Espejo, 2007). Although little is known about the impact of the glaciations in the shore of the Mediterranean, there is clear evidence that Southwestern Europe was subjected to dramatic cooler and drier climatic episodes (Sanchez-Goni et al., 2000; Van Andel, 2002; Espejo, 2007). Thus, the richness of the Mediterranean flora with its unusually high endemism is a reflection of geographical position, geological history and the extent to which species managed to survive the effects of past severe climatic variability (Blondel and Aronson, 1999). The effects of glaciations varied among taxa depending on the differing response of species to the scenario (Weir and Schluter, 2004). In some species, divergence is apparently inhibited (Cracraft and Prum, 1988; Bush, 1994; Riddle, 1996), whereas facilitated in the others (*e.g*. Bermingham et al., 1992; Klicka and Zink, 1997; Avise and Walker, 1998; Knowles and Otte, 2000; Knowles, 2000 and 2001; Ayoub and Riechert, 2004).

Moving farther south into the tropics, much geological evidence indicated that some equatorial regions had been affected by climatic changes that produced extensive glaciers in higher latitudes. Nilsson (1940 and 1949) reported that during the glacial periods, in tropical Africa, the levels of some existing lakes had been much higher and the other extensive lakes in basins were quite dry. However, the tropical regions have often been regarded as a region of serene climate unmarked by major changes during Ice Age, and a refuge for plants and animals that were unable to maintain their temperate habitat ranges in the face of repeated continental glaciations (Livingstone, 1967). Here, our current study is emphasized on investigating basic phylogeographical history of the tropical afro-montane and alpine key plant species with particular emphasis to the tropical East African Mountain massifs.

2.5.1 Basic concepts of nested clade phylogeographical analysis

Modern phylogeographical studies have began with the pioneering work on mtDNA variation (Avise et al. 1979), and the data of the variation was scored in the form of haplotypes that are used to estimate an evolutionary tree and depict the accumulation of mutations in DNA lineages, which yield the array of unique haplotypes. Haplotypes are the lowest units of analysis which were ultimately overlaid upon geography to make phylogeographical inferences. Although visual superimposing of haplotype trees upon geography can be suggestive of phylogeographical events, such method of representations does not constitute a formal estimation or hypothesis testing framework. Because, there is no determination of whether or not enough individuals and geographical sites have been sampled to ensure that the observed patterns could not have arisen by chance alone. Moreover, even there is no formal and explicit interpretative framework for making biological conclusions from the resulting observed patterns. These inadequacies stimulated the development of nested-clade phylogeographical analysis (NCPA) (Templeton et al., 1995). NCPA is therefore a basic tool in intraspecific phylogeography to evaluate the validity of its inferences, through detecting a significant association between clades and geography, and giving biological interpretations using an inference key (Templeton et al., 1995; Templeton 2004).

In principle, NCPA uses the haplotype tree to define a series of hierarchically nested clades (branches within branches) using a set of explicit nesting rules (Templeton et al., 1987; Templeton et al., 1992). Haplotypes are being nested together into mutationally close subsets called one–step clades. The one–step clades in turn are nested into two–step clades, and so on, until a nesting level is reached such that the next higher nesting level would result in only a single clade spanning the entire original haplotype network. When the haplotype network is properly rooted, the oldest clade is known in any given nesting category. Otherwise, it possible to predict using the coalescent theory in such away that the clades on the tips of the tree are highly likely to be younger than the interior clades to which the tips are connected within a population (Castelloe and Templeton, 1994). Therefore, within a nesting category, there are contrasts of interiors versus the tips corresponding to the oldest and the younger clades, respectively.

Although the nested clade approach to phylogeographical inference has much strong, perhaps, at times it has the following serious limitations (Templeton, 2004): (**1**) the NCPA inference is limited by sample size and sample sites, (**2**) it delivers an insufficient genetic resolution to detect an event or process that actually occurred, and (**3**) NCPA makes a pseudo-inference or biological misidentification.

2.5.2 Methods of detecting phylogeographic structures

Empirical studies in ecology and evolution that address questions regarding genetic relatedness among individuals, population structure, phylogenetic relationships, phylogeographic patterns and mapping of quantitative trait loci often depend on accurate assessment of genetic diversity (Avise, 1994, Hills et al., 1996). A series of techniques and genetic markers have been developed to estimate genetic diversity. But no single technique is universally applicable and each available technique exhibits both strengths and weakness. Therefore, the choice of technique is often a compromise that depends on the problem to be tackled and the genetic resolution needed, as well as on financial constraints and the technical expertise available.

The most remarkable progress in attempts of measuring genetic diversity was made in 1966, when the gel electrophoresis was successfully applied to reveal an enormous amount of genetic variation in *Drosophila pseudobscura* (Lewontin and Hubby, 1966) and in humans (Harris, 1966). In plants, the applicability of gel electrophoresis of isozymes for the study of genetic variation was soon demonstrated in cultivated barley (Kahler and Allard, 1970), and *Avena fatua* and *A. barbata* (Marshall and Allard, 1970). Since then, isozymes have been widely used for estimating the amount and distribution of genetic diversity in plant populations. However, as indicated earlier, isozymes are often less informative and possibly more subjective to error than DNA data (Parker et. al., 1998).

A series of remarkable developments in the late 1960s and early 1970s, have helped in the advancement of methods related to the use of molecular markers. The discovery of restriction enzymes (Linn and Arber, 1968, Meselson and Yuan, 1968) and a method for the separation of DNA restriction fragments electrophertically, followed by immobilization on nitrocellulose membranes and detection by radioactive probe (Southern, 1975), have led to the development and application of restriction fragment length polymorphism (RFLP) for accurate measurement of genetic diversity and population structures (Tanksley et al., 1989, Paterson et al., 1991). Restriction fragment length polymorphisms (RFLPs) are the first DNA markers to be used for such scientific applications as genetic mapping, paternity analysis, reproductive biology, and for assessing the evolutionary history of species. Variation in RFLP arises because of the length difference (and hence molecular weight), which, in turn, arises because of mutation, inversion or insertion that alters nucleotide sequences at the restriction site and/or changes the length of restriction fragments (Tanksley et al., 1989).

Depending on the sources of the probes (cDNA or genomic DNA), RFLPs could reveal variation in coding (low-copy number genes) or both coding and non-coding sequences (high copy number sequences). Similarly, depending on the objective of the study, RFLP could be used to investigate variation in nuclear DNA, cpDNA, and mtDNA. RFLPs have such desirable attributes as high reproducibility, relatively high polymorphism, codominant inheritance, selective neutrality and freedom from epistatic (Jones et al., 1997; Karp et al., 1997). However, RFLP is usually too expensive to be routinely applied (Waugh and Powell, 1992), involves radioactivity (requiring more expertise and safety precautions) and requires relatively more DNA sample than PCR-based markers.

In PCR assays, unlike in the RFLP analysis, a small quantity of template DNA is required (Williams et al., 1990). Moreover, the quantity of the amplified DNA is high enough to be detected by staining with fluorescent chemicals and there is no need to develop probes by cloning and detection by radioactive labeling (Williams et al., 1990). This makes the PCR assay, simple and safe compared to RFLP.

Many molecular techniques in DNA amplification require a prior knowledge of the sequences of DNA segment to be amplified and in order to design the two complementary primers (Saiki et al., 1985). This limitation was overcome in 1990 with the advent of random amplified polymorphic DNA (RAPD) (Williams et al., 1990) and arbitrary-primed PCR (AP-PCR) (Welsh and McClelland, 1990). In RAPD, short primers (usually 10 base pairs), with a guanine-cytosine content of at least 50%, are used and amplification products are separated in agarose gels and detected with ethidium bromide staining (Williams et al., 1990). In AP-PCR, slightly longer primers are used and amplification products are separated in polyacrylamide gels and detected with silver staining (Welsh and McClelland, 1990). A slight modification of these two markers, DNA amplification fingerprinting (DAF) was developed in 1991 (Caetano-Annollis et al., 1991). In DAF, short random primers of 5-8 base pairs are used and amplification products are detected with silver staining after electrophoresis in polyacrylamide gels. However, RAPD is more widely used for assessing genetic diversity than AP-PCR or DAF (Karp et al., 1997).

RAPD analysis is fast and easy to perform. It provides high resolution and can be used with very small amounts of DNA (Williams et al., 1990). However, RAPD markers can be sensitive to changes in reaction conditions, resulting in low reproducibility (Schweder et al., 1995). Other potential problems with RAPD analysis include competitive priming (Hallden et al., 1996) and interactions within and between DNA strands during PCR (Rabouam et al., 1999). Other PCR-based markers, such as simple sequence repeats (SSR) or microsatellites (Brown et al., 1996) and inter-simple sequence repeats (ISSR) (Yang et al., 1996; Taramino et al., 1997), are increasingly being used in assessing the genetic diversity and the phylogeographical structures of various plant species. The development of microsatellite has been motivated not only by the need for numerous markers for genome mapping (Roder et al., 1995) but also for the relative ease of genotyping that could lead to build up large datasets.

Microsatellites are tandem repeats of short, 2-6 base pairs DNA sequences (Tautz, 1989). Polymorphism results from the number of repeat units and, hence, length variation. Microsatellites are co-dominantly inherited, show high level of polymorphism and results are quite reproducible (Powell et al., 1996; Jones et al., 1997). However, the design of SSR primers requires prior sequence information and, hence initial cost is high (Gupta et al., 1999). The relatively large investment of time in interpreting some banding patterns across gels, the limits on the number of samples that could be analyzed, the potential for error due to null alleles (Dow and Ashley, 1996), and the likelihood that variation might not be species-specific had restricted the using of microsatellites for genetic analysis (Slatkin, 1995).

Another PCR-based marker system gaining popularity and wide acceptance in genetic diversity analysis and population structures is the amplified fragment length polymorphism (AFLP) (Vos et al., 1995; Vos and Kuiper, 1997). AFLP markers are fragments of DNA from restriction digested genomic DNA that were amplified by PCR in the presence of direct primers (Vos et al., 1995; Karp et al., 1997; Mathes et al., 1998). Thus, AFLP is actually an ingenious combination of RFLP and PCR techniques and is extremely useful for the detection of polymorphism between closely related genotypes. AFLP is believed to combine the advantages of RFLP and RAPD. However, AFLP is technically more demanding and require more DNA than RAPD and it generates dominant markers rather than co-dominant markers (Boivin et al., 1999), rendering this method less useful for studies that require precise assignment of allelic states, such as heterozygosity analyses. However, because of the rapidity and ease with which reliable, high-resolution markers can be generated, AFLPs are emerging as a powerful molecular toolkit. It is a robust, a relatively cheap, easy, fast and reliable, and many markers can be scored for each sample in a single run using an automatic genetic analyzer (Blears et al., 1998). The number of polymorphism detected per reaction is therefore much higher than that revealed by RFLP and RAPD.

The major steps in AFLP analysis are the genomic DNA is digested with two endonucleases and site-specific adapters are then ligated to the DNA fragments. Primers complementary to the adapters and to the restriction sites are designed with two or three selective nucleotides added to the 3’ ends of the primers. Thus during PCR, only the amplified DNA fragments with nucleotides flanking the restriction sites will match the selective nucleotides (Karp et al., 1996). The amplified fragments are radioactively or fluorescently labeled and then separated on sequencing gels.

The key feature of AFLP is its capacity for the simultaneously screening of many different DNA regions distributed randomly throughout the genome. AFLP markers have proved useful for assessing genetic differences among individuals, populations and independently evolving lineages. For a wide range of taxa, AFLP markers have been used to uncover cryptic genetic variation of strains, or closely related species that had been impossible to resolve with morphological or other molecular characters (Heun et al., 1997; Russel et al., 1997; Beismann et al., 1997; Triantaphyllidis, 1997). AFLP methods were shown to be superior to classic systematic methods in nematodes (Semblat et al., 1998), have allowed finer differentiation of microorganisms (Huys et al., 1996; Janssen et al., 1997) and have helped the rapid identification of novel pathogens in epidemiological surveys (Koeleman et al., 1997). For closely related species, AFLP markers have also been used to infer phylogenetic relationships based on measures of genetic distances (Huys et al., 1996; Powell et al., 1996; Tohme et al., 1996; Beismann et al., 1997; Heun et al., 1997; Keim et al., 1997; Semblat et al., 1998). For higher taxonomic levels, phylogenetic inferences based on similarities of AFLP profiles become problematic, because the high variability of AFLP markers reduces similarities between distant taxa to the level of chance. Therefore, the usefulness of AFLP markers rests more on the rapid grouping of closely related lineages, which is crucial for biodiversity surveys and tactical recommendation of conservation strategies.

AFLP markers have found the widest application in analyses of genetic variation, particularly in investigations of population structure and differentiations (Heun et al., 1997; Triantaphyllidis, 1997; Arens et al., 1998; Gonzalez, 1998), and variation within populations (Travis et al., 1996; Semblat et al., 1998; Rosendahl and Taylor, 1997; Winfield et al., 1998). In addition, AFLP markers have been applied to evaluate gene flow and dispersal (Travis et al., 1996; Arens et al., 1998; Majer et al., 1998; Semblat et al., 1998), outcrossing (Gaiotto et al., 1997), introgression

(Tohme et al., 1996) and cases of hybridization (Beismann et al., 1997; Arens et al., 1998). The high resolution of AFLP markers also enables testing for clonal identity between individuals, in which recombination does not exist, and thus permits inferences about sexual versus asexual modes of reproduction (Beismann et al., 1997; Rosendahl and Taylor, 1997; Majer et al., 1998).

Other applications of AFLP markers were in the construction of high-density genetic maps of DNA fragments (Vos et al., 1995). Many studies have already applied the AFLP technique to the mapping of certain plant species, such as *Solanum spp.*, *Zea mays* and *Oryza sativa* (Xu et al., 1999). The AFLP technique has also been used to estimate genetic diversity in maize (Smith et al., 1994), rice (Mackill et al., 1996), soybean (Powell et al., 1996), barley (Russell et al., 1997), sugarcane (Lima et al., 2001), coconut (Teulat et al., 2000), cotton and other species (Abdalla et al., 2001). AFLP markers have been involved in paternity analysis and gene-flow (*e.g. Personia mollis,* Krauss and Peakall, 1998). Several studies have utilized AFLP markers in evolutionary history and phylogeographic structures. Different allelic frequency of AFLP loci have been used to identify as a reliable technique to resolve the taxonomic and evolutionary debates of some flowering plant families (*e.g*. Solanaceae, Kardolus et al., 1998).

In an ideal situation, a chosen marker would have sufficient variation for the problem under study and be able to bring about results that are relatively simple to generate and interpret. A number of studies involving two or more markers in different species (Chan and Sun, 1997; Russell et al., 1997; Bahrman et al., 1999) have established that each marker system has its own advantages and disadvantages. Some techniques are clearly more appropriate than others for some specific applications and usually a combination of complementing methods should be used depending on the objective of the study and availability of resources. At present, cpDNA and AFLP markers are used for generating data presented in our study.

2.6 Basic concept and principle of conservation genetics

Biodiversity is the variety of life. It encompasses the variation among species or other biological elements, such as alleles and gene complexes, populations, communities, ecosystems, landscapes, and biogeographic regions. Biodiversity can be expressed as the variation within a given location or among elements across geographic units. Variation in the diversity of life in turn include different types of species or elements, their relative frequencies, the degree of variation among them, or variation in key processes, such as dispersal, gene flow, interspecific interactions, or ecological successions (Lomolino et al. 2006).

It would be of enormous benefit to basic science and to humanity getting on with the exploration of earth’s biodiversity. So far a lot is known about what one can do to save many habitats and species in natural ecosystems (Wilson, 2005). Since loss of biodiversity is a slow process that largely affect generations and the impact is not always immediately felt, preserving of biodiversity requires radical changes in attitudes and lifestyles of people. The primary factors contributing to the disappearance of ecosystems are directly or indirectly related to human actions and impacts. As the human population is growing rapidly, the impacts are continually increasing. In addition, there are myriad of related factors, such as stochastic effects, which may have environmental, catastrophic, demographic, or genetic factors, *e.g.*, inbreeding depression, and loss of genetic diversity, contributing to the reduction and eventually extinction of the number of species considerably in their natural home.

Since biodiversity is being rapidly depleted due to many direct and indirect acts of the human being, identifying, conserving and managing the diversity of life is becoming one of the greatest challenges in the universe. With the loss of species, biodiversity is diminished at all levels, from genetic and local scales to biogeographic and global ones (Lomolino et al. 2006). Thus, information and awareness regarding the amount and distribution of genetic variation of species are important for efficient management and effective conservation. The goal of conservation is therefore to preserve the species diversity, and the biological and physical resources that will enhance conservation of biodiversity for the long term.

Conservation genetics deals with the genetic factors that affect reduction, extinction, and/or total vanishing risks of biological entities and the necessary management regimes to minimize such effects. Conservation genetics also used to preserve species as dynamic units that can evolve to cope with environmental change and thereby minimize the risk of extermination (Frankham and Briscoe, 2002). Some important concerns regarding the preservation of biodiversity in conservation genetics are thus: species loss of genetic diversity and the ability to evolve in response to environmental changes, and the deleterious effects of inbreeding, like inbreeding depression on reproduction and survival of species.

In Africa in general, and tropical East Africa in particular, human populations advance and transform the existing geographical structures in an alarming rate, and the resulting consequences on the native faunal and floral communities are highly accelerating. The growing pressures from agricultural expansion, wildfire regimes and the unregulated and illegal removal of indigenous plant species and it products are also increasing from time to time. Since the degree of predicting and mitigating the threats to biodiversity of the region depends largely on the abilities to understand and modify the dynamic biogeography of the existing species, awareness of the importance of conservation is crucial. Therefore, it is important to understanding strategies to increase the awareness of the local, regional and national governments as well as the population regarding the wider importance of protecting and preserving biodiversity. In particular, it should be possible to reverse the worrying trend of the expansion of the monocultures of commercial plantations in many parts of the region. Ways and means should be designed to encouraging local communities to plant indigenous tree seedlings in the degraded parts of forests under professional guidance and supervision.

CHAPTER 3 OBJECTIVES

3.1 General objective

The general objective of this study is to investigate the geographical structures of gene pools of some key afro-montane/-alpine plant species in the Mountain massifs of East Africa (Ethiopia, Kenya, Tanzania) in the context of Pleistocene climate oscillations based on molecular data.

3.2 Specific objectives

The specific objectives are to:

- study the colonization history and diversification of an arctic-alpine *A. alpina*. The cpDNA haplotype networks and AFLP markers will be used to address this question.
- analyze genetic structures of *S. merjame* and *C. afromontanum* based on the AFLP DNA markers.
- suggest conservation strategies of these key species based on the observed results.

CHAPTER 4 THE MOUNTAIN SYSTEMS FOR CURRENT STUDY

Twelve high mountains of Ethiopia, Kenya and Tanzania (referred to as East Africa) in this study (Fig. 4.1). Of the 11 mountain systems, six occur in Ethiopia, *i.e.*, Simen Mountains and Mt Choke (northern) and Mt Chilallo, Mt Kaka and Bale Mountains (southern) and Gara Muleta (eastern). The northern-southern distinction is entirely based on the positions of the mountains in relation to the Ethiopian Rift Valley. Similarly, six mountains were used from Kenya (Mt Kenya, Aberdares, Cherangani Hills and Mt Elgon), and Tanzania (Mt Kilimanjaro and Mt Meru). Note that Kenya and Tanzania are referred to as tropical East Africa in this study. All the mountain chains of Ethiopia and tropical East Africa with very few exceptions are the result of volcanic activity and some have been uplifted as part of the Rift system (Berhe et al., 1987; Knox, 1993).

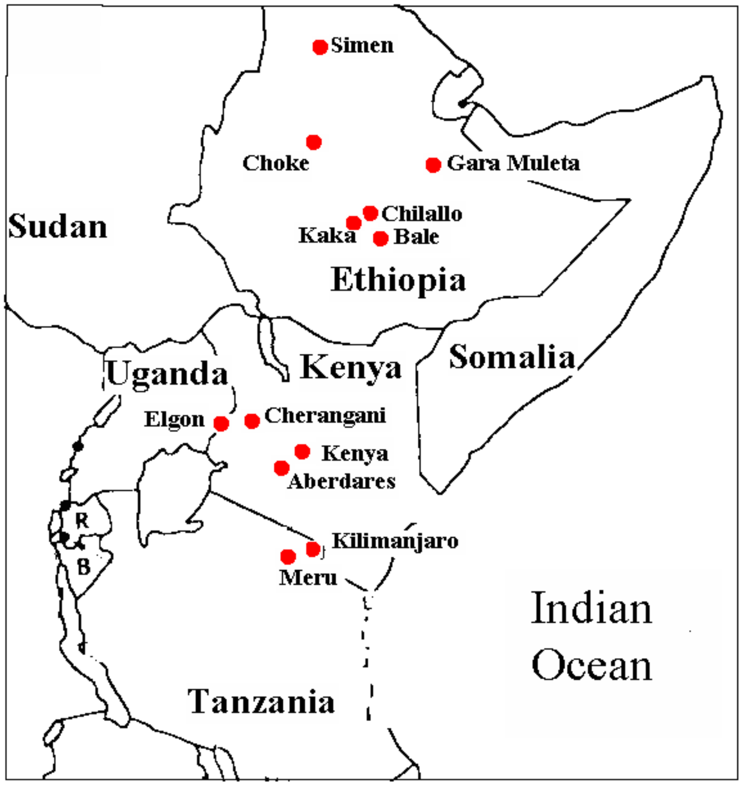

Fig. 4.1 Mountain systems of Ethiopia and tropical East Africa considered in this study.

4.1 The Ethiopian mountain systems

The montane Moorland eco-region of Ethiopia makes up only 2% of the land area of the country. This small area contains 80% of land above 3000 m above sea level (a.s.l.) in the Afro-tropical realm (WWF, 2001). It is believed that the northern and southern massifs of the highlands were formed over 75 million years ago as a large dome propelled upwards by turbulent volcanic forces (Berhe et al., 1987). Eventually the dome was split by the development of the Rift Valley which threads its way north-east between the southern and northern massifs. This volcanic period had ended 4 to 5 million years ago and succeeded by severe climatic fluctuations in the Pliocene and Pleistocene. During the last Ice Age, the higher peaks of the Ethiopian Plateau were glaciated and surrounding areas were covered with montane vegetations. As the climate warmed the broad belts of sub-alpine vegetation contracted, and become restricted to higher altitudes. The ice cover on the peaks of these highlands retreated only a few thousand years ago and therefore vegetation has only relatively recently colonized the areas (Kingdon, 1989). As a result, high levels of endemism are found in the region and the biota demonstrates evolutionary links to both the Palaeoarctic and the Afro-tropical realms. Most plant species of the afro-alpine belt sensu Hedberg (1951) exhibit remarkable morphological adaptations to the extreme climatic conditions of the high altitudes of East Africa. Stretching out across Ethiopia, these pockets of vegetation harbor unique populations of critically endangered animals and several small endemic mammals (WWF, 2001). The afro-alpine ecosystems are unique in their topographic arrangements and their biota but only a few sites are protected in national parks and others due to their physical features such as unsuitability for cultivation and grazing (Fig. 4.2).

Fig. 4.2 Rugged terrains of Simen Mountains, Ethiopia.

The Simen Mountains

The Simen Mountains, are comprised of chains of mountains which are above 4000 m and the highest peak, Ras Dashen rises to 4620 m a.s.l. The latter is the fourth highest peak in the Africa. Geologically, Simen Mountains lie on extremely thick Tertiary volcanic deposits. The soils developed over the rocks are principally nitosols and in some areas lithosols (WWF, 2001). Although Simen Mountains are not too far from the Equator, they lack permanent glaciers but with observed occasional snow on the highest points and the night temperatures often fall below zero (Hedberg, 1997).

Mt Choke

Mt Choke is a range of mountains with elevation generally above 3000 m where some of the peaks rise above 4000 m a.s.l. The total estimated area covered by the mountain is about 215 km^2. The geology of the mountain is of volcanic of the Tertiary era known as the trap series that overlay the Mesozoic sedimentary rocks (Mohr, 1967). In Mt Choke, Olivine basalts often amygdaloidal (with agate and zeolite) are reported to be overlain by basalts and silicic lavas (Mohr, 1971). It has summits of shield volcanoes with a basal diameter of over 100 km which rises to 4052 m, some 1200 m above the surrounding flood volcanoes (Kiefer et al., 2004). The silicic lavas of Mt Choke include trachyte and rhyolitea and the inorganic component of soil, therefore, appears to be derived from the weathering of rocks of this lava (Mohr, 1971). Mt Choke is highly degraded by the surrounding inhabitants because of extensive grazing, cultivation and wood cuttings for firewood, commercial charcoal and for house construction purposes. As a result, the vegetations and wild animals of the mountain are extremely threatened and a robust conservation measures is indispensable at least to maintain the existing flora and fauna and rehabilitate the ecosystem.

Gara Mulata

Gara Mulata is one of the sites of a particular taxonomic and evolutionary significance in the region (Knox, 1993). Gara Mulata was unfortunately receiving heavy uses for grazing and wood-cutting, and even 40 years ago the vegetation was already described as “remnants” (Beals, 1968). The people living in the buffer zone of the western and the northern most parts of the mountain are still extensively cultivating and planting often exotic trees like *Eucalyptus* in few areas and totally remove natural forests from the buffer zone for their daily uses. The most upper saddle of the mountain is flat and extensively grazed by cattle, goat and sheep. People entirely living on the most top of the mountain are isolated from the inhabitants of the buffer lowland zone and are entirely dependent on their herds and firewood that are collected from the mountain forest. Unfortunately, there is no conservation measure either in plan or action for Gara Muleta.

The Bale Mountains

The Bale Mountains belong to the Bale-Arsi massif which forms the western section of the South-Eastern Ethiopian highlands (Friis, 1992). They constitute a prominent part and cover the largest continuous area above 3000 m a.s.l. in Africa. Bale highlands are the extreme south-eastern bastion of the Ethiopian highlands projecting against the Somalian lowlands. The Bale Mountains are located some 850 km north of the equator. The nearest mountain is Mt Kulal (South of the Turkana depression, 550 km distant), followed by Mt Kinyeti (Imatong Mountains, Southern Sudan), Mt Elgon, the Cherangani Hills and Mt Kenya (all approx. 800 km distant). It is worth noting that the northern outliers of the Ethiopian highlands reach equal distances from the Bale Mountains, *e.g*. Ras Dejen is 730 km away, with the important difference that the areas above 3000 m in Ethiopia have much shorter intervals between each other than those in tropical East Africa (Miehe and Miehe, 1994).

The highest elevation of the Bale Mountains is Tulu Dimtu with an altitude of 4377 m a.s.l. It is the second highest peak of Ethiopia and the seventh highest in Africa. Geologically, the Bale massif consists of Tertiary (Oligocene) Trappean lavas which covered the Mesozoic marine sediments and underlying pre-Cambrian rocks after the uplifting of the Ethiopian highlands (Mohr, 1971). During the Plio-Pleistocene rifting phase, the Bale-Aris massif was separated from the Northwestern Ethiopian Mountains by the Rift Valley system and this has also isolated the Southwestern Arabian part of this land mass. The shield volcanoes of the central Bale highlands apparently consist of basalt and trachyte. The Bale highlands consist of a vast lava plateau with at least six volcanic cones, more than 4200 m high, which have been considerably eroded and partly flattened by former glaciations (Miehe and Miehe, 1994). The central rolling plains of these highlands extend between 3800 m and 4050 m a.s.l. and are characterized by numerous glacial lakes and swamps. Some of these apparently dry out periodically and the majority of the lakes in the south-central part of the plateau have no perennial outlet. Bale Mountains are delineated as a National Park mainly for the threatened Ethiopian wolf and mountain Nyala. Historically, since the ice cover on the peaks of these highlands retreated only a few thousand years ago, the colonization of the mountains is relatively recent (Kingdon, 1989).

Mt Kaka

Mt Kaka is adjacent to Bale Mountains and relatively inaccessible to visitors and researchers, because of the absence of motorable road. This mountain is highly influenced by the surrounding inhabitants.

Mt Chilallo

Mt Chilallo is also adjacent and connected to Bale Mountains by continuous highlands.

4.2 The Tropical Eastern African Mountains

The major part of tropical East Africa consists of vast plateau at an altitude of 1000-2000 meters, separated by two series of mainly longitudinal Rift Valleys. Along these valleys lie scattered a number of isolated high mountains, several of which reach altitudes between 3800 and 6000 meters. Most of the Tropical East African Mountains are located on the eastern part of the Great Rift System. Mt Ruwenzori and Virunga volcanoes are along the Western Rift. On the other hand, Mt Elgon (4321 m), Cherangani Hills (3450 m), Aberdares (3999 m) , Mt Kenya (5199 m), Mt Kilimanjaro (5895 m) and Mt Meru (4567 m) lie along the Eastern Rift. Generally, the Eastern Rift Mountains are of volcanic origin, although some have been uplifted through doming and faulting (Knox, 1993), *e.g*. Cherangani Hills. Aberdares was formed through the combined effects of shoulder uplift and fissure and point volcanism to form a high central plateau bounded by two minor peaks (Sattima and Kinangop).

Mt Meru

Mt Meru is an extinct volcanic cone with a single large crater. This mountain is only 90 000-190 000 years old (Evans et al., 1971; Bagdasaryan et al., 1973), and the summit consists of unconsolidated scree. The vegetation reflects the dry conditions, and the alpine zone is not well developed (Hedberg, 1955).

Mt Kilimanjaro

Long before Mt Kilimanjaro was formed there was a gently rolling plain with the remains of a few eroded mountains. About million years ago, the plain buckled and slumped, sinking over a period to form a huge basin known as the Kilimanjaro depression. Mt Kilimanjaro was formed between 500 000 and 750 000 years ago and is a more or less an eroded relic of an ancient volcano with three volcanic centers (Hemp, 2001), *i.e*., (1) Kibo (5895 m) which is connected by its Saddle region to (2) Mawenzi (5149 m), and (3) Shira (3962 m). The latter is the oldest and was the first to collapse and become extinct, rising from the savanna plains at 700 m elevation to a snow-clad summit. Eruptions and lava flow raised Kibo to its maximum height some 45 000 years ago, and it has shrunk only slightly since that time. Uhuru “summit” is the highest peak of a giant oval crater rim of Mt Kilimanjaro with more than three kilometers long by two kilometers wide.

Mt Kilimanjaro is the highest peak in Africa, representing a dormant but not extinct volcano of massive dimensions at the Uhuru peak. It is one of the world largest continental volcanoes and has a diameter of 90 km from northwest to southeast (Hemp, 2001). It is partitioned by deeply incised radial valleys. These valleys, together with some secondary vents, create important refuge for natural flora and fauna.

Mt Kenya

Mt Kenya is the second highest mountain in Africa. It is an extinct volcano and there are permanent glaciers at the highest point.

Aberdares

Aberdares is a chain of mountains with two peaks very close to Mt Kenya. It was formed through the combined effects of shoulder uplift, fissure and point volcanic to form a high central plateau bounded by two peaks.

Mt Elgon

Mt Elgon is found on the national borders of Kenya and Uganda and is a national park.

Cherangani Hills

Cherangani Hills are chains of mountains (more than 100 km long, personal observation) occupying almost all areas of the northwestern Kenya regions as an extension of Mt Elgon from the Western border of the country. The Hills are highly degraded due to human habitation.

CHAPTER 5 MATERIALS AND METHODS

5.1 Selection criteria of the study materials

Three key afro-montane/-alpine species which occur on all mountain systems of the afro-alpine environment of tropical East Africa were selected. These species lack special dispersal structures. These species are described below. A series of field work was carried out in the years 2003 and 2004 where 745 DNA samples from leaves of 90 different populations of these three species (33 populations of *A. alpina*; 26 populations of *S. merjamie* and 31 populations of *C. afromontanum*, Appendix 4.1) were collected from five Ethiopian and six East African mountain systems (Fig. 4.1).

Three plant species were subjected to the current study, *i.e.*, *A. alpina*, *S. merjamie* and *C. afromontanum*. A brief account of each of these species will be given below.

A. alpina (Brassicaceae)

A. alpina L. is purely afro-alpine and grows to 10 – 20 cm high (Fig. 5.1). It is commonly known as alpine or mountain rock cress because it is often found in rock shelters in most of the alpine belts where temperature and moisture conditions are apparently favourable, competition is minimal, and it probably gets extra nutrients from the surrounding areas (Hedberg, 1962 & 1986). This taxon is usually diploid with $2n = 2x = 16$ (Hedberg and Hedberg, 1977; Koch et al., 1999). However, autotetraploid probably due to hybridization was observed in Mediterranean region (Markus Koch, personal communication). *A. alpina* has no highly pronounced morphological variability in it wide distribution areas.

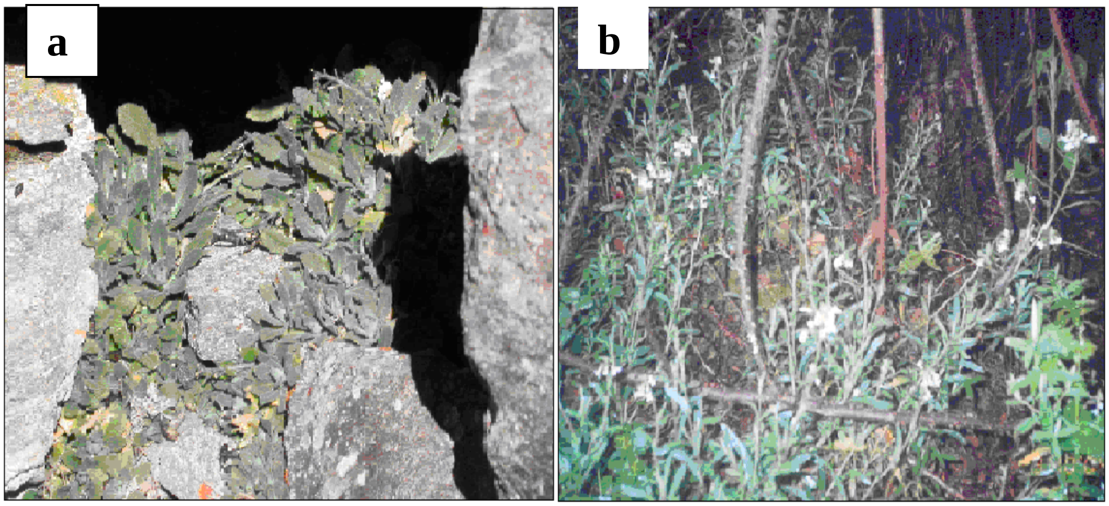

Fig.5.1 *A. alpina* growing in different habitats: (**a**) rocky outcrop and (**b**) in the forest.

Distribution

A. alpina is an afro-alpine plant with an almost worldwide distribution. It exists in all mountain regions of Ethiopia and tropical East Africa, Northern Africa, Cameroon, and Madagascar. Moreover, it has wide ranges of distribution from the northern amphi-Athlantic areas, the European mountains, and the Mediterranean to the Caucasus, Iran and Iraq, and reaches the Arabian Peninsula in the southeast (Fig.5.2). In Ethiopian and the tropical East African mountains, *A. alpina* occurs from 3000 m to 4100 m a.s.l. and the afro-alpine belts. It prefers moist habitats with open gravel, rocky outcrops or in glacier foreland (Koch et al., 2006).

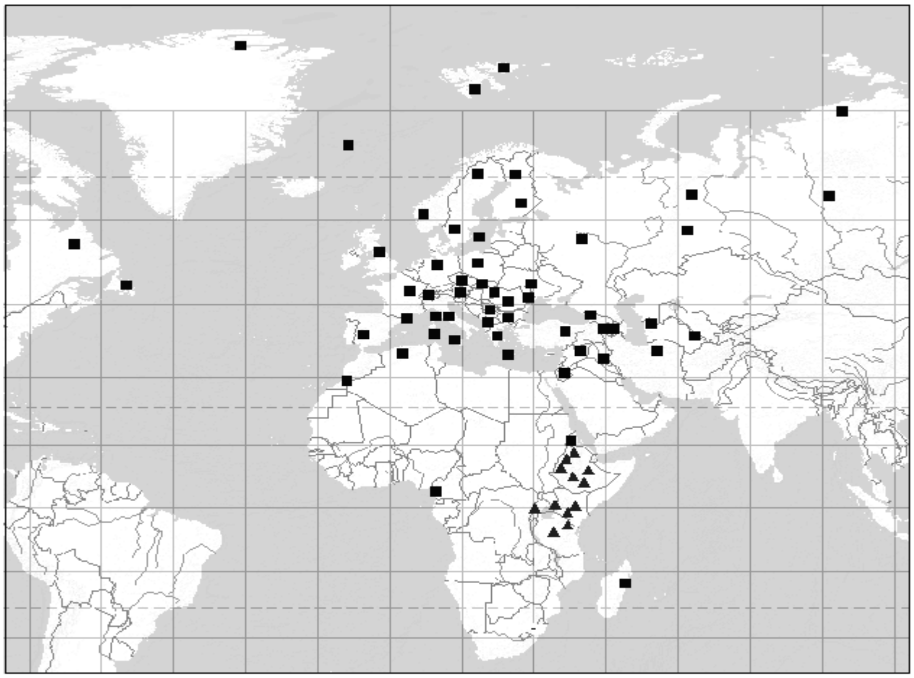

Fig.5.2 Distribution range of *A. alpina*. Triangles denote the plant populations dealt with in the current study.

S. merjamie (Lamiaceae)

S. merjamie is an aromatic herbaceous and perennial plant (Fig. 5.3) and occurs at altitudes from 2200 m –3800 m a.s.l (Hedge, 1974). It grows along roadsides and the edges of forest (Thulin, 1993). The chromosome number of *S. merjamie* is 2n = 6x = 42 (Hedberg and Hedberg, 1977).

This species is a morphologically highly variable with regard to height, leaf size and shape and corolla length (Hedge, 1974; Thulin, 1993). For example, this taxon is stout, broad-leaved and densely pubescent on Bale Mountains below 3800 m and shorter but very stout and densely pubescent with the young inflorescences partly protected by up curved leaves above 3800 m on the same mountains. There is also a remarkable morphological variability in the North and South Ethiopia. In the northern part, it is very dwarf in stature with cleistogamic flowers having large or small corollas, eglandular indumentum and persistent, narrow oblong to deeply pinnatilobed basal leaves. On the other hand, in the southern Ethiopia, this taxon is much less variable and generally tall with large flowers and clearly glandular pilose (Hedge, 1974).

Fig. 5.3 Mature plant of *S. merjamie.*

Geographically, *S. merjamie* is restricted to western and central African regions (Fig. 4.6). It occurs on the highlands of northern Yemen, Eritrea, Somalia, Cameroon, Saudi Arabia, Madagascar, Ethiopia and East African Mountains (Hedge, 1974; Thulin, 1993). *S. merjamie* has similar distribution to *S. nilotica* (Hedge, 1974) but they are not closely related. The latter is obviously linked to the Eastern Cape species. Both species are very frequent on the high mountains of Ethiopia and Tropical East Africa.

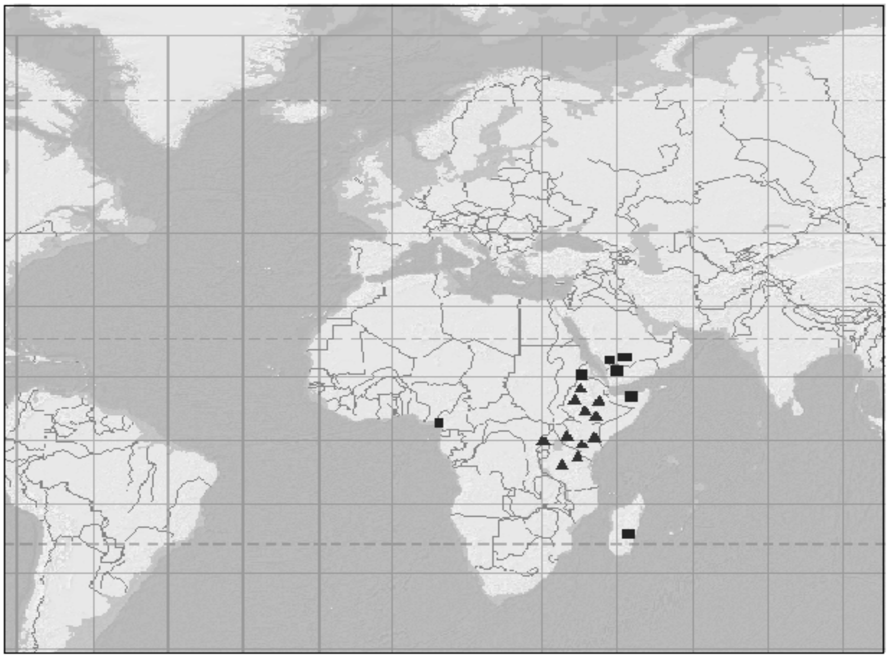

Fig. 5.4 Distribution of *S. merjamie*. Triangles denote the plant populations dealt with in the current study.

C. afromontanum (Caryophyllaceae)

C. afromontanum (Fig. 5.6) is a common in open or lightly shaded and disturbed sites in afro-montane vegetation belts sensu Hedberg (1951) and has an altitudinal range of 1850 m – 4400 m a.s.l.

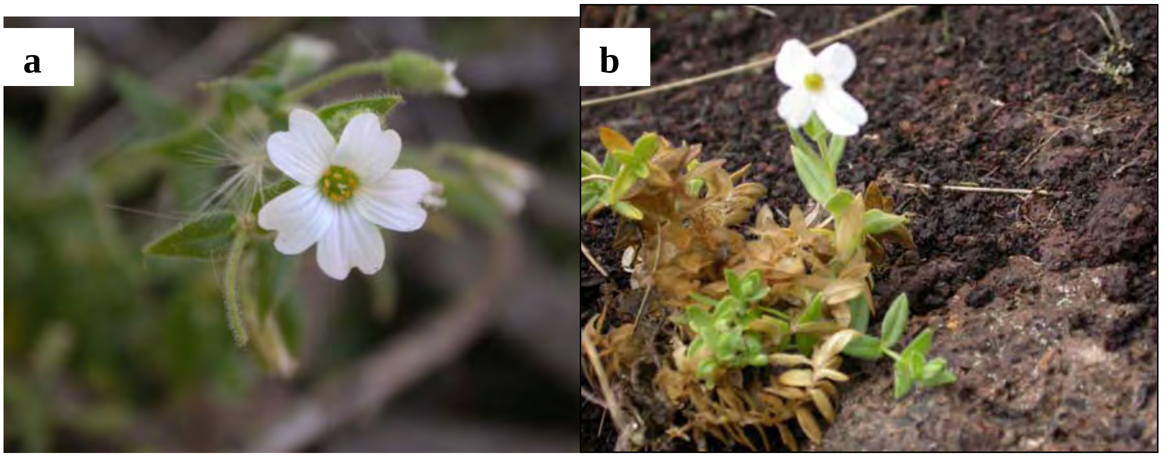

Fig. 5.6 *C. afromontanum:* (**a**) flower and (**b**) habit.

It is a polyploid with 2n = 6x = 36, *e.g*. specimens collected from Bale Mts, Elgon, Aberdares, Mt Kenya and Mt Kilimanjaro, and 2n = 8x = 48 for specimen from Mt Kilimanjaro (Hedberg and Hedberg, 1977).

C. afromontanum is a pilose and glandular small tufted herb, with usually long spreading and ascending slender branches, and procumbent stems. Its leaf shapes vary from ovate to lanceolate, and rarely oblong-elliptic with rounded base (Agnew, 1974). Its flowers are tetramerous or pentamerous with white petals much longer than sepals. *C. afromontanum* is an African endemic taxon (Fig. 5.7). It occurs on the mountains of Ethiopia, Kenya; Tanzania, Uganda, Madagascar and Sudan.

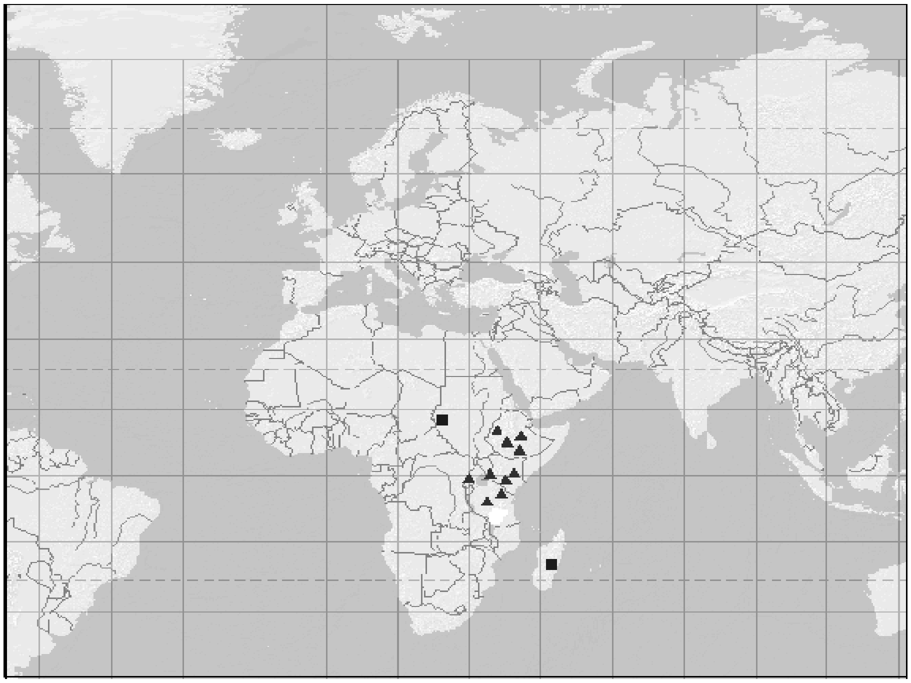

Fig. 5.7 Distribution of *C. afromontanum*. Triangles denote the plant populations dealt with in the current study.

5.2. DNA sampling strategy

Whenever possible, three populations from different localities on each mountain system were considered for this study. Eleven individuals per population per mountain system were collected (Appendix 4.1) although slight modifications were required in some cases where the populations in some species have low density. A visionary line transect was established and healthy leaves of each individual plant at every 25 m distance (modified in some cases) were collected into silica gel filled tubes and labeled accordingly. An individual of a randomly chosen plant from each population was sampled twice and one of these samples was designated "X", blind test for control. The "X' list was kept unknown until the laboratory experiment was completed. One plant from each population was also selected and pressed as a voucher and kept for future reference in the National Herbarium of Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia.

5.3. DNA extraction

The entire molecular laboratory works and the data analyses were performed at National Centre for Biosystematics (NCB), Natural History Museum and Botanical Garden, University of Oslo, Norway.

For DNA extraction, a total 24 specimens were selected for one species from a mountain system (see Appendix 4.1). As a result, the number of individuals used for AFLP for *A. alpina* is 258, 252 for *S. merjame* and 228 for *C. afromontanum*.

Genomic DNA extraction of the three species under investigation was carried out in two ways. First, DNA was extracted by using Qiagen DNeasy 96® Plant Kit (Qiagen Hilden, Germany) following the manufacturer's instructions for *A. alpina*. Second, a hexadecyl trimethylammonium bromide (CTAB) procedure (Doyle and Doyle, 1987) was followed to extract DNA of *S. merjamie* and *C. afromontanum.* Each of these methods is briefly outlined below.

5.3.1 Qiagen DNeasy 96® Plant Kit for DNA isolation

DNA extraction was performed following the instructions of the DNeasy 96® Plant Mini Handbook (Qiagen Series, 2002) because it is a simple procedure and relatively yields a good quality DNA. About 10 mg of silica dried leaf specimen was collected with one tungsten carbide bead in 1.2 ml collection microtubes and ground to a fine powder by Mixer Mill (MM 301, Retsch Gmbh and Co, Haan, Germany) for 5 min at 22 Hz. The resulting fine powder was then collected by centrifugation of the microtubes for 2 min at 8000 rpm, thoroughly resuspended in 400µl of the working lysis solution and incubated at 65°C for 10 to 20 min to increase the DNA yield.

The collection microtubes were subjected to 5 min centrifugation at 6000 rpm to pellet the cell debris and the supernatants were transferred to new collection microtubes, followed by the addition of 130µl Buffer AP2 to each lysate. The microtubes were shaken vigorously for up to 15s and centrifuged at 3000 rpm to collect any solution from the internal part of the caps and the samples were then incubated for 10 min at -20°C. Thereafter, about 400µl of each supernatant was carefully transferred into new collection microtubes and 600µl of Buffer AP3/E was added to each sample. Then the microtubes were once again shaken vigorously for about 15s and centrifuged up to 3000 rpm, to collect any solution from the caps. Then, 1ml of each sample was transferred to the DNeasy 96 plates, each plate was sealed using an AirPore Tape Sheet (QIAGEN Group, USA), to avoid sample cross-contamination and then centrifuged at 6000 rpm for 4 min until the lysate completely passed through the membrane and collected in each well of the DNaesy 96 Plates. Following this, 800µl Buffer AW was added to each sample after carefully removing the old tape sheet. To dry the DNaesy membranes, each DNaesy 96 Plate was sealed with a new AirPore Tape Sheet and centrifuged at 6000 rpm for 15 min for complete removal of residual ethanol. The tape was removed and 100µl Buffer AE was added to elute the DNA into each sample placed in the correct orientation on a rack of elution microtubes. Subsequently, each plate was sealed and incubated for 1 min at room temperature (15-25°C), followed by centrifugation at 6000 rpm for 2 min. This step was repeated with a further addition of 100µl Buffer AE and the elution microtubes were sealed with new caps and stored at -4°C for a few days.

5.3.2 CTAB procedure for DNA isolation

For CTAB DNA extraction, about 10 mg of silica dried leaf samples was powdered by grinding in a Mixer Mill (MM 301, Retsch Gmbh and Co., Haan, Germany) with one tungsten carbide bead in a 2ml eppendorf tubes for 5 min at 22 Hz. The powder was then collected by centrifugation of the tubes for 2 min at 8000 rpm. To approximately 50 mg of leaf powder 700µl CTAB solution (12.1g Tris, 5.8g Na_2 EDTA, 81.9g NaCl, 20g CTAB and 4g PVP dissolved in a liter of water) containing 200 µl of β-mercaptoethanol per 100 ml was added to the mixture and incubated at 60°C for about 30 min. An equal volume of chloroform/isoamylalcohol (24:1) mixture was added and mixed gently for a few minutes. Samples were centrifuged for 10 min at 13000 rpm and 500µl of the supernatant was transferred to labeled tubes. Then, 350µl of isopropanol was added and the contents of the tubes mixed gently for a few minutes. The DNA was then collected by centrifugation for 10 min at 13000 rpm; the pellet was resuspended in 1000µl of 70% ethanol for 5 min and kept in fridge without agitation. The ethanol was decanted and the pellet was vacuum-dried for 10 to 15 min. The pellet was dissolved for 30 min at room temperature in 100µl of TE (10mM Tris HCl, 1mM EDTA pH 8.0 buffer) and 2.5µl RNase (100mg/ml). The extracted DNA was vortexed and allowed to stand at room temperature for at least 30 min in order to digest the RNA and resuspend the DNA. Finally, the resulting DNA was stored at 4 °C for a few days or -20 °C for long term.

5.4. DNA sequencing and AFLP fingerprinting

The extracted DNA samples were subjected to both sequencing and AFLP fingerprinting.

5.4.1 DNA sequencing

cpDNA was sequenced using the dideoxy method from six individual samples of *A. alpina* from each mountain system, *i.e.*, two individuals from each sampled population or six individuals from three populations per mountain massif in total 66 individuals from 11 mountain systems (Appendix 4.2).

Determination of annealing temperature

In order to determine the optimal primers annealing temperature for *A. alpina*, five different temperature grades, *i.e.*, 47°C, 49°C, 51°C, 53°C, 55°C and 57°C were programmed on an automated RoboCycler (StrataGene, La Jolla, CA, USA). Two specimens per population were selected randomly to run the selective PCR for each annealing temperature category using the selected universal primers. The PCR was carried out in a total volume of 10µl reaction mixture consisting the following: PCR buffer, 1.2µl of 2.5mM $MgCl_{2,}$ 0.4µl dNTP (0.4mM, total concentration; Applied Biosystems, Foster City, CA, USA), 0.4µl of 10 µM primer 1 (universal primers c and d, Table 5.1, Taberlet et al., 1991), 0.4µl of 10 µM primer 2 (primer e, Taberlet et al., 1991 and primer f, Dobeš et al., 2004, respectively), 0.1µl RedTaq PCR reaction buffer 10X (Sigma, Saint Louis, Missouri, USA) and 1:10 diluted 2µl DNA template of unknown concentration. The resulting product was subjected to 1% agarose gel electrophoresis in 1X TBE (tris-borate-EDTA) buffer at 90V for 20-30 min. Based on the results, 57°C was identified for the universal primers c and d, while 55°C was optimal for primers e and f for effective amplification of *trn*L intron and *trn*L-*trn*F intergenic spacer of the cpDNA non-coding regions, respectively.

Polymerase chain reaction

The *trn*L intron and *trn*L-*trn*F intergenic spacer (IGS) of the chloroplast DNA regions were amplified by PCR using the forward universal primer 5'-CGA AAT CGG TAG ACG CTA CG-3' and the reverse primer 5'-GGG GAT AGA GGG ACT TGA AC-3' (Table 5.1, primers c and d, respectively, Taberlet et al., 1991), which anneals in the first and second exon of the *trn*L gene, respectively. Sequences comprised the complete *trn*L gene. For amplification of the *trn*L primer 5'-GGT TCA AGT CCC TCT ATC CC-3' (primer e, Taberlet et al., 1991) and the *trn*F IGS primer 5'-GAT TTT CAG TCC TCT GCT CTA C-3' (primer f, Dobeš et al., 2004), which anneals in the second exon of the *trn*L gene and the *trn*F gene, respectively. Amplified sequences included the complete IGS and that of the *trn*F gene.

Table 5.1 Universal primer combinations and sequences for amplification of the non-coding regions of chloroplast DNA of *A. alpina.*

Universal primers	Primer sequence	Author
1. *c*	5’-CGA AAT CGG TAG ACG CTA CG-3’	Taberlet et al., 1991
2. *d*	5’-GGG GAT AGA GGG ACT TGA AC-3’	Taberlet et al., 1991
3. *e*	5’-GGT TCA AGT CCC TCT ATC CC-3’	Taberlet et al., 1991
4. *f*	5’-GAT TTT CAG TCC TCT GCT CTA C-3’	Dobeš et al., 2004

PCR was performed for both non-coding regions in a total volume per sample of 10µl containing 4.2µl pure water, 1µl of 1X PCR buffer (supplied with the enzyme), 1.2µl of 2.5mM $MgCl_2$, 0.4µl of 10mM dNTP (Applied Biosystems, Foster City, CA, USA), 0.4µl of 10µM each primer, 0.1µl of 0.5U AmpliTaq Polymerase (Perkin-Elmer) and 2µl of unknown concentration of template DNA. Amplifications were performed using a PTC-200 Thermal Cycler (MJ Research, Watertown, MA, USA). The PCR program consisted of 3 min at 95°C (primary denaturation of the DNA double helix) followed by 35 cycles each comprising 30 s at 95°C (denaturation), 30 s annealing at 57°C for *trn*L intron and at 55°C for *trn*L-*trn*F IGS, and 60s at 72°C (amplification), followed by a final extension for 7 min at 72°C. Then PCR products were checked on 1% agarose gel for the DNA band equality.

Sequencing the PCR product

Cleaning the product

The PCR product from each sample was mixed with 2µl of 1:10 diluted 10mM exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, USB Corp., Cleveland, OH, USA) and put on the PCR machine (MJ Research, Watertown, MA, USA) for effective purification of the products at 37°C for 20 min in order to activate the enzymes acting on the components in the reaction mixture, and then at 80°C for 15 min to remove the enzymes and other traces of the components. After the completion of the purification reaction steps, each of the resulting products was diluted with 10µl purified water and ready for cycle sequencing.

Cycle sequencing reaction

Each purified PCR product was sequenced in both forward and backward directions in two different reactions using the TaqDyeDeoxy Terminator Cycle Sequencing Kit comprises fluorescent dye-labelled terminators and AmpliTaq DNA polymerase (ABI Applied Biosystems, Foster City, CA, USA). The reaction volume for each individual sample was 10µl consisting of 2µl of 3.2µM BigDye V 1.1, (Applied Biosystems, Foster City, CA, USA) 1 µl of 1x10 PCR buffer, 3µl pure water, 1µl of 10µM primer 1 and 2 (Table 5.1) and 3µl diluted PCR product. Primer 1 and 2 were the original amplification primers, except for the reverse *trn*L-*trn*F IGS primer, which was modified with an additional cytosine to its 3' end (Dobeš et al., 2004).

Ethanol precipitation

Ethanol precipitation of the DNA sample rinses the cycle sequencing products from salts, excess dNTP and other unnecessary trace of chemicals before loading on the ABI sequencer. In this study, the ethanol precipitation method was employed as follows: to each product, 1µl sodium acetate (3M, pH 5.2) and 25µl cold ethanol (70% conc.) were added and the mixture was kept on ice for 10 min. The specimen was then centrifuged at 5500 rpm for 15 min at 4°C and the supernatant was completely removed. The pellet was washed by resuspending in 70% ethanol followed by centrifugation at 5500 rpm for 5 min. This step was immediately followed by instant drying of the tubes by dry vacuum centrifugation (Applied Biosystems). Finally, the DNA pellets were dissolved in 12 µl HiDi (foramide dye) before loading on an automatic capillary sequencer.

Electrophoresis

Electrophoresis was carried out on an automatic ABI 3100 capillary sequencer (ABI 3100 PRISM GeneScan version 3.7 software, PE Applied Biosystems, Foster City, USA). Both strands were sequenced and in most cases the complete sequence could be read in forward and backward directions.

5.4.2 AFLP fingerprinting

Amplified fragment length polymorphism (AFLP) was performed as described by Vos et al. (1995) for the three plant species. The overall PCRs process for AFLP fingerprinting used in this study consisted of four distinct steps:

1. Digestion of genomic DNA using restriction enzymes.
2. Ligation reactions, the restriction DNA fragments annealed with the adaptors. In this reaction, the base change at the restriction fragments incorporated into the adapters prevents restoration of the restriction site.

3. Pre-selective amplification reaction, in which template DNAs are amplified in the presence of primers complementary to adaptors, recognition site and one selective nucleotide.
4. Finally, the selective fragment amplification where the products of the pre-selective amplification were further amplified in the presence of specific primer combinations and three fluorescence dye labeled selective nucleotides.

The selective amplification is based on the recognition of unique nucleotides flanking the restriction site. The principle of selective amplification can be used to adjust the number of fragments that are amplified in a single PCR reaction. Two separate selective rounds of PCR were carried out. In the pre-selective, only one selective nucleotide was used, whereas in the selective, three or rarely two selective nucleotides were used. In practice, this results in several fragments being amplified, which can be separated by electrophoresis. The final step of AFLP study is the gel analysis, which is the separation of amplification products of the restriction fragments by automatic capillary sequencing machine.

Restriction-ligation reactions

Restriction and ligation reactions were conducted in order to ligate restricted fragments of a DNA sample with adapters (commercially available short oligonucleotides, Table 5.2). The reaction mixtures consist of enzyme master mix-1 (10X T4 Ligase buffer, 0.5M NaCl, 1mg/ml BSA, 40U/µl *Eco*RI, 5U/µl *Mse*I, 5U/µl T4 DNA ligase and ddH$_2$0, up to 1µl equals to 0.405 µl/sample); enzyme master mix-2 (10X T4 Ligase buffer, 0.5M NaCl, 1mg/ml BSA, 10µM *Mse*I adapter pair, and 10µM *Eco*RI adapter pair) and 5.5µl (unknown quantity) DNA template from each sample. Prior to their addition to the reaction mixture, the adapter pairs (*Mse*I and *Eco*RI,

Table 5.2) were denatured at 94°C for 5 min and annealed by slow cooling to room temperature. The mixture was then incubated at 37°C for 3h and stored at 4°C until used.

Pre-selective PCR

The restriction and ligation reaction products were diluted 10 times with distilled purified water. Pre-selective PCR was performed using 1.5µl of the 10X diluted mixture (the diluted restriction-ligation products) added to 11µl mixture containing 10X PCR buffer, 25mM $MgCl_2$, 10mM dNTP, 10mM *Eco*RI and 10mM *Mse*I primers (Table 5.3 for the primers), and 5U/µl AmpliTaq Polymerase (Perkin-Elmer). PCR reactions were carried out on GeneAmp PCR System 9700 thermocycler with the following duration and temperature parameters: 2min at 72°C 30 cycles of 30 s denaturing at 94°C, 30 s annealing at 56°C, and 2 min extension at 72°C, was ending with 10min. at 72°C to complete extension. To verify successful amplification, the resulting PCR product was checked by electrophoresis on a 1% agarose gel by loading 5µl of the pre-selective product. After checking for the presence of a smear of fragments of often 300-600 bp in length, the amplification product was then diluted 20 times in purified water and ready to be used as a template for the subsequent selective amplification reactions.

Screening of primer combinations

Twelve primer combinations were tested in selective PCR on four individual samples per population of the different mountain systems. The three different *Eco*RI (E + NNN/NN) and *Mse*I (M + NNN/NN) primer combinations, each with two or three selective nucleotides at the 3' end were selected specifically for the three plant species under investigation, *i.e.*, for *A. alpina* 6-FAM-E + AGT/M+ CAA, VIC-E+ AAG/M + CA, NED-E + AGC/M + CT; for *C.*

afromontanum 6-FAM-E + AGA/M + CTC, VIC-E + AGG/M + CAT, NED-E + ACC/M + CAG; and for *S. merjamie*: 6-FAM-E + ACA/M + CAT, VIC-E + ATG/M+ CTA, NED-E + AGA/M+ CTG, Table 5.3, and were also chosen based on the clarity of the produced bands and were used for subsequent selective PCR of all the sample to be analyzed. Reproducibility and consistency of each primer combination was further checked by carrying out the whole AFLP protocol twice using various numbers, *i.e*., 8, 16, 32, and 48 of samples that belonged to different populations from various study areas.

Selective PCR

The selective amplification reaction was carried out using three of the selected primer combinations for each plant species from 12 different alternative primer combinations using the whole series of AFLP protocols on four individual DNA samples belonging to different populations of the study areas. For selective PCR, the pre-amplified and diluted template was added to a reaction mixture containing 1.25µl of the final concentrations of 10X PCR buffer, 1.25µl 2.5mM $MgCl_2$, 0.10µl 1mg/ml bovine serum albumin, 1.0µl 10mM dNTP, 0.10µl 10mM E + NNN/NN primer and 0.25µl 10µM M+ NNN/NN primer marked with a fluorescent dye, specific for each of the three plant species (Table 5.3), and 0.10µl 5U AmpliTaqGold Polymerase (Perkin-Elmer). PCR reactions were performed with the following selective PCR parameters: 10 min at 95°C, 13 cycles of 30 s denaturing at 94°C, 1 min annealing temperature by decreasing from 65-56°C and 1min elongation at 72°C, followed by 23 cycles for 30 s at 94°C, 1 min at 56°C, 1 min at 72°C; and for 10 min final extension at 72°C.

Electrophoresis

Each individual sample containing, 2.0µl 6-FAM, 2.0µl VIC and 4.0µl NED labeled selectively amplified PCR products were mixed with 11.7µl formamide and 0.3µl GeneScan Rox 500 internal size standard and then denatured at 95°C for 5 min and put on ice immediately for another 5 min. Electrophoresis was run on an automated ABI 3100 capillary sequencer. Blind samples (X-lists) and replicates were routinely included to test for contamination and reproducibility.

Table 5.2 Adapters and their sequences

Adapter	Sequence
*Mse*I-1	5'-GACGATGAGTCCTGAG-3'
*Mse*I-2	3'-TACTCAGGACTCAT-5'
*Eco*RI-1	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI-2	3’-AATTGGTACGCAGTCTAC-5’

Table 5.3 Sequences of the primer combinations used for the AFLP analysis. The bold bases in the primer combination sequences are the two/three selective nucleotides at the 3’ end that have been screened for the selective PCR reactions.

Pre-selective primer		Sequence
*Mse*I + **N**		5'-GATGAGTCCTGATAA**C**-3'
*Eco*RI + **N**		5'-GACTGCGTACCAATTC**A**-3'
A. alpina		
Fluorescence dye	Selective primer	Sequence
6-FAM	*Eco*RI + AGT	5'-GACTGCGTACCAATTC**AGT**-3'
	*Mse*I + CAA	5'-GATGAGTCCTGATAA**CAA**-3'
VIC	*Eco*RI + AAG	5'-GACTGCGTACCAATTC**AAG**-3'
	*Mse*I + CA	5'-GATGAGTCCTGATAA**CA**-3'
NED	*Eco*RI + AGC	5'-GACTGCGTACCAATTC**AGC**-3'
	*Mse*I + CT	5'-GATGAGTCCTGATAA**CT**-3'

C. afromontanum		
Fluorescence dye	Selective primer	Sequence
6-FAM	*Eco*RI + AGA	5'-GACTGCGTACCAATTC**AGA**-3'
	*Mse*I + CTC	5'-GATGAGTCCTGATAA**CTC**-3'
VIC	*Eco*RI + AGG	5'-GACTGCGTACCAATTC**AGG**-3'
	*Mse*I + CAT	5'-GATGAGTCCTGATAA**CAT**-3'
NED	*Eco*RI + ACC	5'-GACTGCGTACCAATTC**ACC**-3'
	*Mse*I + CAG	5'-GATGAGTCCTGATAA**CAG**-3'

S. merjamie		
Fluorescence dye	Selective primer	Sequence
6-FAM	*Eco*RI + ACA	5'-GACTGCGTACCAATTC**ACA**-3'
	*Mse*I + CAT	5'-GATGAGTCCTGATAA**CAT**-3'
VIC	*Eco*RI + ATG	5'-GACTGCGTACCAATTC**ATG**-3'
	*Mse*I + CAT	5'-GATGAGTCCTGATAA**CAT**-3'
NED	*Eco*RI + AGA	5'-GACTGCGTACCAATTC**AGA**-3'
	*Mse*I + CTG	5'-GATGAGTCCTGATAA**CTG**-3'

5.4.3 Band scoring

Raw data collected from the capillary sequencer, was aligned and its pattern visualized using the ABI PRISM GeneScan Analysis Software version 3.1 (Applied Biosystems). Here the GenScan reveals fluorescence peaks indicating the presence of amplified restriction fragment and, the most intense and clear polymorphic peaks were eventually checked individually. Subsequently, the GenScan files were imported into GENOGRAPHER (version 1.6.0; available at http://hordeum.oscs.montana.edu/genographer), where individual peaks of the fragments often in sizes ranging from 50-500 base pairs, were scored under optimum intensity threshold. However, when clear peaks were found beneath the lower bound of the threshold value, they were also scored and included in the data matrix. The resulting data were two groups based on the presence or absence of a peak and recorded as 1 or 0 matrix, respectively, and then exported into Microsoft Excel for further analyses.

5.5. Data analyses

This part consists of DNA sequencing and AFLP data analyses. The DNA sequencing analysis was carried out only for *A. alpina,* and the AFLP data analyses were carried out for all study samples of the three species, *A. alpina, S. merjamie* and *C. afromontanum.*

5.5.1 Sequencing analysis

Sequences were edited and manually aligned using GeneTool 2.0 (BTI Software, Edmonton, Canada). For carrying out the phylogenetic analysis, these data were combined with the previous dataset of the range-wide phylogeographic investigation comprising haplotypes from the whole range of the species to see how the current data fit into previously published phylogeography (Koch et al., 2006). A haplotype network was plotted using the software Network 4.112 (Bandelt et al., 1999). Among the African sequences, the only observed gaps were in a stretch of several cytosine nitrogen base of the DNA sequences or Cs (3-6) followed by several thymine nitrogen base of the sequences or Ts (9-12). It was assumed that the mutation rate for length variation in this region was higher than for substitutions, and the homoplasy was more likely for these characters. Therefore, the African part or the final network was plotted after excluding the gaps and then manually adding haplotypes differing by the number of Cs and Ts, considering these length variants of the haplotypes defined by the nucleotide sequences. A phylogenetic tree was constructed based on the maximum parsimony criterion using the software TNT (Goloboff, 1999). A traditional search was performed starting with ten random addition sequences and using the TBR branch swapping.

Gaps that were composed of several base pairs were recoded as single characters, as were gaps consisting of single base pair steps, while insertions in the poly-C and poly-T stretch were weighed down to 0.5. Sequences of *Aubrieta deltoidea* and *Draba aizoides* were used as outgroups (obtained from Koch et al., 2006). A strict consensus tree was computed and support for the different clades was estimated from 1000 bootstrap replicates. A neighbour-joining tree was also plotted using Juke and Cantor's (1969) distance and the program TREECON version 1.3b (Van de Peer and Wachter, 1994). Gaps were coded just as for the maximum parsimony analysis, but all weighed equally and support assessed with 1000 bootstrap replicates.

For the phylogeographic analyses, the seven African individuals from Koch et al. (2006) data were included. Genetic diversity was estimated for each of the 11 mountain regions as haplotype diversity (h) and nucleotide diversity (π; Nei, 1987). The level of genetic differentiation among phylogeographic groups and among mountains within groups was quantified by Analysis of Molecular Variance (AMOVA) using the program Arlequin 3.01 (Excoffier et al., 2005). Because the phylogeographic pattern was clear on the map, testing alternative groupings is considered to be unnecessary. Significance of differentiation at the different levels was estimated from 10000 permutations. Both $F_{ST,}$ an estimate of differentiation based on haplotype frequencies only, and $\Phi_{ST,}$ an estimate taking into account the molecular distance between haplotypes (number of pair-wise differences), were also estimated. These two estimators are correspond to G_{ST} and N_{ST}, respectively (Pons and Petit, 1996). In case of correspondence between haplotype phylogenies and their geographic distribution, estimates for N_{ST} (Φ_{ST}) will be greater than the G_{ST} (F_{ST}) values (Pons and Petit, 1996; Petit et al., 2005). A permutation test in the program PERMUT (available from *http://www.pierroton.inra.fr/genetics/labo/Software*) was used to test the significance of the difference between the two estimates (Burban et al., 1999).

As an alternative approach, the statistical association between genealogy and geography was investigated using a nested clade phylogeographical analysis (NCPA, Templeton, 2004), and a simplified network was constructed including only haplotypes occurring in Africa and grouped the haplotypes into nested clades according to the algorithm described by Templeton et al. (1987). This nested designed, together with the geographical locations of the samples (coordinates), was analysed by means of permutation tests at the different nesting levels with the software GeoDis (Posada et al., 2000). Templeton's modified inference keys (Templeton, 2004) were used to infer the processes likely to be involved in the statistically significant associations observed.

To investigate the demographic history of the populations, the mismatch distribution (the distribution of the number of pair-wise differences between haplotypes) was estimated for the African lineage. This distribution is usually multimodal in samples drawn from populations at demographic equilibrium, but unimodal in populations having experienced a recent demographic expansion (Rogers and Harpending, 1992). Assuming a model of sudden expansion under which the population size increased from N_0 to N_1 t generations ago, it is possible to estimate the parameter τ (*Tau*) = 2μt from the mismatch distribution, where μ is the mutation rate per DNA fragment. The software Arlequin 3.01 estimates this parameter by a generalized non-linear leastsquare approach, according to Schneider and Excoffier (1999). The validity of the stepwise expansion model is tested by a parametric bootstrap approach, which is also used to estimate confidence intervals (CIs) for the estimated parameters.

5.5.2 AFLP data analysis

Amplified fragment length polymorphism data analyses were carried out for all study samples of the three species, *Arabis alpina, Salvia merjamie* and *Cerastium afromontanum,* to test phylogeographic structures and evaluate the conservation status in the highlands of Ethiopia and Tropical East Africa.

A principal co-ordinate analysis (PCO) of all populations matrices that belonged to the three study plant species was calculated and plotted by choosing Dice similarity coefficient in the program package NTSYS-pc 2.0 (Rohlf, 1997; programs DCENTER, EIGEN) with simple matching as a similarity measure to evaluate the similarities among the AFLP marker phenotypes. This is mainly because the Dice similarity coefficient takes similarity into account only in the presence of DNA fragments. A neighbour-joining analysis of Nei-Li distance measurement (Nei and Li, 1979) based on genetic distance matrix was computed using the software TREECON 1.3b. The trees were midpoint rooted and support for the branches was estimated with 1000 bootstrap replicates.

Genetic diversity within local samples, among local populations and within regions was estimated as the average number of pair-wise differences between individuals (Kosman et al., 2003) using the R-script AFLPdat (Ehrich, 2006) and Arlequin 3.01 software for the data analysis. The levels of differentiation among local populations and regions were estimated in an AMOVA using the software Arlequin 3.01. As genetic distance, the number of pair-wise differences between individuals was used. Significance of differentiation at the different levels was estimated from 10000 permutations.

Additionally, the population structure was examined by genetic admixture analyses using the software BAPS (Bayesian Analysis of Population Structure) version 3.2 (Corander et al., 2005) and Structure version 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 in which each individual belongs. The program Structure implements a model based clustering method in which a logarithmic probability for the data was calculated given a number of clusters, and assigned the specimens to these clusters probabilistically using MCMC estimation and the likelihood of the data estimated in different runs for various number of groups (K) was compared, finally the optimal value of K was identified. Structure program at Bioportal centre, University of Oslo (*http://www.bioportal.uio.no*), was used to analyze the data with number of clusters, K 1–10 and 10 replicates of each K's value were run for different selections of samples with a burn-in period of 200000 and 1000000 iterations. Then 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 an R-script.

CHAPTER 6 RESULTS

This part has been organized in such a way that the cpDNA sequence data of *A. alpina* are first presented and followed by AFLP data of *A. alpina* itself and the remaining species.

6.1 cpDNA of *A. alpina*

6.1.1 Haplotype relationships and geographic distribution

The length of the aligned cpDNA sequences from the 66 selected individuals of *A. alpina* was 745 base pairs (bp). There were 14 variable sites, comprising eight substitutions, and six insertions and deletions (indels). All indels were in the stretch of several C's (3-6) followed by several T's (9-12). Seven of the substitutions and all length variation were parsimony informative. Nine different haplotypes were detected in this study (Fig. 6.1 and Table 6.1). Two of them, haplotypes 04 and 19, were identical to haplotypes observed by Koch et al. (2006) and the remaining seven were new. Koch et al. (2006) have also found two additional African haplotypes, haplotypes 05 and 18 which were not observed in the current material (Fig. 6.1). The sequences of the new haplotypes have been deposited in GenBank (accession numbers EF449508 - EF449514). Combining the current data with those of the world-wide analysis of Koch et al. (2006) resulted in an alignment with 26 variable sites and 25 indels (excluding outgroups). Nine substitutions and 11 indels were parsimony informative in the combined data set.

The haplotype network of the combined data set confirmed the general structure reported by Koch et al (2006). It emerged that there were three major groups, the African, the Asian and the

European groups (Fig. 6.1). The new haplotypes were placed mainly in the African group (A, B, C, D, E and F), thereby increasing its diversity and one haplotype was placed in the Asian group (G).

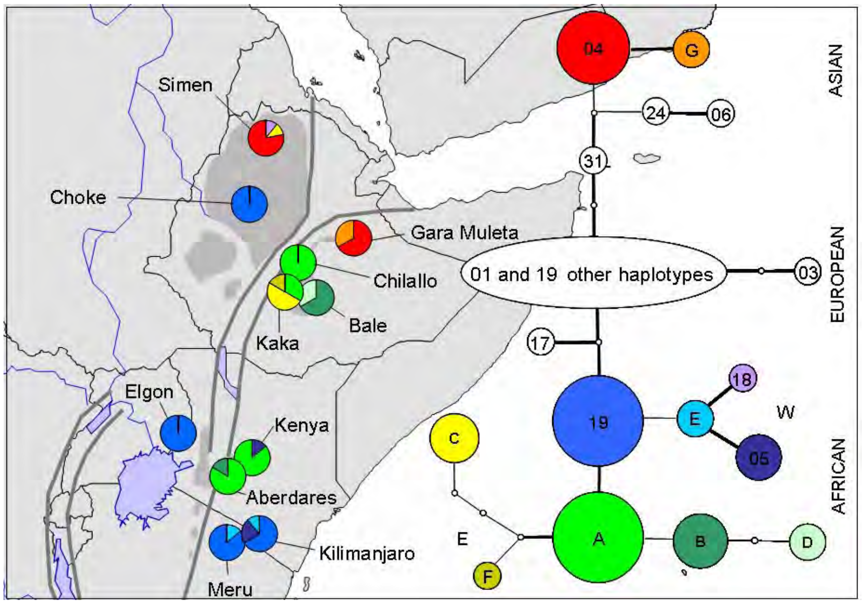

Fig. 6.1 Map of the cpDNA haplotype network corresponding to the study areas. For each mountain massif, a pie diagram shows the observed haplotypes and the colours in the diagram correspond to the colours of the haplotypes in the network. The area shaded in grey indicates mountains and highlands. The thick dark-grey lines show the Great Rift Valley. On the haplotype network, the three different lineages are shown and the letters W and E indicate the African sublineages. The size of the haplotypes is drawn proportional to their frequencies. Thick lines represent substitutions, thin lines insertions or deletions and small circles hypothetical intermediate haplotypes. The European lineage was represented in a simplified way and haplotypes not observed in Africa were not coloured.

The maximum parsimony analysis using the combined matrix yielded 22 most parsimonious trees with a length of 215 steps. On the consensus tree, the African and the Asian haplotypes formed distinct clades with moderate bootstrap support (61% and 68%, respectively; Fig. 6.2). Haplotype 17 from an individual sampled in Lebanon by Koch et al (2006) was placed in a sister position to the African clade, but the bootstrap support was only 50%. Within the African clade, haplotypes A, B, C, D and F formed a sub-clade (bootstrap support 51%). The topography of the neighbour-joining tree was identical to the maximum parsimony tree, but the bootstrap support for the sub-clade containing haplotype A was exactly 60% (Fig. 6.3).

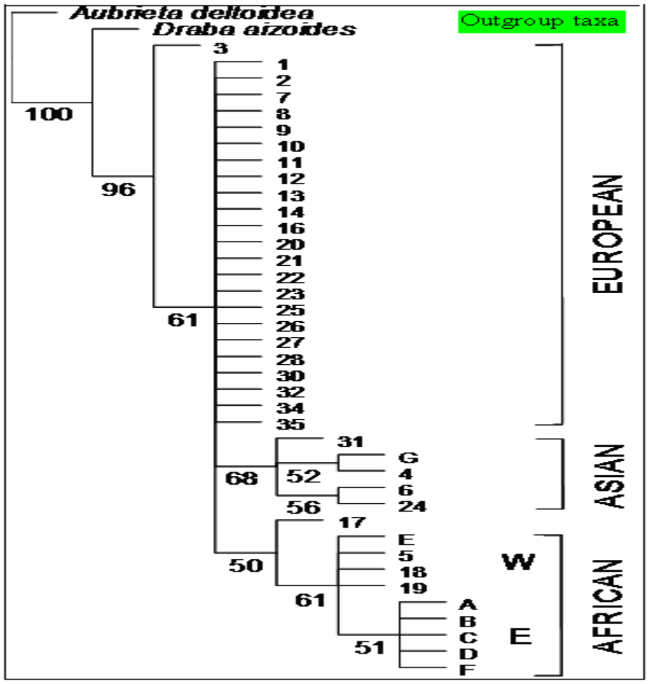

Fig. 6.2 Consensus tree summarizing the 22 most parsimonious trees. Bootstrap support in percentages is given close to the branches. The three different lineages are shown and the letters W and E indicate the African sublineages.

The Asian and the African clades, as well as the sub-clade containing haplotype A were statistically supported by these analyses (Fig 6.2). In addition, a second weakly supported sub-clade in Africa joining haplotypes 05, 18 and E was suggested (56% bootstrap support on the NJ-tree, Fig. 6.3). In order to get a robust estimate for the shallow phylogeny, a maximum parsimony tree was also estimated excluding indels, which resulted in identical topography. The Asian lineage was restricted to the northernmost and easternmost mountain regions in Ethiopia, situated on opposite sides of the Great Rift Valley (Simen Mts and Gara Muleta, Fig. 6.1). Haplotype G, a derived haplotype of the Asian lineage, occurred only in Gara Muleta. In the Simen Mts, three haplotypes (C, 05 and 18) from the African lineage were found as well.

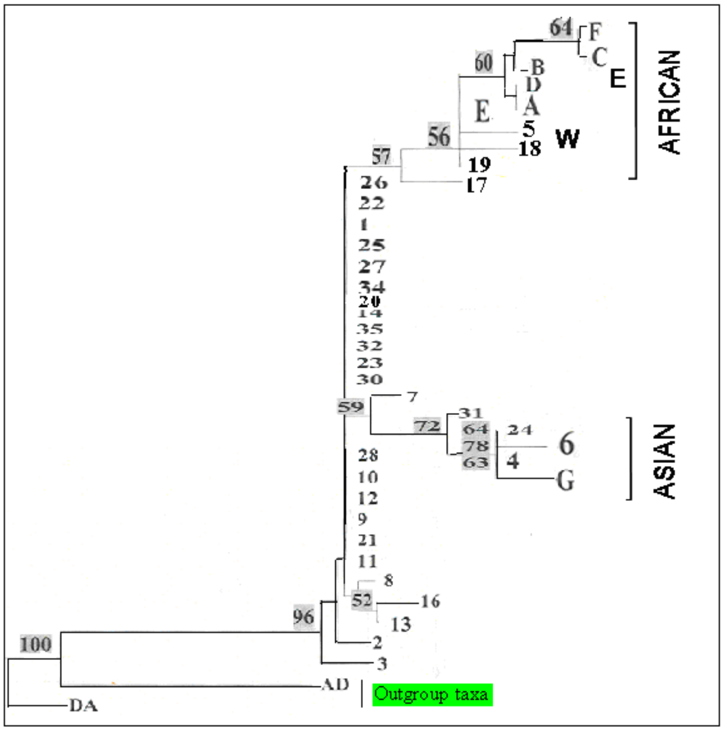

Fig. 6.3 Neighbor-joining tree of samples of *A. alpina* based on Nei and Li’s (1979) distances between cpDNA haplotypes. Trees were mid-point rooted. Bootstrap values estimated from 1000 replicates above 50% are also indicated.

The haplotypes of the African lineage could be divided into two groups with largely non-overlapping geographic distributions, corresponding to the division observed in the network, the phylogenetic analyses (Fig. 6.1). One of these phylogeographic subgroups consisted of the weakly supported clade containing haplotype and will hereafter be referred to as group A (the common haplotype A and its derived haplotypes B, C, D, and F; Fig. 6.1). The other contained the rest of the African haplotypes and will hereafter be referred to as group 19 (the common haplotype 19 and its derived haplotypes E, 05, and 18). Despite the fact that this group was not resolved and did not constitute a clade, its topology on the haplotype network and its distinct geographic distribution support it as a phylogeographic group.

Group A was found mostly on the East of the Rift Valley, while group 19 occurred on the Western side. In the south of the study area, however, group 19 extended eastwards across the Rift Valley to Mt Meru and Mt Kilimanjaro (Fig. 6.1). Haplotype 05 could be found on Mt Kilimanjaro and also on Mt Kenya where it once co-occurred with haplotypes of group A, extending group 19 further northwards. In the north, the group A extended westwards across the Rift Valley with the derived haplotype C (found on Mt Kaka and in the Simen Mts) and overlapped with group 19 in the Simen Mts (where it also co-occurred with the Asian lineage). The Simen Mts were thus the only locality where haplotypes from all three lineages were found.

6.1.2 Phylogeography and demographic history

Phylogeography

Phylogeography has become a powerful approach for elucidating contemporary geographical patterns of evolutionary subdivisions within closely related species (Arbogast and Kenagy, 2001). It has been used primarily to examine geographical structuring of intraspecific lineages, when typically individual species are sampled from certain geographical ranges, the genomic DNAs are characterized for each individual species, either through sequencing or by other molecular techniques. The resulting data are thus used to infer a phylogeny which reflects the evolutionary relationships of the individuals. The affiliation between the resulting gene trees and the geographical location of individual is therefore elucidating the geographical distribution of major gene lineages or monophyletic clades.

Table 6.1 Regional sample sizes (n), number of different haplotypes identified, haplotype diversity (h) and nucleotide diversity (π).

Region	n	Number of haplotypes	Haplotype diversity (h) SD)	Nucleotide diversity, π (SD) in %	Haplotypes										
					19	18	5	E	A	B	C	D	F	4	**G**
Simen Mts*	9	3	0.42 (0.19)	0.59 (0.37)		1*					1			7	
Mt Choke	6	1	0.00 (0.00)	0.00 (0.00)	6										
Gara Muleta	6	2	0.53 (0.17)	0.07 (0.08)										4	2
Mt Chilallo	6	1	0.00 (0.00)	0.00 (0.00)					6						
Mt Kaka	6	3	0.73 (0.16)	0.36 (0.26)					2		3		1		
Bale Mts	6	2	0.53 (0.17)	0.14 (0.13)						4		2			
Mt Elgon	6	1	0.00 (0.00)	0.00 (0.00)	6										
Mt Kenya*	7	1	0.29 (0.19)	0.12 (0.11)			1*		6						
Aberdares	6	2	0.33 (0.22)	0.09 (0.09)					5	1					
Mt Kilimanjaro*	9	2	0.56 (0.17)	0.12 (0.11)	6		2*	1							
Mt Meru	6	2	0.33 (0.22)	0.05 (0.06)	5			1							

*In addition to the 66 samples analysed in the present studies, seven haplotypes from Koch et al. (2006) are included.

Table 6.2 Population pair-wise differentiation among regional groups as estimated with AMOVA. F_{ST} estimated from cpDNA (*trn*L intron and *trn*F intergenic spacer of the *trn*L-*trn*F region) sequences based on haplotype frequencies only in the lower part and Ø$_{\mathrm{ST}}$ estimated pair-wise differentiation by taking into account the molecular distance between haplotypes in the upper part.

	Simen	Choke	Gara Muleta	Chilallo	Kaka	Bale	Elgon	Kenya	Aberdares	Kilimanjaro	Meru
Simen		**0.756**	0.160	**0.776**	**0.646**	**0.689**	**0.756**	**0.776**	**0.750**	**0.739**	**0.739**
Choke	**0.833**		**0.969**	**1.000**	**0.600**	**0.800**	0.000	**1.000**	**0.857**	-0.000	-0.000
Gara Muleta	0.025	**0.733**		**0.972**	**0.854**	**0.914**	**0.969**	**0.972**	**0.954**	**0.951**	**0.951**
Chilallo	**0.833**	**1.000**	**0.733**		0.429	**0.680**	**1.000**	0.000	**-0.000**	**0.8571**	**0.8571**
Kaka	0.418	**0.633**	0.367	0.450		0.200	**0.600**	0.429	0.357	**0.565**	**0.565**
Bale	**0.567**	**0.733**	**0.467**	**0.733**	0.367		**0.800**	**0.680**	**0.533**	**0.753**	**0.753**
Elgon	**0.833**	0.000	**0.733**	**1.000**	**0.633**	**0.733**		**1.000**	**0.857**	-0.000	-0.000
Kenya	**0.833**	**1.000**	**0.733**	0.000	0.450	**0.733**	**1.000**		-0.000	**0.857**	**0.857**
Aberdares	**0.667**	-0.000	**0.567**	-0.000	0.262	**0.512**	**0.833**	-0.000		**0.750**	**0.750**
Kilimanjaro	**0.667**	-0.000	**0.567**	**0.833**	**0.467**	**0.567**	-0.000	**0.833**	**0.667**		-0.200
Meru	**0.667**	-0.000	**0.567**	**0.833**	**0.467**	**0.567**	-0.000	**0.833**	**0.667**	-0.200	

Values revealing significant differentiation at the $P = 0.05$ level in 10000 permutation tests are in bold, for both F_{ST} and Ø$_{\mathrm{ST}}$.

Genetic diversity estimates for each mountain region are presented in Table 6.1. Nucleotide diversity was highest in the Simen Mts with $\pi = 0.59\%$. Among the remaining sites, nucleotide and haplotype diversities were highest on Mt Kaka with $\pi = 0.36\%$ and $h = 0.73$. On three mountains, only a single haplotype was found (Mt Choke, Mt Chilallo and Mt Elgon). For the AMOVA analyses, the populations were divided into three main phylogeographic regions according to the geographic distributions of the Asian lineage (Simen in the north and Gara Muleta in the east) and the two African haplotype groups (group A: Mt Chilallo, Mt Kaka, Bale Mts, Mt Kenya and Aberdares; group 19: Mt Choke, Mt Elgon, Mt Meru and Mt Kilimanjaro; Fig. 6.1). Population pair-wise differentiation among regional groups as estimated with AMOVA indicated significant differences (Table 6.2). High differentiation was also observed among these three main regions. The percentage of variation among regions was considerably higher when taking into account molecular variation (71.5%) than when analyzing only haplotype frequencies (51.2%; Table 6.3). According to the G_{ST}/N_{ST} test, this difference was significant with $p = 0.014$, confirming that at this scale, related haplotypes were grouped geographically. Only a small proportion of variation was found between individual mountains within the three regions (6.0% and 13.6%, respectively; Table 6.3).

Table 6.3 Genetic differentiation among phylogeographic groups and mountains within groups as estimated with AMOVA.

Source of variation	Percentage of variation (haplotype frequencies)		Percentage of variation (no. of pair-wise differences)	
Among three phylogeographic groups	51.2	p = 0.0001	71.5	p = 0.0001
Among mountains within groups	13.6	P < 0.0001	6	p < 0.0001
Within mountains	35.2		22.5	
Between group A and 19	52.3	p = 0.009	48.8	p = 0.008
Among mountains within groups	15.7	p = 0.0001	17.5	p = 0.0001
Within mountains	32.0		33.7	
Among mountains in group 19	3.7	p = 0.36	7.2	p = 0.24
Within mountains	96.3		92.8	
Among mountains in group A	42.6	p = 0.0001	39	p = 0.0002
Within mountains	57.4		61	
Eastern group without Bale: among	24.4	p = 0.012	32.1	p = 0.007
Within mountains	75.6		67.9	
Between mountains in the N. group	2.1	p = 0.3	5	p = 0.2
Within mountains	97.9		95	

Calculations were performed using haplotype frequencies only and taking into account the molecular variation as number of pair-wise differences between haplotypes. Significance of differentiation was estimated from 10000 permutations using the software Arlequin 3.01.

The differentiation between the two groups belonging to the African lineage was somewhat lower. Contrary to expectations based on the nearly distinct distribution of the two groups of haplotypes (Fig. 6.1), there was slightly more variation among regions for the estimates according to haplotype frequencies only (Table 6.3), indicating no significant phylogeographic structure. This can, however, be explained by a large amount of homoplasy in length variation, which represented a considerable part of the total variation in both African groups. There was no differentiation among mountains within the northern and the southwestern group (group 19). In the eastern group (group A), the proportion of variation among mountains was significant; however, the G_{ST}/N_{ST} test was not. The two haplotypes from the Bale Mts (B and D) were observed almost only here, making this locality distinct (Fig. 6.1). But differentiation was significant within this group even when the Bale Mts were excluded (32.1% of variation among mountains, p=0.007).

The nested clade phylogeographic analysis revealed a significant association between genealogy and geography only in three clades (Fig. 6.4). For clade 1-2, this association could not be attributed to a particular process (inconclusive outcome, Table 6.4). For clade 2-1, inference key indicated gene flow with isolation by distance, and for the total network, past fragmentation and/or long-distance colonization (Table 6.4).

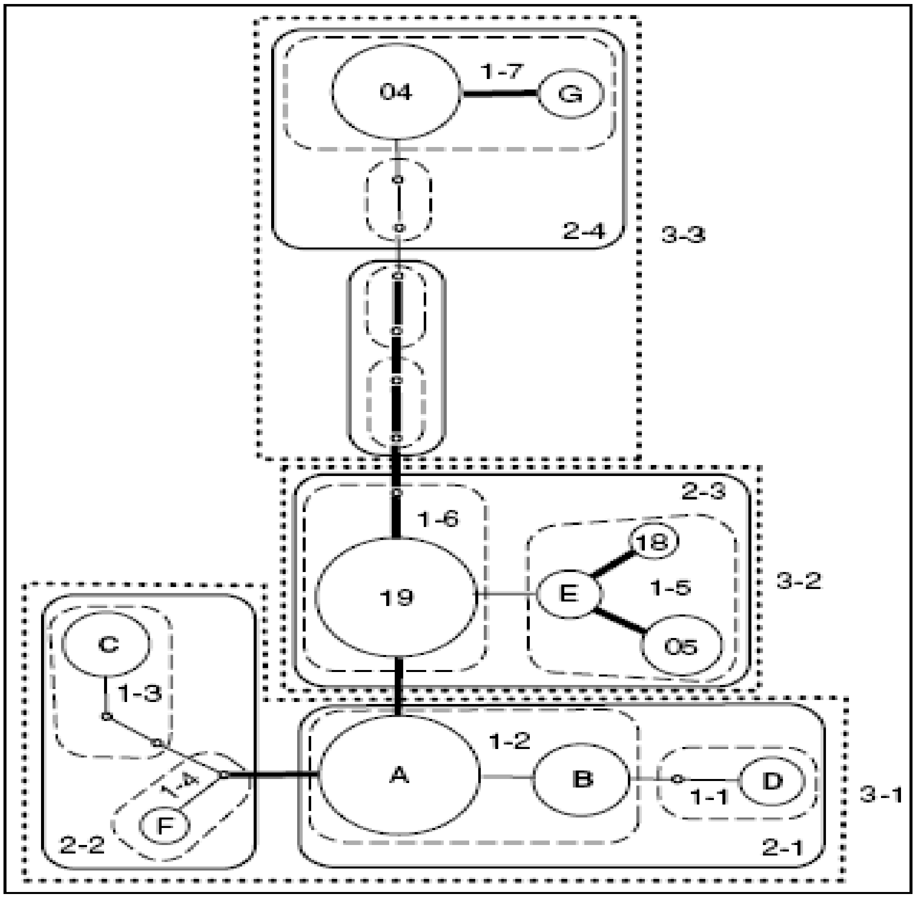

Fig. 6.4 Nested design of the haplotype network used for the nested clade phylogeographic analysis. Thin dashed lines indicate one-step clades, lines reveal two-step clades and thick dotted lines show three-step clades. Only haplotypes observed in Africa were included.

Table 6.4 Clade with their biological interpretation from the NCPA according to the modified inference key (Templeton, 2004).

Nesting clade	Chain of inference	Inference key conclusion
1-2	1, 2	Inconclusive outcome
2-1	1, 2(c+d), 3, 4	Restriction gene flow with isolation by distance
Total cladogram	1, 2(a+d), 3(a+b), 5, 15	Past fragmentation and/or long distance colonization

Only clades with some significant values from the permutation tests are shown, and the inference chain, as well as the conclusions from the inference key, is reported.

Demographic history

Because it is likely that length variation in the poly-C and poly-T stretch mutates at a higher rate than the rest of the sequence, this segment was excluded from the estimation of the mismatch distribution. For the whole African clade, the sudden expansion model was rejected with $p<0.001$, which is consistent with the subdivision into two distinct phylogeographic groups. The mode of the distribution was one, as expected from the nearly equal frequencies of the two most common haplotypes A and 19, which differed by one substitution (Fig. 6.5). The analysis for each phylogeographic group was also carried out separately, although there was very little variation in each group (Fig. 6.4). Both distribution patterns support the sudden expansion model. The parameter $\tau = 2\mu t$ was estimated as 0.368 for group A (CI 0.024–0.530 for $\alpha = 0.1$). For group 19 the estimate was 3.0 (CI 0.399–3.187 for $\alpha = 0.1$), but this value does not correspond to expectations from the total amount of variation observed (the shape of the observed distribution) in this group, which was quite similar to that of the eastern group or group A and will be disregarded in the following discussion as an artefact of the small sample size and low level of variation.

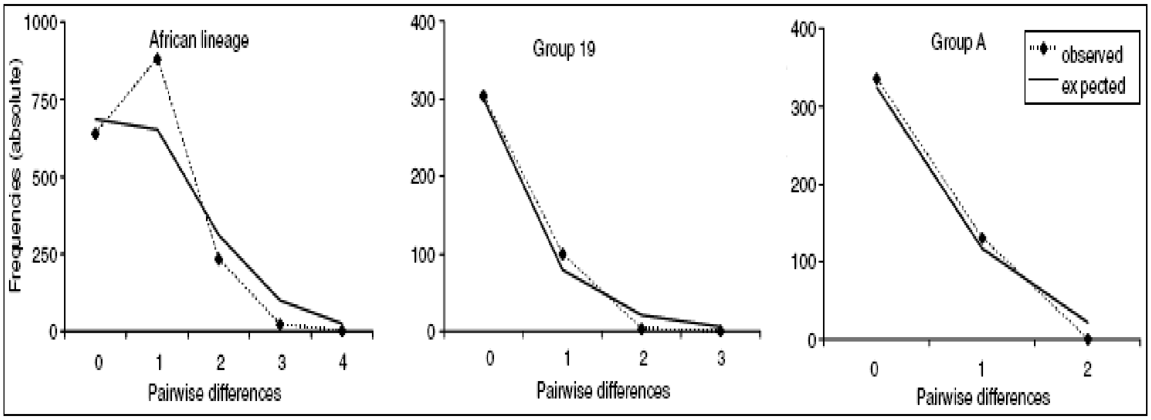

Fig. 6.5 Mismatch distribution for the whole African lineage and for the African phylogeographic groups 19 and A. The black diamonds indicate observed values, whereas the line shows expected values under a model of sudden expansion.

6.2 AFLP fingerprinting results

In the following sections, details of AFLP fingerprinting of *A. alpina*, *S. merjamie* and *C. afromontanum* will be presented.

6.2.1 *A. alpina*

The total number of individual samples of *A. alpina* used for the AFLP analysis from 12 mountain systems was 224 and 30 replicates, which resulted in 254 polymorphic markers with greater than 99% reproducibility.

Principal coordinate analysis

Principal coordinate analysis is used to classify individuals of populations into genetically defined phylogeographic groups corresponding to their geographical regions. Thus, on the PCO plot, the samples were divided into at least four clusters (Fig. 6.6). The first axis (31.8% of variation) separated Simen and Gara Muleta in northern and eastern Ethiopia, respectively, from most of the other study regions. Two populations from Elgon and three individuals from Chilallo were placed close to Simen and Gara Muleta on the PCO plot. The plants from Meru had an intermediate position. The second axis (9.1% of variation) separated mostly the Ethiopian plants from those from the southern mountain massifs. The third axis (6.8% of variation) separated markedly the individual samples of Choke from the rest (Fig. 6.7). In this axis two groups were clearly shown on either side of the axis.

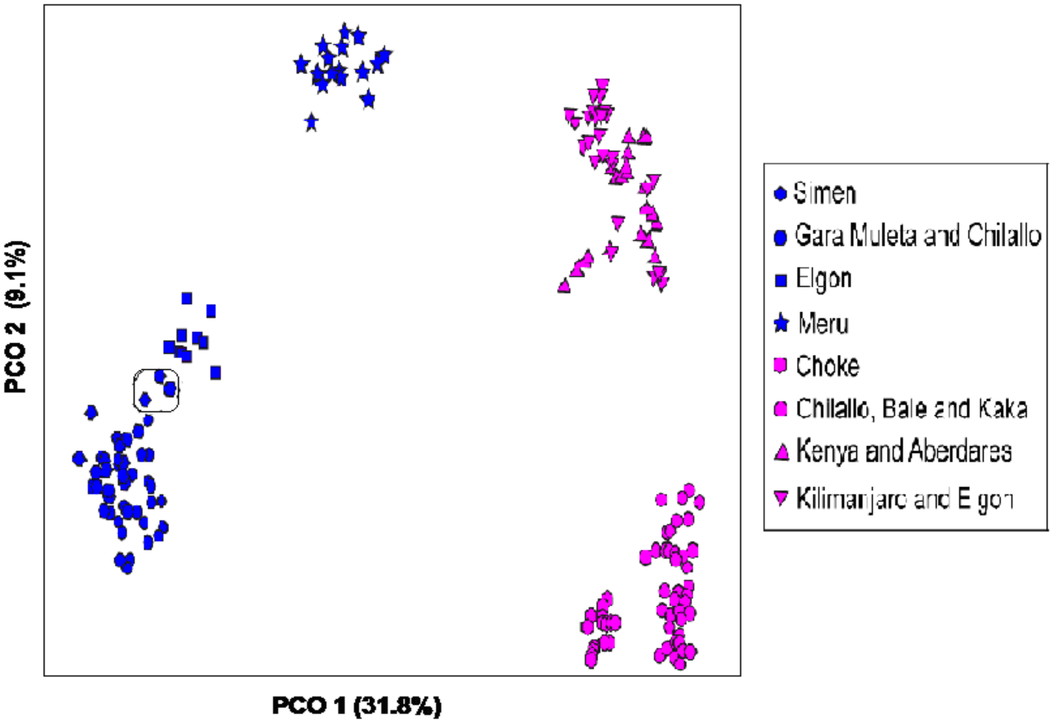

Fig. 6.6 Plots of the first two axes from principal coordinate analyses of the AFLP dataset obtained for *A. alpina*. The percentage of variation explained by each axis is indicated. Colors identify main genetic groups and shapes of symbols identify subgroups as determined by Structure, identical to those in figure.610 a. The circle highlights the three individuals from Chilallo, which belong to the same genetic group as the individual from Gara Muleta.

Neighbor-joining analysis

In the N-J analysis, mid-point rooting resulted in an unresolved division between Gara Muleta-Simen (and the related individuals from Elgon and Chilallo), Meru, and the other mountain regions (Fig. 6.8). All these three main groups had high bootstrap support. The strong genetic structure was also reflected in high bootstrap support for several regional groups, such as southern Ethiopia (Bale, Kaka and Chilallo), Choke, Kilimanjaro or the two distinct groups from Elgon.

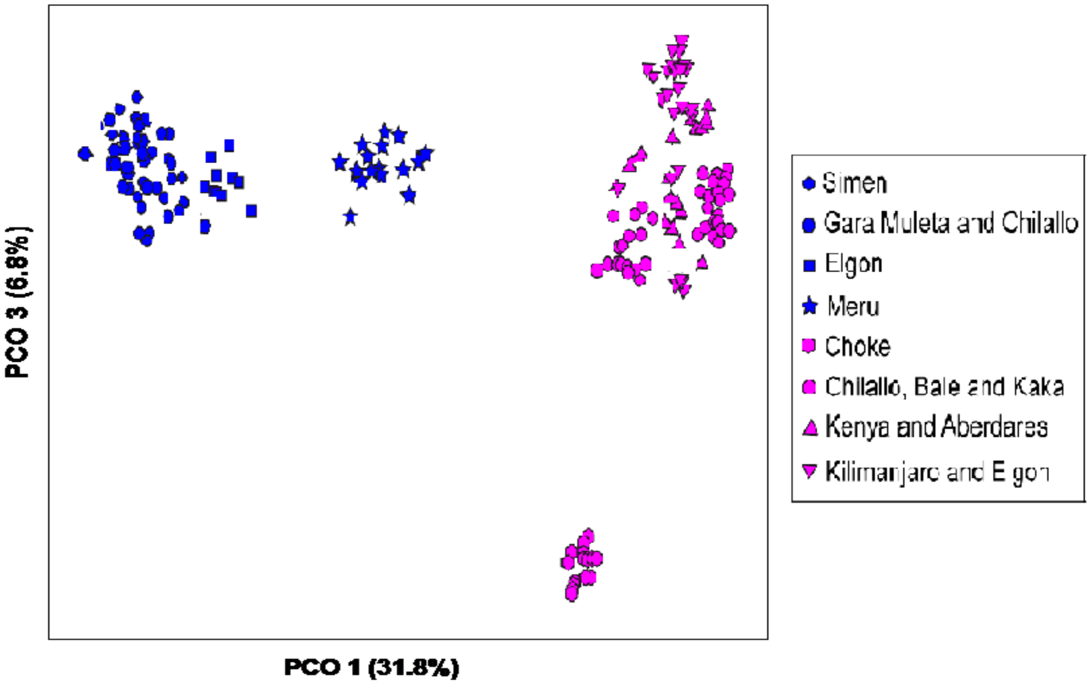

Fig. 6.7 Plots of the first and the third axes from principal coordinate analyses of the AFLP data obtained from *A. alpina*. The percentage of variation corresponding to each axis is indicated. Colors identify the genetic groups and shapes of symbols identify subgroups as determined by Structure in figure 6.10 a.

Genetic diversity analysis and AMOVA

The level of genetic diversity within the populations of *A. alpina* ranged from 0.002-0.111. Genetic diversity within local samples was highest in population from Chilallo in southern Ethiopia, where plants from both main groups (Kaka and Bale Mts) were observed. Thus in the presence of the three divergent samples from Chilallo, the average intra-population diversity was 0.051 (SD = 0.052, Table 6.14) and the regional genetic diversity was 0.104, which are relatively higher than in their absence (Table 6.5). Otherwise, the diversity was highest in the two divergent mountain regions in Ethiopia, Simen and Gara Muleta and lowest on Mt Kenya and in Choke (Table 6.6). The results indicate that the regional diversity was highest on Mt Elgon (0.146), where populations belonging to both main groups occurred.

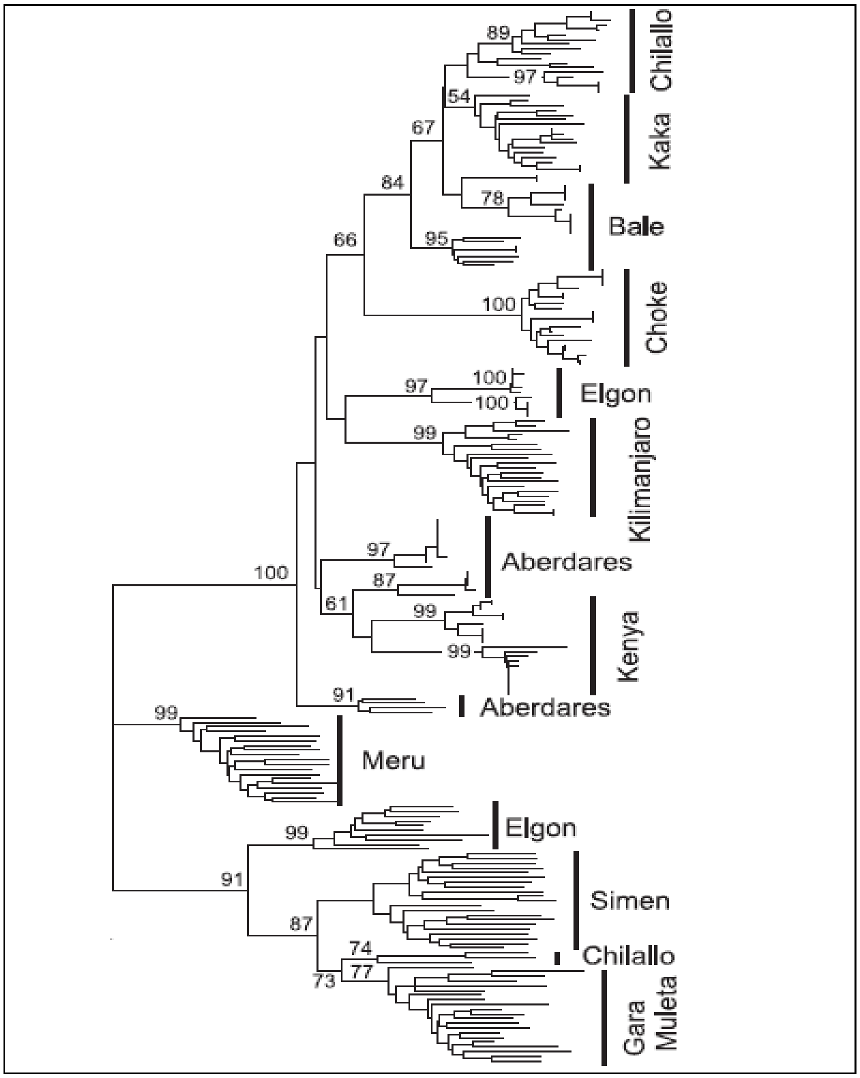

Fig. 6.8 Neighbor-joining trees of individual samples of *A. alpina* based on Nei and Li’s (1979) distances between individual AFLP types. Trees were mid-point rooted. Bootstrap values estimated from 1000 replicates above 50% are also indicated.

Table 6.5 Genetic diversity (average proportion of pair-wise differences) in *A. alpina* by region in the absence of the divergent individuals of Chilallo. The averages and standard deviations were estimated (as intra-population diversity) within localities and within regions.

Region	Intra-population diversity (SD)	Regional diversity
Simen	0.081 (0.026)	0.102
Gara Muleta	0.066 (0.012)	0.067
Choke	0.032	0.032
Southern Ethiopia	0.051 (0.052)	0.104
Elgon	0.045 (0.017)	0.146
Kenya	0.030 (0.028)	0.088
Kilimanjaro	0.049	0.049
Meru	0.060	0.060

Table 6.6 Genetic diversity (average proportion of pair-wise differences) in *A. alpina* by region. The averages and standard deviations were estimated (as intra-population diversity) within localities and within regions.

Region	Intra-population diversity (SD)	Regional diversity
Simen	0.081.(0.026)	0.102
Choke	0.032	0.032
Gara Muleta	0.066.(0.012)	0.067
Southern Ethiopia*	0.035.(0.016)	0.081
Elgon	0.045.(0.017)	0.146
Kenya	0.030.(0.028)	0.088
Kilimanjaro	0.049	0.049
Meru	0.060	0.060

***** represents the regional genetic diversity estimate of the three divergent individuals of Chilallo, which revealed on the figure 6.6 of PCO analysis were excluded from the individual samples of southwestern Ethiopian (Chilallo, Kaka, and Bale Mts) group.

Table 6.7 Genetic differentiation of the AFLP dataset for *A. alpina* with AMOVA.

Group	Percentage of variation	
Between two major groups	40.7%	
Among localities within groups	38.4%	
Within local samples	20.9%	
Between two major groups	36.7%*	p=0.027
Among subgroups within groups	32.8%	
Within subgroups	30.5%	
Among localities in group 1	68.9%	
Within local samples	31.1%	
Among localities in group 2	56.3%	
Within local samples	43.7%	

The data were divided into two major groups: group 1 comprised Simen, Gara Muleta, Meru and part of the individuals from Elgon, and group 2 the rest of the localities, as suggested by the Structure analysis. Subgroups correspond to the eight clusters identified by the further analysis with Structure. The three divergent individuals from Chilallo were excluded from this analysis.

The genetic differentiation obtained with AMOVA between the two major groups, among localities within groups and within local samples were 40.7%, 38.4% and 20.9%, respectively, and are all significant (p<0.001). On the other hand, significant differentiation (p=0.027) was also revealed between two major groups (Table 6.7). Moreover, the AMOVA analysis showed that 68.9% of variation was among local samples in one of the two major groups (Gara Muleta, Simen, Meru and Elgon, blue on Fig. 6.10 a) and 56.3% in the other (Table 6.7).

Genetic mixture analyses

Similar to the results of PCO and N-J analyses, clear patterns were identified by genetic mixture analyses (Structure and BAPS). Results from genetic mixture analysis with the software Structure 2.1 for the *A. Alpina* total dataset indicate that convergence was good only for K=2. In another words, the results from Structure were consistent over 10 runs only for the value of K=2 (Fig. 6.9).

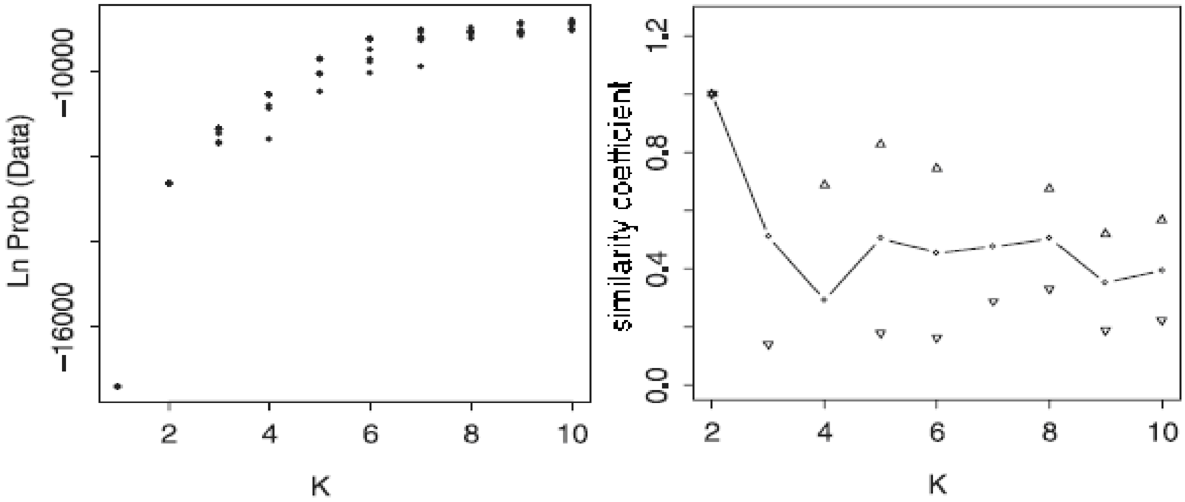

Fig.6.9 Structure analysis of the AFLP dataset for *A. alpina* with the program Structure 2.1. The plots on the left side show the estimated likelihood for values of or each number of groups (K) ranging from one to10 runs. The plots on the right show the average similarity between runs for each calculated value of K according to Rosenberg et al. (2002). Circles represent the mean of or the average similarity coefficient for all pair-wise comparisons among the 10 runs, whereas triangles indicate an interval ± the standard deviation. A similarity value above 0.85 corresponds to a generally similar structure (Rosenberg et al., 2002).

The results from Structure analysis indicate a division in the dataset into the two main groups along axis 1 on the PCO plot (with Meru in the same group as Gara Muleta and Simen, some individuals of Elgon, and still the three divergent individuals of Chilallo were attributed to the “blue” on the map Fig. 6.10 a). Structure analyses of the two main groups separately resulted in eight distinct subgroups, *i.e.*, 4 subgroups on each side; thus 8 groups as optimal subdivision, which indicated with different shapes on the map in figure 6.10 a. Interestingly, the spatial distribution of the subgroups was very impressive in that, four subgroups were in Ethiopian highlands and the other subgroups were in the tropical East African (Kenya and Tanzania) mountain regions.

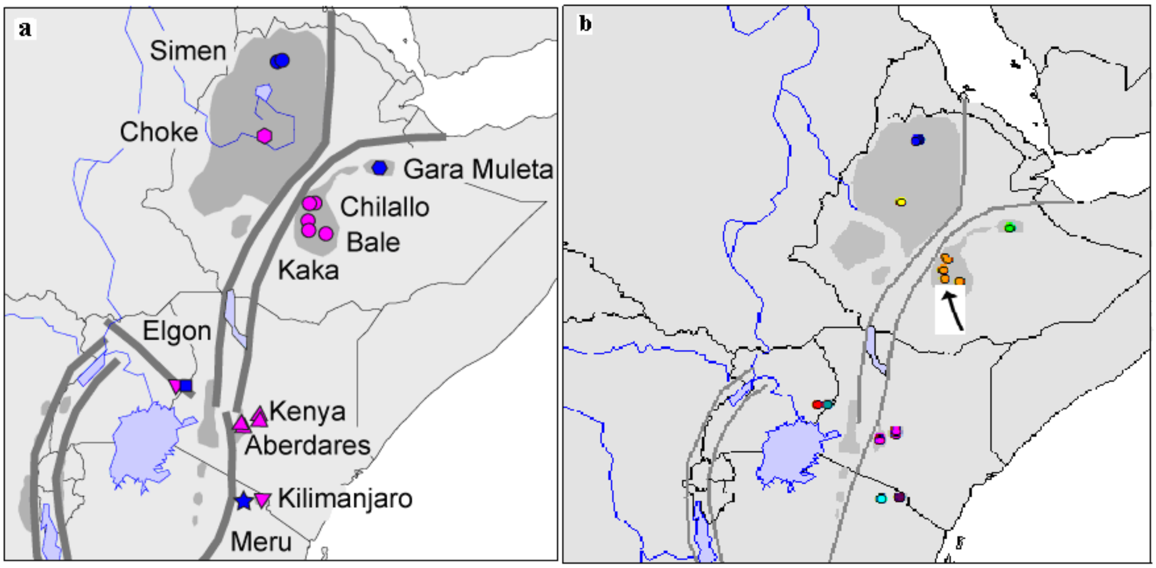

Fig. 6.10 (**a**) Sampling localities and distribution of genetic groups in *A. alpina* as determined by Structure analysis of the AFLP dataset. Colors identify main groups and shape of symbols identifies subgroups. (**b**) The nine identified genetic groups with BAPS from individual samples of *A. alpina* AFLP dataset. The arrow indicates three individuals of a population from Chilallo which were attributed to the same cluster as the populations from Gara Muleta. The dark grey lines indicate the Great Rift Valley and the grey shaded areas depict highlands (rough contour of areas above 2000 m).

BAPS is a program to infer the optimal number of clusters as well as the cluster in which each individual belongs, and in this analysis, it identified nine distinct groups or clusters. These clusters are identical to the Structure subgroups, except that Kilimanjaro was separated from Elgon (the difference was the subdivision of the group containing one population from Elgon and the populations from the Kilimanjaro, Fig. 6.10 b). Except for the three divergent individuals from Chilallo, which were assigned to the group from Gara Muleta, all individuals sampled in one locality were attributed to the same cluster.

6.2.2 *S. merjamie*

The AFLP analysis of 217 individual samples from 31 populations of *S. merjamie* resulted in 225 or 81.12 % polymorphic markers with 1.7 % error rate and reproducibility of above 99 %. The results of the analysis are presented below.

Principal coordinate analysis

Two different principal coordinates (PCOs) were plotted. The first PCO plot was for populations belonging to all the mountain systems of Ethiopia and tropical East Africa and revealed highly divergent groups, which co-occurred in some localities. Thus, the first axis (45.7 % of variation) separated genetically diverged "cryptic species" from Bale, Simen and Kaka from the rest of the mountains (Fig.6.11). The second axis (13.5 % of variation) separated tropical East African Mountains, *i.e.*, Mt Kilimanjaro, Meru, and Aberdares and Kenya, with the exception of some populations from Elgon-Cherangani ranges (Fig.6.11).

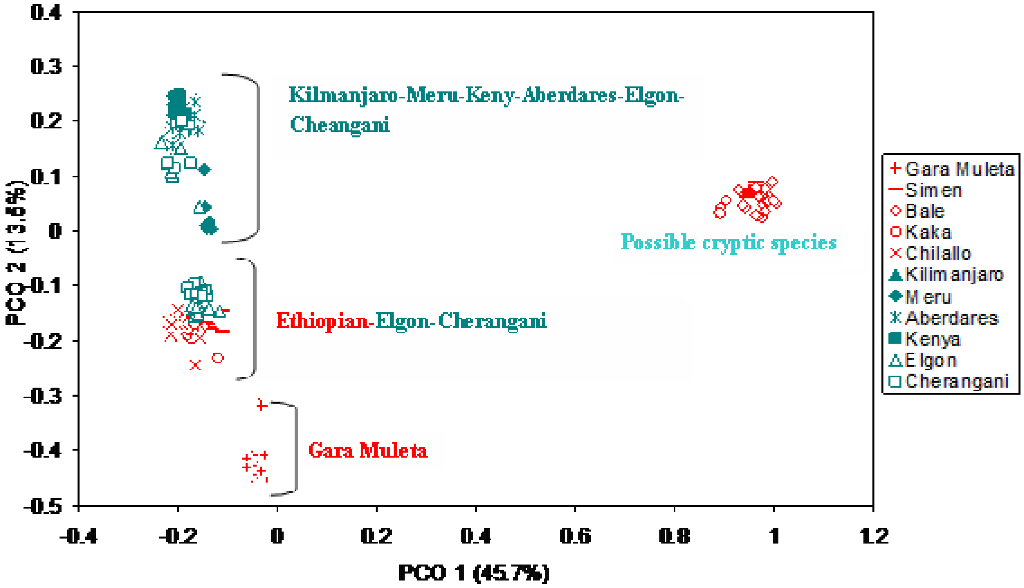

Fig.6.11 Principal Coordinate Analysis for *S. merjamie* samples from all study regions (Axes: 1 and 2).

Populations from most Ethiopian mountains (Simen, Kaka, Bale and Chilallo) clustered with plants from Elgon-Cherangani. With the exception of very few individuals from Simen which mixed with the southern mountains, the third axis separated (7.7 % of variation) the tropical East African populations from the Ethiopian highlands (Fig.6.12).

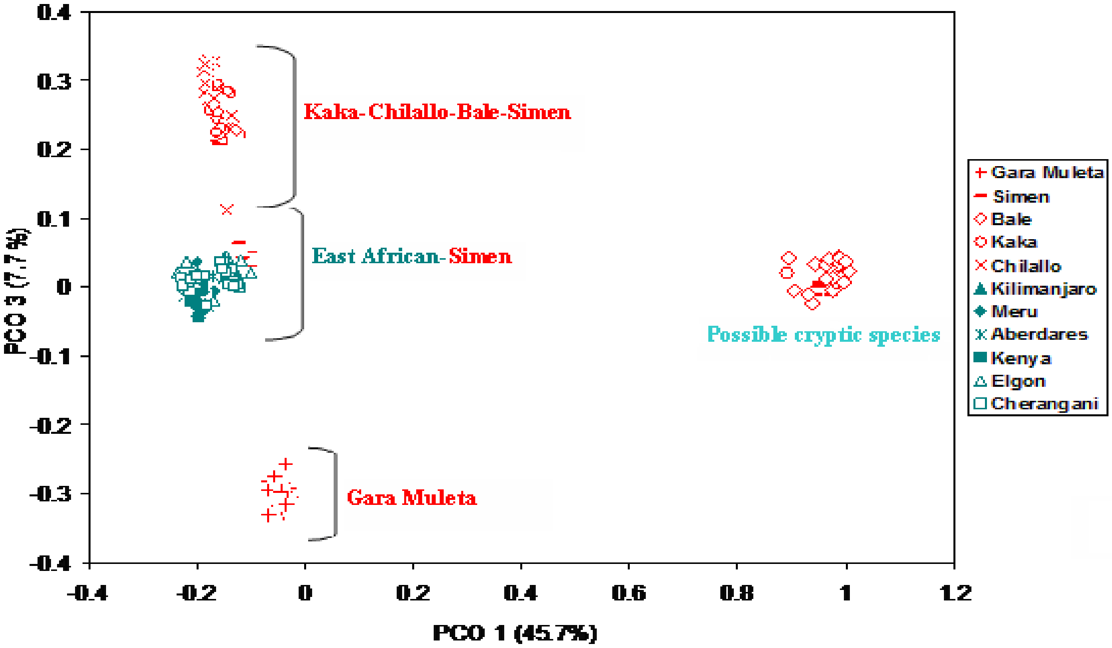

Fig.6.12 Principal Coordinate Analysis for *S. merjamie* samples from all study regions (Axes: 1 and 3).

In the second PCO plot without the ‘cryptic species’, the populations of tropical East African Mountains tend to cluster together with some linking elements from the Simen Mountains (Fig.6.13). In both PCO plots, the populations of Gara Muleta (27.5 % of variation) were distinctly isolated from the rest of the mountain systems and had the most divergent position (Fig.6.11 and Fig.6.13).

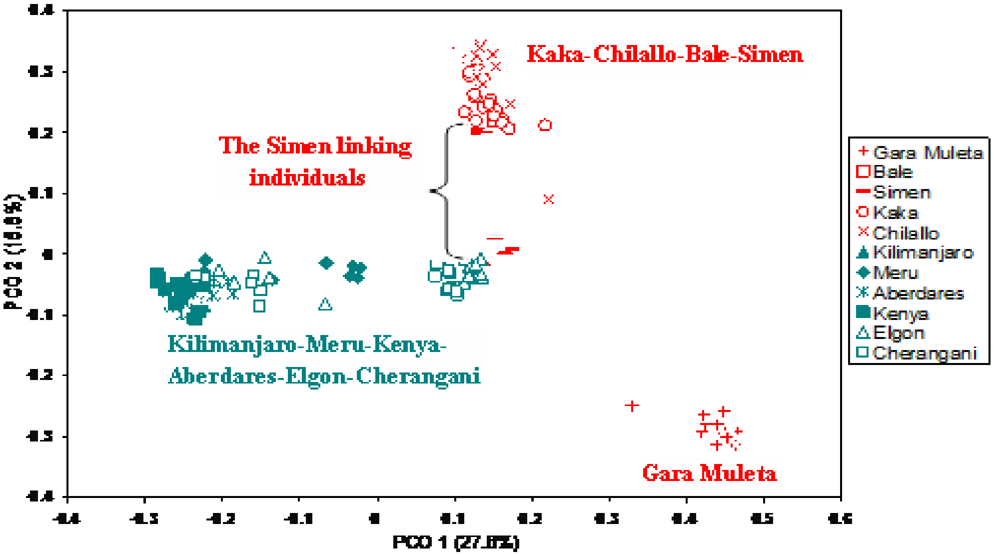

Fig. 6.13 Principal Coordinate Analysis of *S. merjamie* without the highly divergent plants of Bale, Simen and Kaka (Axes: 1 and 2).

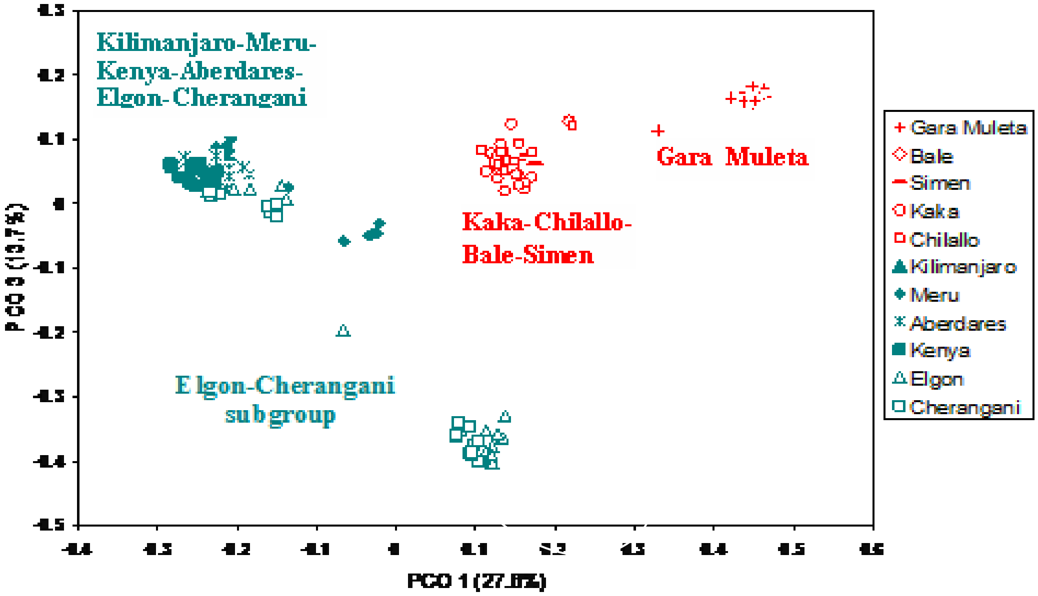

Fig.6.14 Principal Coordinate Analysis of *S. merjamie* without the highly divergent plants of Bale, Simen and Kaka (Axes: 1 and 3).

Neighbor-joining analysis

In the Neighbor-joining tree (Fig.6.15), the ‘cryptic species’ has formed a distinct clade which corresponds to the first PCO plots (Fig. 6.11) and revealed a remarkable relationship among them. When this ‘cryptic species’ is excluded from analysis, the PCO (see Fig.6.13 and Fig. 6.14) and the resulting N-J tree (Fig. 6.16) appears largely congruent.

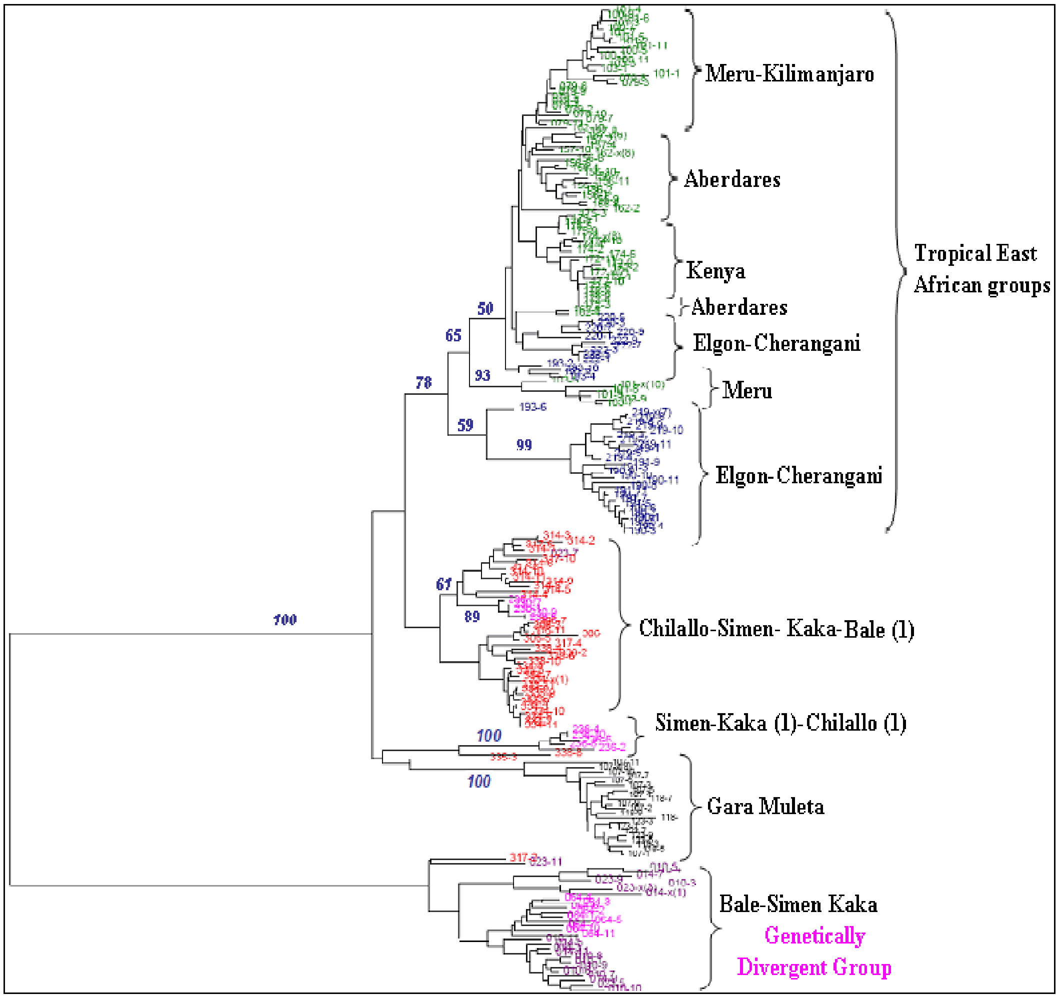

Fig. 6.15 A mid-point rooted neighbor-joining tree from AFLP data based on the Nei and Li (1979) pair-wise distance method indicating the highly genetically divergent individuals of *S. merjamie* cryptic species from Bale, Simen and Kaka mountains of Ethiopia. Bootstrap values above 50% that estimated from 1000 replicates are indicated.

Gene diversity and analysis of molecular variance

As indicated in Table 6.8, gene diversity analysis revealed that the highest average heterozygosity was in Simen (0.389) and the lowest is from Chilallo (0.007) in the presence genetically divergent individuals (Table 6.8). On the other hand, when the data were analyzed after excluding the most divergent samples, big value of diversity was still recorded from Simen (0.117), while the lowest diversity in the latter case was from Mt Kilimanjaro (0.023; Table 6.9). No remarkable difference could be observed among other mountains.

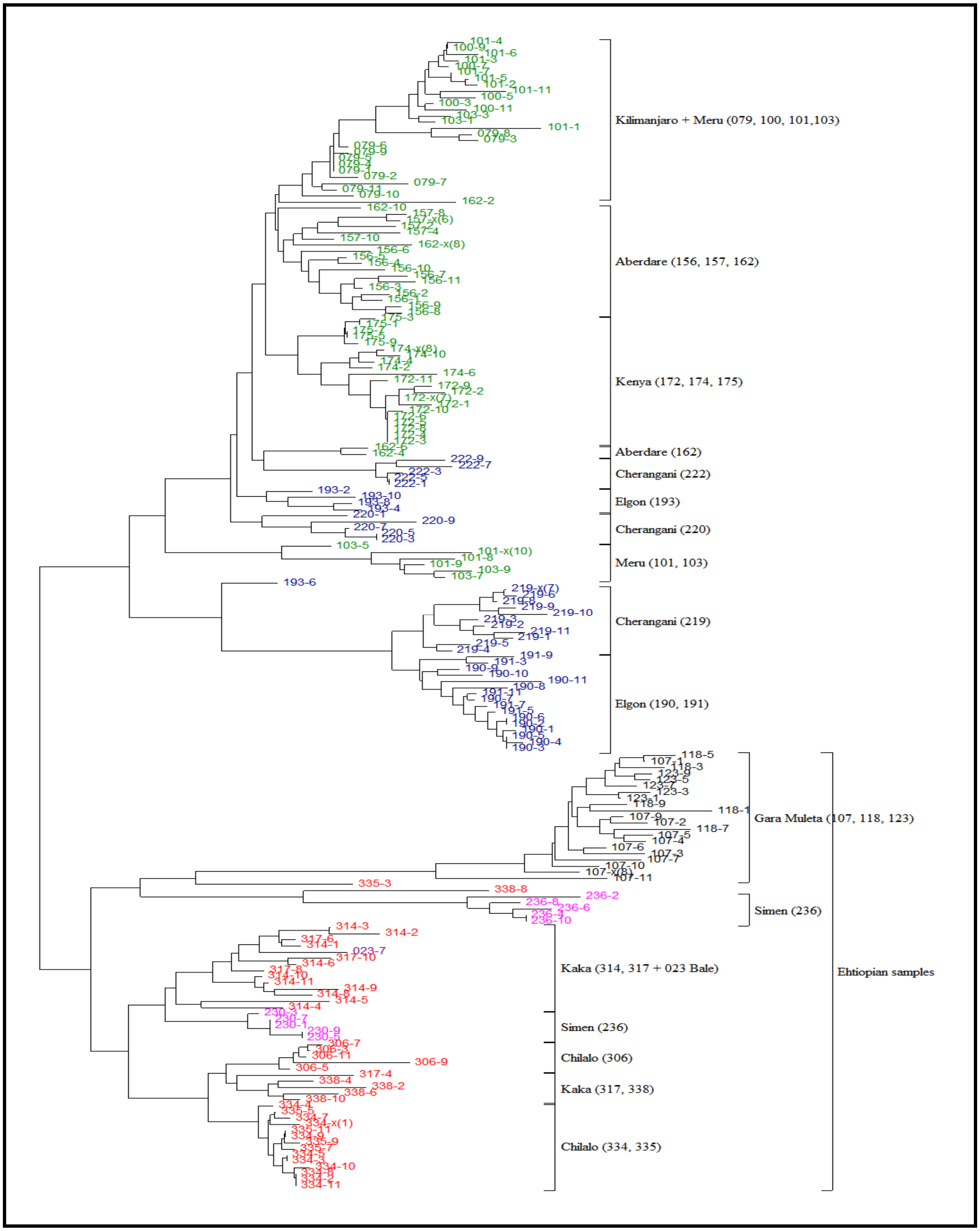

Fig.6.16 A mid-point rooting neighbor-joining tree following the Nei and Li (1979) method from AFLP data of *S. merjamie* sampled populations from Ethiopian and East African mountain massifs without genetically divergent individuals from Bale, Simen and Mt Kaka.

Table 6.8 Average intra-population gene diversity of *S. merjamie*.

Region	No. of individuals	Proportion of variable markers	Average gene diversity
Aberdares	23	0.240	0.055
Bale	23	0.769	0.146
Cherangani	25	0.324	0.108
Chilallo	5	0.018	0.007
Elgon	26	0.276	0.093
Gara Muleta	24	0.364	0.139
Kaka	13	0.204	0.052
Kenya	23	0.280	0.102
Kilimanjaro	11	0.102	0.030
Meru	24	0.409	0.124
Simen	20	0.880	0.389

Table 6.9 Average intra-population gene diversity of *S. merjamie* without the most divergent samples

Region	No. of individual	Proportion of variable markers	Average gene diversity
Aberdares	21	0.204	0.050
Cherangani	21	0.267	0.100
Chilallo	20	0.160	0.031
Elgon	21	0.244	0.068
Gara Muleta	21	0.187	0.041
Kaka	19	0.253	0.072
Kenya	21	0.129	0.030
Kilimanjaro	11	0.089	0.023
Meru	21	0.218	0.060
Simen	10	0.240	0.117

The regional gene diversity per population is very high in Bale (0.320) and Kaka (0.282), and too low in Kenya 0.006 (Table 6.10). However, when Bale is excluded from the analysis, the diversity became high on Mt Meru 0.065 (Table 6.11) in Tanzania. In Structure groups, the average gene diversity was relatively high in Simen-Chilallo-Kaka group (0.069) and low in Simen subgroup 0.025 (Table 6.12 and Fig. 6.17). Application of AMOVA to estimate genetic differentiation showed that the largest proportion of genetic variation was found among groups at 68.5%, while within population the variation was 31.5% (Table 6.13).

Table 6.10 Gene diversity (average number of pair-wise differences) per population of *S. merjamie*

Region	Population number	No. of individual	Proportion of variable marker	Gene diversity
Aberdadares	AFR 156	11	0.089	0.031
	157	5	0.067	0.033
	162	5	0.133	0.060
Bale Mts	AFR 010	10	0.258	0.087
	014	6	0.204	0.089
	023	5	0.707	0.320
Cherangani Hills	AFR 219	11	0.071	0.029
	220	5	0.076	0.034
	222	5	0.040	0.018
Mt Chilallo	AFR 306	5	0.044	0.019
	334	10	0.031	0.009
	335	5	0.084	0.035
Mt Elgon	AFR 190	11	0.089	0.024
	191	5	0.049	0.024
	193	5	0.116	0.052
Gara Muleta	AFR 107	11	0.147	0.043
	118	5	0.076	0.036
	123	5	0.044	0.021
Mt Kaka	AFR 314	10	0.120	0.043
	317	5	0.667	0.282
	338	5	0.124	0.055
Mt Kenya	AFR 172	11	0.044	0.010
	174	5	0.053	0.024
	175	5	0.013	0.006
Mt Kilimanjaro	AFR 079	11	0.089	0.023
Mt Meru	AFR 100	5	0.031	0.014
	101	11	0.196	0.065
	103	5	0.116	0.063
Simen Mts	AFR 004	9	0.209	0.064
	230	5	0.018	0.009
	236	5	0.058	0.025

Table 6.11 Gene diversity (average number of pair-wise differences) per population of *S. merjamie* excluding the highly divergent samples from Mt Kaka, Bale and Simen Mountains

Region	Population number	No. of individual	Proportion of variable markers	Gene diversity
Aberdadares	AFR 156	11	0.089	0.031
	157	5	0.067	0.033
	162	5	0.133	0.064
Cherangani Hills	AFR 219	11	0.071	0.029
	220	5	0.076	0.034
	222	5	0.040	0.018
Mt Chilallo	AFR 306	5	0.044	0.019
	334	10	0.031	0.009
	335	5	0.084	0.035
Mt Elgon	AFR 190	11	0.089	0.024
	191	5	0.049	0.024
	193	5	0.116	0.052
Gara Muleta	AFR 107	11	0.147	0.043
	118	5	0.076	0.036
	123	5	0.044	0.021
Mt Kaka	AFR 314	10	0.120	0.043
	317	4	0.124	0.064
	338	5	0.124	0.055
Mt Kenya	AFR 172	11	0.044	0.010
	174	5	0.053	0.024
	175	5	0.013	0.006
Mt Kilimanjaro	AFR 079	11	0.089	0.023
Mt Meru	AFR 100	5	0.031	0.014
	101	11	0.196	0.065
	103	5	0.116	0.065
Simen Mts	AFR 230	5	0.018	0.009
	236	5	0.058	0.025

Table 6.12 Average gene diversity in the seven Structure groups of *S. merjamie*. Group represents the Structure groups, n stands for the number of individuals for each group, and category indicates the colour plates differentiating the Structure groups, the proportion of polymorphic markers and the average gene diversity.

Group	Category	n	Proportion of variable markers	Average gene diversity
Simen-Chilallo-Kaka	🟨	44	0.324	0.069
Meru	🟩	21	0.218	0.059
Gara Muleta	🟦	21	0.187	0.041
Kilimanjaro-Aberdares-Elgon-Cherangani	🟪	47	0.351	0.064
Kenya	🟩	21	0.129	0.029
Elgon-Cherangani	🟥	27	0.156	0.038
Simen	🟧	5	0.058	0.025

The gene diversity and variable markers of *S. merjamie* from all the groups were represented in a histogram (Fig. 6.17) in order to more clarify the relationship between these parameters. It clearly demonstrates that there is a direct correspondence between the proportions of variable markers and average gene diversity.

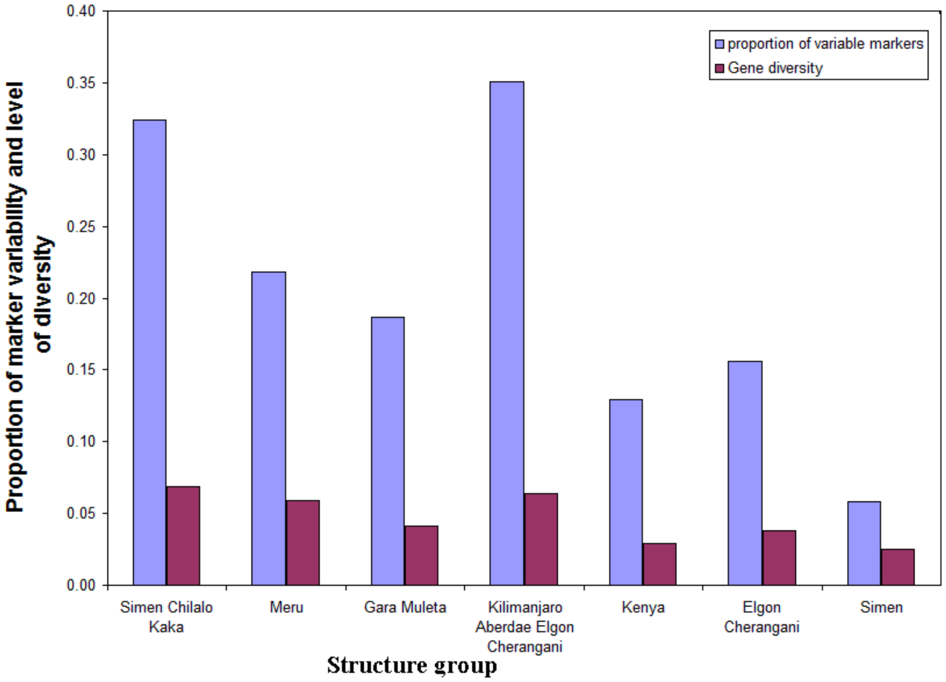

Fig.6.17 Gene diversity and proportion of variable markers of *S. merjamie* among the seven Structure groups.

Table 6.13 Genetic differentiation estimated with AMOVA among seven major Structure groups of *S. merjamie* that suggested by the Structure analysis. Differentiation at all levels was significant at p≤0.0001.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	6	2031.91	13.08	68.51
Within populations	179	1076.55	6.01	31.49

Significance of differentiation was estimated from 10 000 permutations using the software Arlequin 3.01

Genetic mixture analyses

The results of the genetic mixture analyses confirmed the absence of clear regional structuring of the sampled populations of *S. merjamie*. After excluding the ‘cryptic species’, Structure analysis also revealed seven highly mixed groups (Table 6.12). However, BAPS identified eight but regionally intermingled groups in which at least three of the divergent groups corresponded with the Structure groups, *i.e.*, Meru, Gara Muleta and Elgon-Cherangani. Moreover, similar to the PCO, the BAPS findings were also strongly consistent with the N-J analysis, where the ’cryptic species’ still excluded (Fig. 6.18).

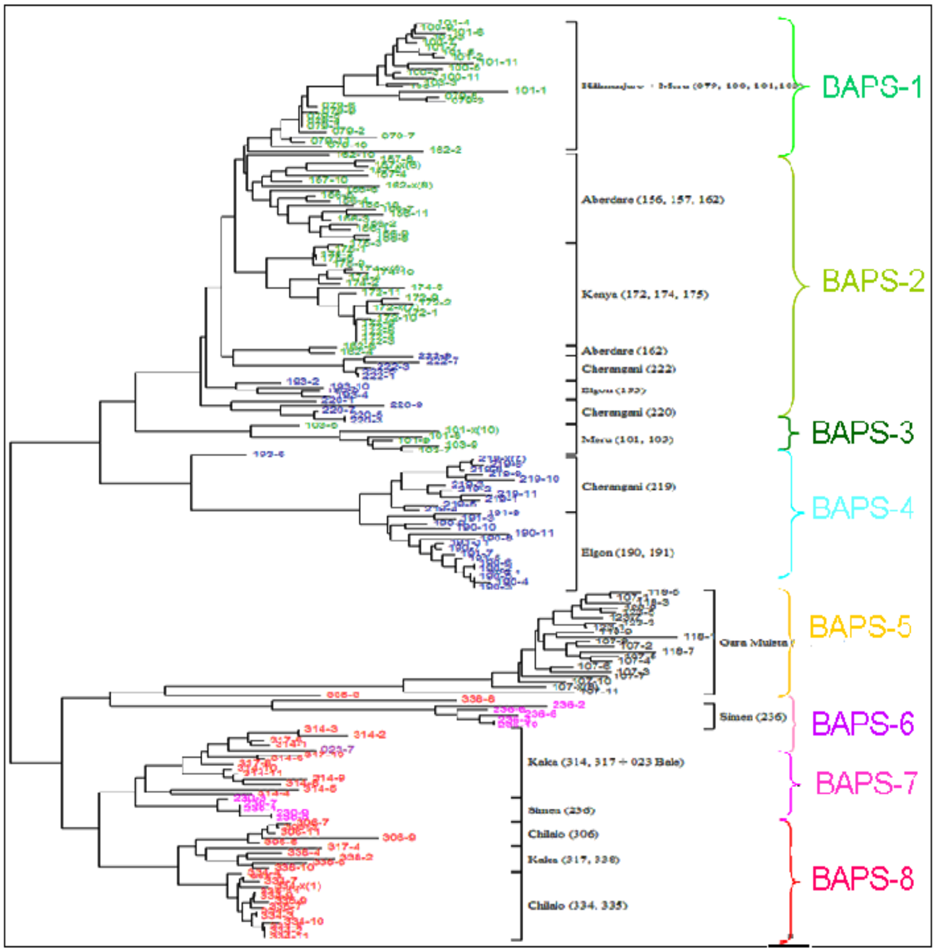

Fig. 6.18 Neighbor-joining tree and the corresponding BAPS analysis of *S. merjamie* from the AFLP dataset.

6.2.3 *C. afromontanum*

The AFLP analysis of 187 individual samples from 26 populations of *C. afromontanum* in 10 mountain systems of Ethiopia and tropical East Africa generated 180 or above 96% polymorphic DNA markers using 38 replicates with an error rate of only 1.5%. The resulting phylogeographic structure of this species was very much complicated, and it is not convenient to calculate diversity per mountain region. However, intra-populations gene diversity for the five identified Structure groups and gene diversity from average number of pair-wise differences of individuals per population from each mountain region were calculated. Moreover, to avoid extreme subdivision of populations belonging to the same mountain region, individuals in a population that belonged to another group (as in Aberdares and Meru; 141-4 and 092-10, respectively), were excluded.

Principal coordinate analysis

The PCO plots divided the samples into about three geographically very mixed groups (Fig. 6.19): Group-1 (Simen-Gara Muleta-Bale-Chilallo-Meru-Elgon-Cherangani); Group-2 (Aberdares-Cherangani); and Group-3 (Kenya-Elgon-Meru-Aberdares-Bale-Kaka-Chilallo). That is, the first axis (31.9% of variation) separated most samples of Group-1 from Group-2 and 3. All samples from Simen and Gara Muleta were entirely grouped under the first cluster (Group-1). It appears that the resulting divisions of the first axis are the most divergent groups.

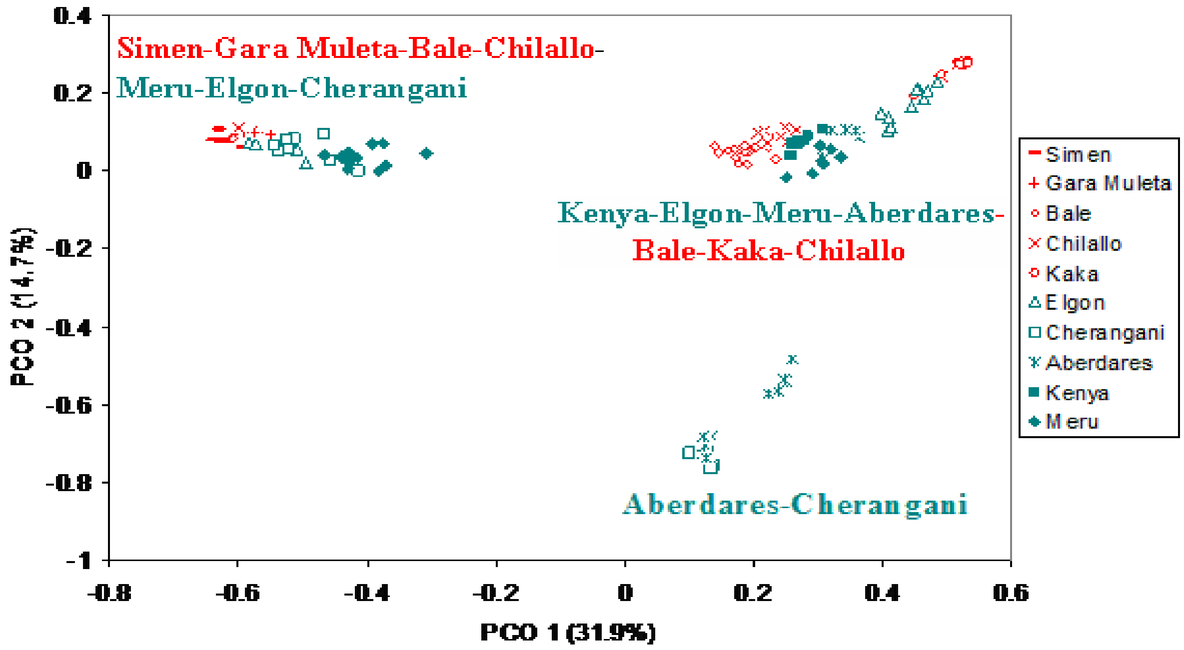

Fig. 6.19 Principal Coordinate Analysis of *C. afromontanum* (Axes: 1 and 2).

The second axis separated most samples of Aberdare and few of Cherangani Hills from the other mountain systems of tropical East Africa (14.7% of variation). On the other hand, axis two separated a third group consisting only Aberdares and Cherangani. However, axis three showed incomplete division of the large group of the south and the north samples into two where both of these are found on Ethiopia and tropical East African mountains. Note that a few Chilallo and Aberdare individuals are found as a linking element (Fig. 6.20).

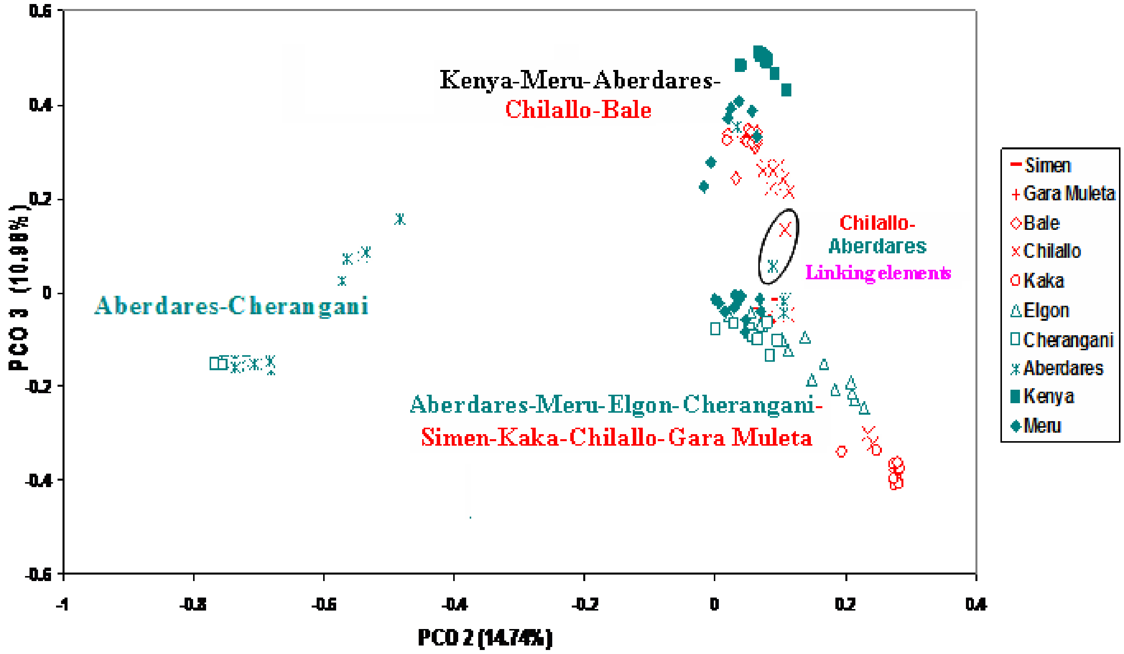

Fig. 6.20 Principal Coordinate Analysis of *C. afromontanum* (Axes: 2 and 3).

Neighbor-joining analysis

In the neighbor-joining-analysis, mid-point rooting was generally consistent with the clusters already identified by the PCO analyses.

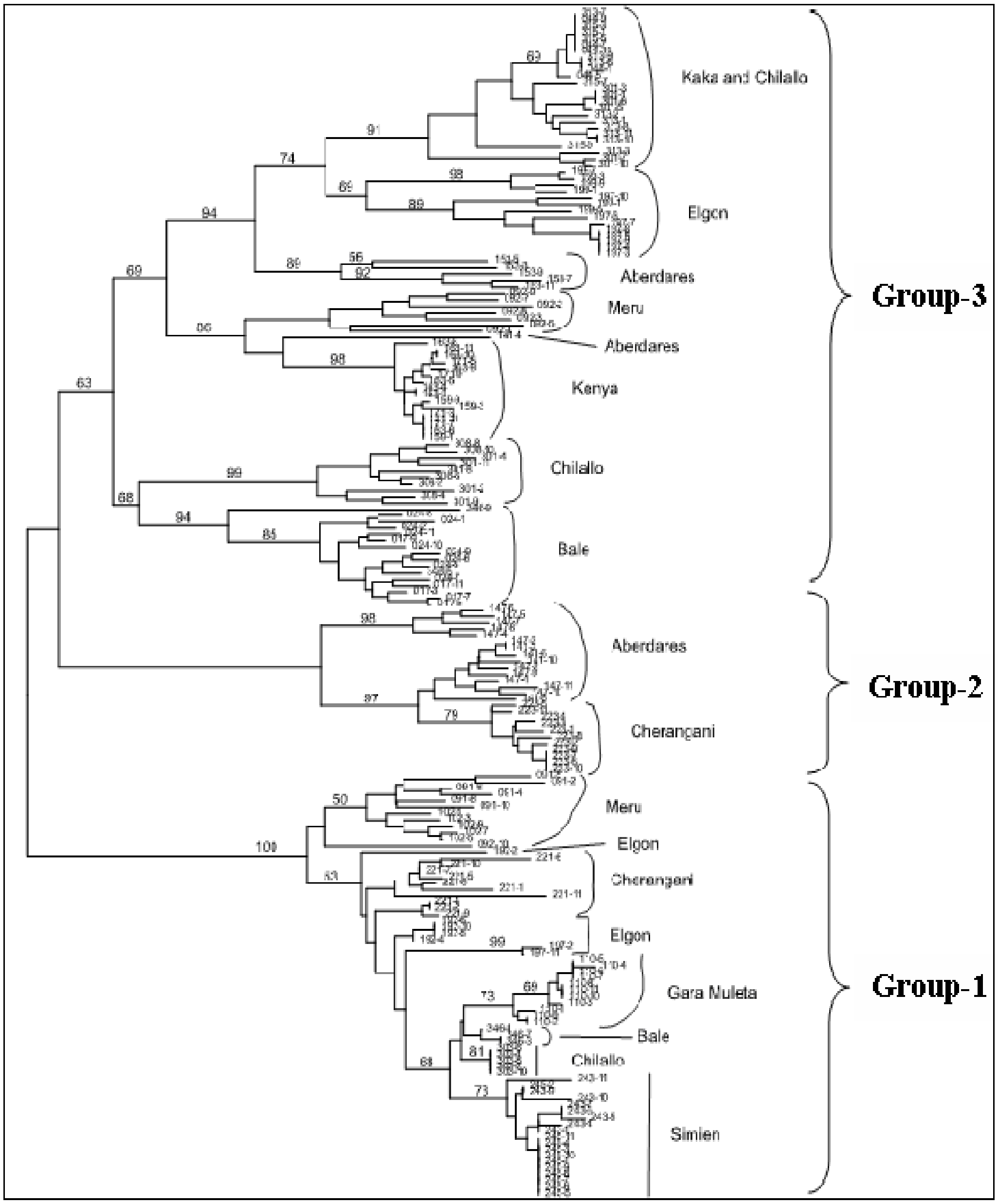

Fig. 6.21 Neighbor-joining tree of *C. afromontanum* samples based on Nei and Li’s (1979) distances between individual AFLP markers. Trees were mid-point rooted. Bootstrap values estimated from 1000 replicates are indicated. Group-1, Group-2, and Group-3 are corresponding to the PCO analysis (see Fig. 6.19).

Genetic diversity and AMOVA

The highest value of gene diversity (average number of pair-wise differences) was observed in individuals from Mt Meru (0.127) and very low gene diversity has characterized those from Simen Mts and Gara Muleta. Relatively low genetic diversity was also recorded in Chilallo, Bale and Elgon populations (see Table 6.14). In the five Structure groups, the average intra-population gene diversity was relatively high in Chilallo-Bale group (0.049, Table 6.15) and followed by Aberdares-Cherangani (0.047). As is the case with AMOVA, the lowest genetic diversity was again recorded for the group comprising the Simen and Gara Muleta populations (0.014) by Structure analyses. It is to be noted that genetic differentiation following AMOVA was found to be 64.13% among the five main groups, 5.12% within groups and 12.87% variation within populations of a group (Table 6.16).

Table 6.14 Gene diversity (average number of pair-wise differences) per individual of each population of *C. afromontanum*. Mountains or the sampling regions, number of population, total number of individuals for each region, and gene diversity are indicated

Region	Population number	No. of individual	Gene diversity
Aberdares	AFR 141	4	0.031
	147	11	0.091
	153	5	0.061
Bale Mts	AFR 017	5	0.034
	024	9	0.040
	346	5	0.044
Cherangani Hills	AFR 221	10	0.029
	223	11	0.019
Mt Chilallo	AFR 301	11	0.038
	303	5	0.000
	308	5	0.033
Mt Elgon	AFR 192	5	0.014
	197	11	0.017
	199	5	0.046
Gara Muleta	AFR 110	11	0.010
Mt Kaka	AFR 044	5	0.004
	313	9	0.032
	315	5	0.026
Mt Kenya	AFR 159	3	0.011
	163	8	0.013
	171	5	0.008
Mt Meru	AFR 091	6	0.042
	092	7	0.127
	102	5	0.021
Simen Mts	AFR 243	8	0.017
	245	11	0.001

Table 6.15 Average gene diversity in the five structure groups of *C. afromontanum* with: Structure groups, n represents the number of population for each group, gene diversity and SD stands for standard deviation.

Group	n	Average gene diversity ± SD
Kaka-Chilallo-Elgon-Aberdares	7	0.031 ± 0.018
Meru-Kenya	4	0.040 ± 0.058
Chilallo-Bale	5	0.049 ± 0.021
Aberdares-Cherangani	3	0.047 ± 0.038
Simen-Gara.Muleta-Chilallo-Bale-Cherangani-Elgon-Meru	10	0.014 ± 0.013

Table 6.16 Genetic differentiation calculated using AMOVA among the five Structure groups of *C. afromontanum*

Source of variation	d.f	Sum of squares	Variance component	Percentage of variation
Among groups	4	2173.16	14.27	64.13
Among populations within groups	24	824.73	23.00	5.12
Within populations	156	446.91	2.86	12.87

Differentiation at both levels was significant with $p<0.0001$ and d.f. represents degree of freedom. Significance of differentiation was estimated from 10 000 permutations using the software Arlequin 3.01.

Genetic mixture analyses

Patterns identified by genetic mixture analyses were not obvious. Similarly, the results obtained from Structure were not reliable due to the absence of a unique value for any K, which in turn indicated that the similarity among the groups was too low for all K values. BAPS analysis of population structure identified nine clusters (Fig. 6.22), corresponding to the groups differentiated by the N-J analysis, except that some Aberdares samples were intermixed with the most divergent group (see the arrow).

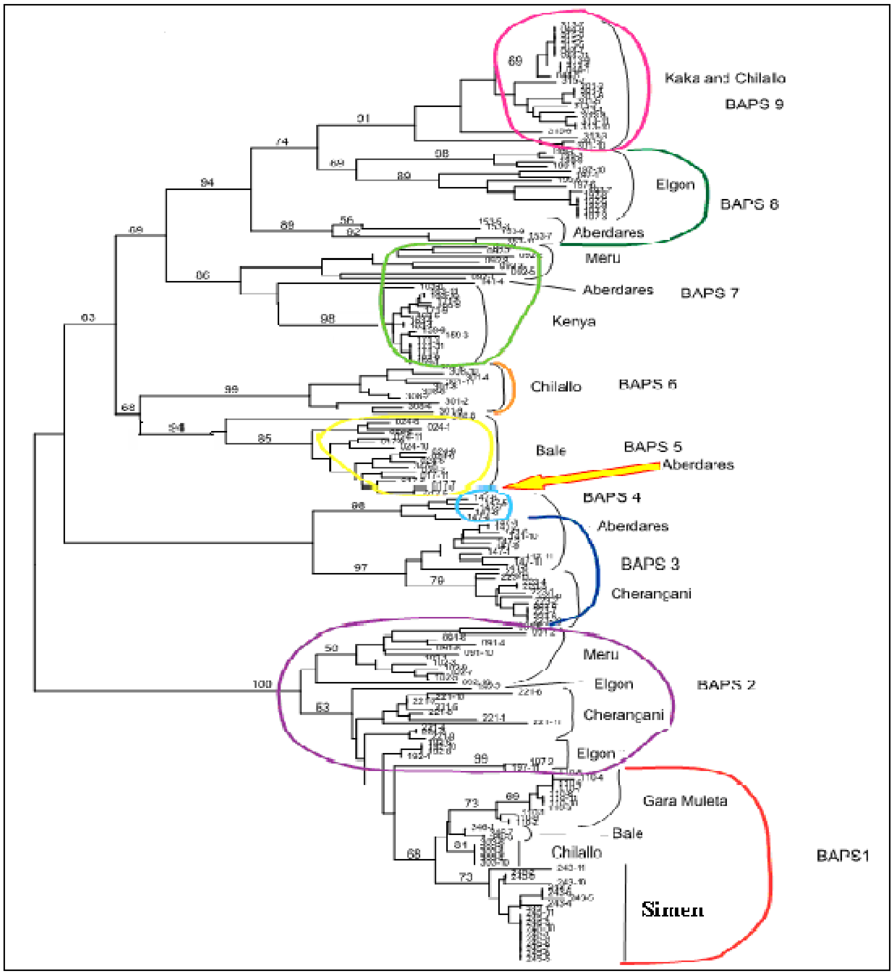

Fig. 6.22 The encircled groups with different colours represent the BAPS outcome corresponding to the N-J analysis of individuals from *C. afromontanum*. The yellow arrow indicates one of the subgroup of the highly divergent populations of Aberdares in BAPS and N-J analyses.

Analysis from Structure at K=5 found five Structure groups: Group-1 (G-1) is Simen-Gara Muleta-Chilallo-Bale-Cherangani-Elgon-Meru; G-2 is Chilallo-Bale; G-3 is Chilallo-Kaka-Elgon-Abredares; G-4 is Cherangani-Aberdares; and eventually G-5 is Kenya-Meru as shown in the map (Fig. 6.23 a). These groups are very consistent with the N-J tree and the results of BAPS although the latter subdivided several groups. On the other hand, the three Structure groups that were obtained from Structure at K=3 (see Fig. 6.23 b) were strongly consistent with the three groups revealed by axes 1 and 2 of PCO analyses.

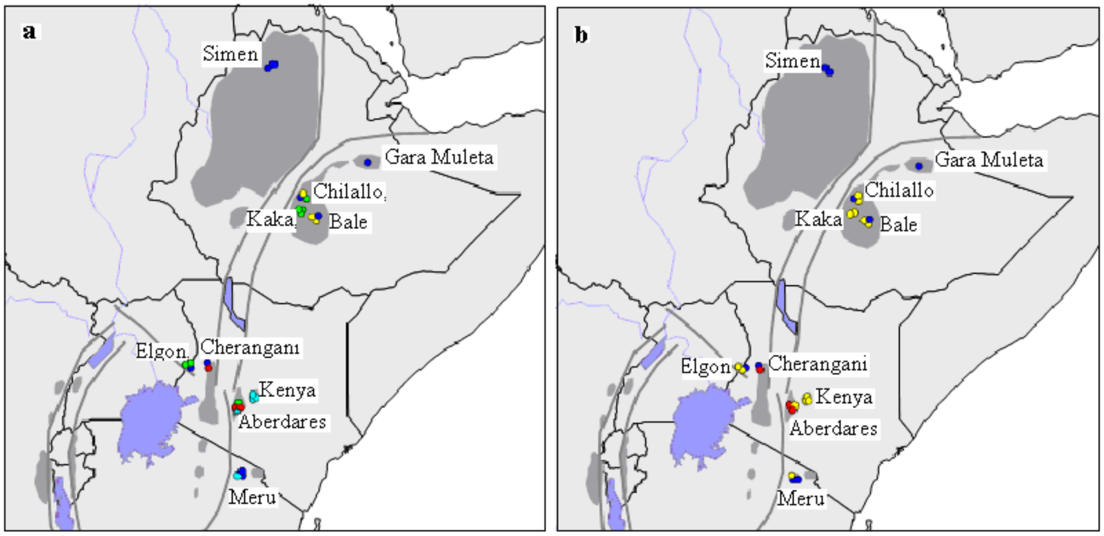

Fig. 6.23 The sampling localities and the distribution of genetic groups obtained with the Structure 2.1 from the AFLP dataset of *C. afromontanum*. **(a)** The five structure groups obtained from K=5, six out of 10 runs: G-1 is consisting of Simen-Gara Muleta-Chilallo-Bale-Cherangani-Elgon-Meru; G-2 is Chilallo-Bale; and G-3 is Cherangani-Aberdares; G-4 is Chilallo-Kaka-Elgon-Abredares; and G-5 is Kenya-Meru-Aberdares. **(b)** The three structure groups obtained from K=3, four out of 10 runs corresponding the PCO divisions in figure 6.19: G-1 is the blue colour consisting of Simen-Gara Muleta-Chilollo-Bale-Elgon-Cherangani-Meru; G-2 is the yellow colour comprising Chilallo-Bale-Elgon-Kenya-Aberdares-Meru; and G-3 is Aberdares-Cherangani with the red colour.

CHAPTER 7 DISCUSSIONS

7.1 *A. alpina*

7.1.1 Pleistocene colonization of tropical East African mountain systems by *A. alpina*

In the previous study, the haplotype distribution and the cpDNA network analyses give clear evidence that East Africa was colonized by two independent lineages, which originated in the Balkans/Middle East (Koch et al., 2006). Subsequently, they spread further south via the coastal mountain ranges of the Arabian Peninsula and by intermountain dispersal within East Africa. It is likely that haplotypes of the African clade were the first to reach East Africa for several reasons: (**1**) the clade has the highest diversity in the region and extends further to the south, (**2**) haplotype 19 has also an internal position in the network, possibly indicating its older age, and (**3**) Later, the Asian lineage, *i.e.*, with its derived haplotype 04, migrated southwards and probably reached Ethiopia.

Our current results obtained from various analyzing approaches were also confirmed the presence of two divergent lineages of *A. alpina* in Africa in consistent with the above finding reported by Marcus Koch et al. (2006). In addition to the widespread African lineage, which is restricted to eastern Africa, some haplotypes of the Asian lineage were also detected, but confined largely to the northernmost and the easternmost mountains in this study (Simen Mts and Gara Muleta in Ethiopia, respectively). Interestingly, the Simen Mts and Gara Muleta are situated on opposite sides of the Great Rift Valley, suggesting either two separate immigration events from the Arabian Peninsula or recent long-distance dispersal of the Asian lineage.

The Simen Mts and Gara Muleta are the localities closest to the Arabian Peninsula (Fig. 2.1). Total number of the observed haplotypes belonging to the Asian lineage was only two (haplotypes 04 and G), which differed by one substitution (Fig. 6.1). The diversity of this Asian clade in Africa was thus considerably lower than for the African clade (nine haplotypes). All together, the occurrence of this clade close to the Arabian Peninsula and its low diversity support the conclusion of Koch et al. (2006) regarding the arrival of this clade to Africa.

The African lineage of *A. alpina* was widespread and represented in all localities, except Gara Muleta, and within this lineage a subdivision into at least two phylogeographic groups (group A and group 19) were found with largely distinct geographic distribution. These groups were found on opposite sides of the Rift Valley in Ethiopia (except for haplotype C which also occurred in the Simen Mts), and extended southwards on their respective sides. This pattern, especially the distinct groups in the Ethiopian highlands on different sides of the Rift Valley, is consistent with the expectations assuming a larger and more continuous afro-alpine habitat during cooler periods of the Pleistocene (Messerli et al., 1977; Flenley, 1979; Gottelli et al., 2004). However, group 19 in the south extended across the Rift Valley to Mt Meru and Mt Kilimanjaro on the eastern side, and one haplotype of this group also occurred further north in Mt Kenya, where it co-occurred with the group A. Thus, despite the distinct ranges occupied by the three phylogeographic groups, secondary contacts were observed both in the south (Mt Kenya) as well as in the north (Simen Mts, Fig. 6.1). These secondary contacts were each represented by one haplotype found at a locality dominated by another group. It is interesting to notice that the three haplotypes forming the secondary contacts were derived haplotypes, contrary to what would have been expected from the observed haplotype frequencies. Each of them occurred only in one or two geographically rather close locations.

7.1.2. Phylogeography of *A. alpina* in tropical East African Mountains

A. cpDNA haplotypes

Both the G_{ST}/N_{ST} test and the NCPA showed that there was a significant phylogeographic structure within Africa. The G_{ST}/N_{ST} test detected a significant association between phylogeny and geography only between the Asian and the African clade, and not between the two phylogeographic groups within the African clade, probably because of a considerable amount of homoplasy in length variation within this clade. The NCPA indicated that the significant phylogeographic pattern among the three main clades in the nested design of the network could be explained by past population fragmentation and/or long-distance colonization. For the distinction between the Asian and the African clade, past population fragmentation was suggested by the long branch separating the two clades on the network. This is consistent with the interpretation of Koch et al. (2006) that the two clades have evolved in different regions, and co-occur in Africa as a result of secondary contact resulting from a later immigration of haplotypes of the Asian clade. According to Templeton’s (2004) inference key conclusion, the low divergence between the two African clades (3-1 and 3-2 in the nested design of figure 6.4) indicated colonization or perhaps recent fragmentation.

B. AFLP data

Compared to the Northern Atlantic groups, the Ethiopian and tropical East African phylogeographic groups of *A. alpina* were highly differentiated and geographically localized

(Ehrich et al., 2007). However, unlike the mountains of central Europe, the structure was not strictly consistent with geography. Two divergent groups were observed on Mt Elgon, and a few divergent individuals from Chilallo were also intermingled with the populations of the eastern isolated mountain in Ethiopia. Despite the long isolation of the East African “sky islands’ plant populations from their European counter-parts, almost all Ethiopian and tropical East African *A. alpina* populations harbored genetic diversity at a level similar to that of the populations in the European mountain massifs (see also Ehrich et al., 2007).

The phylogeographic structure of *A. alpina* populations in the African mountains was highly fragmented, but the local populations harbored much genetic diversity as could be expected from isolated refugial populations. In another words, the fragmented “sky islands” of the tropical East African mountains were acting as secluded refugial areas for the few immigrant plant populations, such as *A. alpina* of the northern hemisphere during the past glaciations. This resulted in the maximum differentiation as well as variation among the local populations, indicating that *A. alpina* populations had dispersed very little once they established themselves on the particular mountains (Ehrich et al., 2007). Similar investigations on the same plant species using cpDNA sequencing data showed two very divergent lineages in Africa Mountain massifs, which are the Asian lineage in Simen and Gara Muleta (Ethiopia), and the African lineage in the remaining Ethiopian and East African highlands (Koch et al., 2006; Assefa et al., 2007). These findings are in line with the distinct AFLP genotypes observed in the same localities, *i.e.*, in Simen and Gara Muleta. Moreover, the high AFLP diversity observed in Simen is consistent with the observation that this was the only mountain region, where cpDNA haplotypes from the three lineages were recorded.

C. Phylogroups of cpDNA versus AFLP

Contrary to the cpDNA, the AFLP data suggest that individuals of the Asian lineage dispersed southwards and reached Elgon, where they co-occur (but in different local populations) with plants related to the population from the African lineage on the other side of the Rift Valley in tropical East Africa. The latter belonged to the same Structure group as the population from Mt Kilimanjaro (see Fig. 6.10 a). Comparing genome-wide genetic pattern as provided by AFLP to patterns from cpDNA, which is mainly maternally transmitted, allows distinguishing gene flow mediated by seeds from gene flow mediated by pollen. Because all individuals from Elgon had the same cpDNA haplotype (Assefa et al., 2007), but belonged to two highly divergent AFLP-based genetic groups, indicating pollen from the Asian lineage might have dispersed southward. However, because *A. alpina* is insect pollinated and produces little pollen, this hypothesis can be considered very unlikely. Alternatively, the similarity to all cpDNA haplotypes on Mt Elgon might indicate the phenomenon of cpDNA introgression in the area. The current results from the AFLP analysis also showed that some individuals that are closely related to the populations from Gara Muleta had spread to Mt Chilallo. However, no cpDNA data were available for the three divergent individuals from Chilallo. Thus, dispersal of the small and light seeds by strong winds seems more plausible mechanism for long-distance dispersal among mountain massifs with subsequent introgression of *A. alpina, e.g.*, cpDNA in Elgon into the plants which originated from Simen or Gara Muleta, in tropical East African. Simen is the only mountain region where cpDNA haplotypes from both divergent lineages were recorded (Koch et al., 2006; Assefa et al., 2007), which is consistent with the high AFLP diversity observed in the same region (Table 6.5 and Table 6.6).

7.1.3 Role of the Rift Valley as a phylogeographic barrier in *A. alpina*

The long tropical East African Rift Valley extending from the most northeastern part of Ethiopia through Afar to the southeastern part of Mozambique had significant historical influence on geographical distribution of the African flora and fauna, and had contributed to the shape of their current distribution, especially for migrating species. The effect of its geographical position was investigated by the current study on *A. alpina* in Ethiopian and tropical East African mountain massifs. The significance of the Rift Valley, as a phylogeographic barrier has been repeatedly documented for animals, notably the Ethiopian wolf *Canis simensis* (Gottelli et al., 2004), the ostrich (Freitag and Robinson, 1993) and the wildbeest (Arctander et al., 1999).

To our knowledge, this study is the first to document a similar phylogeographic pattern in plants. Phytogeographic studies have previously documented a stronger link of the Northeastern parts (see Fernandes et al., 2006) and Simen Mountains of Ethiopia to the Arabian Peninsula than to the Bale Mountains (Gillett, 1955; Puff and Nemomissa, 2001), confirming the strong barrier effect of the Rift Valley. However, the Rift Valley had not been an absolute barrier against some dispersal across it as has been inferred from genetic data of the Ethiopian wolf population (Gottelli et al., 2004). The understanding of the Rift Valley as a significant, but permeable, barrier to dispersal may be applicable to afro-alpine plants as well. In Ethiopia, the maximum elevation in the Rift Valley is about 1600m. Thus, assuming that the afro-alpine vegetation belt was 1000-1500m lower during cooler periods in the Pleistocene, it would not have reached the bottom of the valley. The possibility that high-altitude grassland reached the highest parts of the floor of the Rift Valley at some time during the Pleistocene has, however, been suggested by Yalden and Largen (1992). However, the Rift clearly represented distinct and a discontinuity in the alpine habitat for most of the Pleistocene and this could explain the obvious difference between the Northwestern and the Southeastern Highlands in Ethiopia.

The distinct phylogeographic groups observed in the Ethiopian highlands on different sides of the Rift Valley strongly support the existence of a larger and more continuous afro-alpine habitat during cooler periods of the Pleistocene, which, however, was divided by the Rift Valley. In addition, haplotypes belonging to the same group were observed on adjacent Southern mountains (Meru and Kilimanjaro versus Kenya and Aberdares), confirming the assumption of gradual dispersal during periods when the afro-alpine habitat was more widespread than today.

Despite the clear overall phylogeographic structures in African *A. alpina*, that indicate gradual expansion, isolation and rather strong dispersal barriers, the present results suggest several long-distance dispersal events. At least three of these events seem to have enabled the species cross the Rift Valley: **(1)** the Asian lineage: the Simen Mts and Gara Muleta (or two independent immigrations from the Arabian Peninsula), **(2)** phylogeographic group A: from Mt Kaka to the Simen Mts, **(3)** phylogeographic group 19: across the Rift Valley in the south (that the current data do not allow to exclude gradual dispersal over the Rift Valley in the past, a scenario that, however, considered unlikely for ecological reasons). These observations suggest that in addition to more or less gradual or step-wise range expansion during cooler periods of the Pleistocene, long-distance dispersal events have also been important for colonization of isolated mountain tops. These events were not identified by NCPA, confirming that they were not the main factors shaping the genetic pattern that observed today. Long-distance dispersal parallel to the Rift

Valley may also have been necessary for the dispersal of the phylogeographic groups between Ethiopia and the southern mountain massifs.

7.1.4 Demographic history of *A. alpina*

The divergence between the three major lineages of *A. alpina* has been dated to the period of Pleistocene about 500 000 years ago (Koch et al., 2006), which is relatively recent compared to the other previous estimates (Hedberg, 1969 & 1970). Thus, it is likely that the African lineage has experienced several cycles of range expansion and contraction following Quaternary climate changes before reaching its present distribution. The divergence between the two African sublineages was about four times less than between the European and African lineages (1.02 versus 4 base pairs in net number of pair-wise differences between groups, excluding length variation), indicating an age in the order of magnitude of 125 000 years, which is a more recent subdivision. The mismatch distribution is consistent with a population expansion in both phylogeographic groups and the power to reject such a model was low. The estimate for $\tau = 2\mu t$ was 0.368 for group A, indicating an expansion which is twice as recent as for the European lineage ($\tau = 0.7$, Koch et al., 2006). It could not be estimated in a meaningful way for group 19, but the level of variation and the graphs of the mismatch distribution were very similar to that of the eastern group or group A indicating similar ages.

For the European clade, Koch et al. (2006) suggested an expansion predating the Last Ice Age, possibly during the previous Ice Age. For the African lineage, the smaller estimate of τ may indicate expansions on each side of the Rift during the Last Ice Age. Prior to the inferred expansion during the interglacial warm period, *A. alpina* may have been restricted to at least two refugia, one on the western side of the Rift Valley or in the Kilimanjaro-Meru area in the south, and one on the eastern side of the Rift, possibly in Ethiopia. Considering the distribution of diversity within lineages, the refugium of group 19 may have been in the Kilimanjaro-Meru region in the south, and the refugium of group A in Ethiopia. Although these estimates may suggest roughly where in history to place an event, it is important to remember that they are very imprecise and that CIs are large.

7.2 *S. merjamie*

7.2.1 Possible ‘cryptic species’ and a complex phylogeography

Both PCO plots and neighbor-joining analysis revealed a strong structure among the sampled populations of *S. merjamie* from the Ethiopian and tropical East African Mountains (Fig. 6.11/12 and Fig. 6.15). Within the Ethiopian populations, the differentiation of within individuals genetically into very divergent taxon may indicate that similar biological phenomenon could be the case in afro-alpine environment where climate is harsh. In fact, this genetic data should be supplemented by morphological data to reach at robust conclusion. The cleistogamous nature of this taxon may have contributed to observed sharp genetic distinctions of the ‘cryptic species’ by limiting gene flow.

Generally, the phylogeographic structure of *S. merjamie* in the mountain systems of tropical East Africa is very complex. Furthermore, certain populations have shown noticeable regional diversity, *e.g.*, the Simen genetic group (Table 6.8/9) probably indicating a long isolation of the group from the rest of the mountains.

Populations from Gara Muleta formed distinct groups with a relatively low genetic diversity compared to the Simen populations (Table 6.8) which may indicate long time isolation followed by genetic bottleneck or recent divergence of the group from the source populations. The examination of the hierarchical partitioning of genetic variation by AMOVA demonstrated that differentiation amongst the Structure groups was highly significance at $p<0.0001$ and its variance components was 13.08 (Table. 6.13), while the within population variance component was 6.01

with high level of significance. These confirm a clear genetic differentiation of populations among and within populations of a mountain.

An independent analysis of two different regions [*psb*E-*pet*L, and *rpl*32-*trn*L (UAG)] of cpDNA sequence isolated from the same leaf specimens produced very consistent results (Popp et al., in preparation) with PCO. The two main genetically divergent groups recovered by axes 1 and 2/3 PCO plots of the AFLP dataset (Fig. 6.11 and Fig. 6.12) are strongly supported by the cpDNA sequencing results.

7.3 *C. afromontanum*

7.3.1 Phylogeography

The phylogeographic structure of *C. afromontanum* is also very complex and the genetic diversity per population is dominated by very divergent individual populations from most mountain systems (Table 6.15). All plants from Mt Kaka and Mt Kenya belonged to a single genetic group, whereas in the six remaining mountain systems individuals from divergent groups were observed.

The PCO plots roughly divided the populations into three divergent groups. The neighbor-joining analysis also revealed similar patterns of divisions, with several partitions that probably resulted from the highly divergent nature of the sampled populations in most mountain massifs. Thus, it was observed that a single mountain region showed deep differentiation into two or more very small sub-groups (Fig. 6.21). Even though the results from Structure group seem to be unreliable due to the absence of a unique value for any K, one category of the groups (at K=3) divided the populations into three meaningful classes: Group-1, Group-2 and Group-3, which in turn exactly corresponds to the three groups obtained from the PCO plot (Fig. 6.19).

Recently, DNA sequencing results from the two commonly used cpDNA regions, *i.e*., *psb*E-*pet*L and *rpl*32-*trn*L (UAG), Popp et al., in preparation, recovered the three genetically divergent groups following PCO analyses of the AFLP dataset. The clustering of each individual population in various groups and subgroups showed by the PCO and phylogenetic analyses did not have any defined patterns, and the genetic groups obtained do not definitely correspond to geographical distribution. In this study, the divergent lineages of populations of different mountain systems and the observed complexity phylogeogrphic patterns may be attributed polyploidy.

CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

Regarding its migration and colonization history, the results from the cpDNA sequencing analysis suggested the following scenario for *A. alpina* in Ethiopian and East African Mountain systems. During an early glaciation in the Pleistocene, around 500 000 years ago, the first *A. alpina* colonized Africa from the north, possibly from the Middle East via the mountains bordering the Red Sea. A first range expansion was followed by several cycles of range contractions and expansions. During the interglacial warm period, the African lineage survived in two different refugia situated on the western and on the eastern sides of the Rift, in agreement with the recent fragmentation among the two African clades indicated by NCPA. In the following cool period, corresponding to the last glaciations in the northern Hemisphere, the two phylogeographic groups expanded from these refugia following the increasing area of the afro-alpine habitat on each side of the rift, but also occasionally across the Great Rift Valley.

At the same time populations belonging to the Asian clade spread southwards along the mountains bordering the Red Sea and immigrated into northern and eastern mountain massifs of Ethiopia. Thus, this data indicate that the cooler periods of the Pleistocene may have played a crucial role for the dispersal of afro-alpine plants between isolated mountain tops. However, despite the considerable barriers imposed by lowland habitats surrounding the highlands, in particular the GRV, occasional long-distance dispersal also occurred among the isolated ‘sky islands’, possibly by wind or birds.

In general the genetic structure observed in Africa highlights a paradox present throughout this particular study of *A. alpina*. On one hand, high differentiation among geographically close populations (*e.g*. between Meru and Kilimanjaro, between the two populations on Elgon) indicates that once established in a region, *A. alpina* disperses little. However, some genetically defined groups in Africa had disjunctive geographical distributions, suggesting occasional events of long distance dispersal. Altogether, the results strongly suggest variation for traits relevant to dispersal and colonization ability in *A. alpina*.

Comparing the overall analyses of the Africa *A. alpina*, AFLP data revealed much more differentiation among mountain massifs than the cpDNA sequencing data, which distinguished only three phylogeographic groups, of which one was subdivided. This may be explained by the sample size or the relatively low mutation rate, and thus low resolution of the presently analyzed cpDNA markers.

In cpDNA sequencing analysis, Chilallo, Bale and Kaka in Ethiopia, and Kenya and Aberdares in tropical east Africa belonged to the same phylogeographic subdivision, but there were some differences between the two geographical regions for AFLP data profiles, possibly reflecting restricted dispersal over lowlands in recent times. Similarly, whereas populations from Elgon, Kilimanjaro, Meru, and Choke formed the other group within the African lineage for cpDNA data, on the other hand, in the AFLP data Meru and Choke had clearly divergent positions, in accordance with their remote geographical locations within the group. However, contrary to the cpDNA data, the AFLP data placed Kenya and Aberdares rather close to Elgon and Kilimanjaro.

This may indicate some gene flow across the Great Rift Valley, not reflected in the small sample sizes analyzed for cpDNA sequencing.

On the basis of the current analyzed data of *S. merjamie*, no genetic variability showed clear geographical structuring or well defined phylogeographic groups, rather the resulting structures were so complex and found difficult for suggesting any past historical scenario of the species among the Ethiopian and tropical East African mountain massifs.

In another development, few morphological data analysis have been carried out on the basis of very small sample size from the floral and vegetative structures, *i.e.*, the length of corolla, shape of leaf, plant height, leaf length and width, but the results could not reveal any obvious variability along altitudinal gradient within and among mountains, particularly no significant variation was observed in the northern, southwestern and the eastern Ethiopian mountains (Bale, Simen and Gara Muleta, respectively), where the highly genetically divergent individuals were observed from the AFLP analyzed data.

Similarly, the phylogeogrphic structures of *C. afromontanum* were very complex but the results from the different datasets (cpDNA and AFLP data) were still congruent. The role of polyploidy cannot be discounted with regard to this complex phylogeographic pattern. It is to be noted that a preliminary chromosome count has yielded two ploidy levels in this taxon in the mountain systems of tropical East Africa. Further cytogenetic studies may shade light on the geographic structures of the gene pools of *C. afromontanum*.

Generally, extensive morphological and cytogenetic studies may provide answers to the observed genetic data of *S. merjamie* and *C. afromontanum.* High biological diversity due to cryptic species has been reported for plant species inhabiting extreme habitats (Grundt et al., 2006) and afro-alpine environment may not be an exception.

8.2 Recommendations

Climate change is the variation in either the mean state of the climate or in its variability, persisting for an extended period. It encompasses temperature increases, sea-level rises, changes in precipitation patterns and increased frequencies of extreme weather events.

Change in global climate conditions seem to come in cycles of several thousands of years. Past changes in the global climate resulted in major shifts in species spatial distributions and marked reorganization of biological communities, landscapes, and biomes and these changes occurred in a landscape that was not as fragmented as it is today and with little or no pressures from human activities. Any major change in the current climate is coupled with other human pressures and is stressing biodiversity far beyond the levels imposed by the global climatic change that occurred in the recent evolutionary past. Therefore, the human component needs to be incorporated when dealing with the impacts of climate change on biodiversity – that is, activities aimed at mitigating and adapting to climate change in which biodiversity considerations are essential.

Global climate change has emerged as a major environmental issue over the past 20 years and it has been established that climate change is one of the threats to biodiversity. What is less often stressed is the fact that in most resource-poor, developing countries of sub-Saharan Africa, large-scale habitats and biodiversity losses means that species with potentially great economic importance may become extinct before they are even discovered. Additionally, with current state of affairs it is even impossible to assess the impacts of climate change on biodiversity. It is only if we know the current state, composition and extent of the natural flora and fauna that we could possibly monitor any potential change in the long run. This should be of major concern to the all concerned bodies that are expected to develop strategies for promoting synergy among activities to protect and preserve biodiversity at the national, regional and international level, including activities to combat desertification and land degradation, and activities for the conservation of and sustainable use of biodiversity.

There are ecological, economic, ethical and aesthetic reasons to conserve biodiversity. It would be of enormous benefit to basic science and humanity getting on with the exploration of earth’s biodiversity. So far a lot is known about what one can do to save many habitats and species in natural ecosystems. As the loss of biodiversity is a very slow process with effects accumulating through several generations and the negative impact is not always immediately felt, preserving of biodiversity requires radical changes in attitudes and lifestyles of people. Environmental degradation and loss of biodiversity is directly related to the daily activity of the different part of societies everywhere in the world. The difficulty in many of the biological conservation attempts is the concentration of the poverty and biological diversity in the developing countries, where both almost entirely depend on the other. The central problem and the main bottle-neck in formulating a workable conservation strategy is thus how to raise the poor to an endurable level of quality of life, while preserving as much of the natural world as possible.

The potential consequences and repercussions to humankind of the deterioration of the existing biodiversity are not yet fully understood. Out of the countless species of the planet Earth, barely about 1.8 million species of animals, plants and micro-organisms have been discovered and characterized (Wilson, 2005). This highlights a significant margin of uncertainty and reflects the extent of our unawareness about the riches of the planet.

Particularly in tropical East Africa, large amounts of biodiversity are being destroyed before it even becomes known. Surprisingly, human activity is the cause of such huge destructions in the continent and still its impact is accelerating from time to time. Thus the last remnants of many ecosystems are not just being reduced in area, rather they are eliminated completely altogether.
It is encouraging to know that efforts are being made to save the rich biodiversity of tropical East Africa, through which a number of mountains have been recognized by UNESCO as biodiversity hotspots, *e.g.*, the Simen Mts in Ethiopia, Mt Kenya and Aberdare ranges in Kenya, and Mt Kilimanjaro in Tanzania, which resulted in relatively a good conservation status of the regions. In addition, a limited number of mountains in Ethiopia, Kenya and Tanzania are regarded as national reservation centers (as national parks, areas for hunting game, sanctuaries and forest reserves), where various plant and animal species are protected to varying degrees. There are also some naturally protected areas that are difficult for people to reach for cultivations or undesirable to occupy.

Mountains and highlands of the Ethiopia and tropical East Africa naturally serve as water catchments areas. Most of them are snow-capped mountains, *e.g.*, Mt Kenya, Aberdare ranges and Mt Kilimanjaro, and several tributaries from almost all huge mountains in the region pour their water into the nearby rivers, which are sources of drinking water for hundreds of millions of people. These mountains are therefore very important ecosystems for the people, their livestock and wildlife, and have significant impacts on many economic sectors including agriculture, tourism and energy.

Before three decades large sections of the East African mountains were deemed suitable for commercial plantations of fast-growing species of pines from the Northern Hemisphere and eucalyptus from the Southern Hemisphere. These exotic plantations were intended to provide timbers for the then emerging local building industry and hands fuel for the steam engine, *e.g.*, Mt Kenya and Aberdare National Park (Maathai, 2005). As a result, the local floras considered to be economically less valuable and subjected to uncontrolled clearance. In addition, increasing population pressure and the consequent rise in the demand for arable land lead to wholesale conversion of more virgin indigenous forests into farmland. The ever increasing demand for commercial timber, domestic firewood and land for expanding population are the major factors responsible for continuous forest clearance in many parts of the world and much more so in East Africa. This is the main cause for continuous disappearance of irreplaceable local biodiversity. As indicated above, the matter is even more complicated by the fact that the indigenous forest trees are being replaced by exotic monoculture of trees, which normally are not forests and are characterized by lack of the capacity to conserve rainwater. When rain falls in commercial plantations much of the rainwater is lost running downstream. As a result, many rivers and lakes in the region are either drying up or have their water levels greatly reduced. When rainwater runs downstream through lands that are extensively cultivated it causes massive soil erosion and sometimes flooding. Kenya and Ethiopia have had a good share of flooding in recent years, mainly due to upstream deforestation.

Wildlife management is directly linked to good management of their habitats. One of the major problems in wildlife management in the region is the human-wildlife conflict. This happens when animals are forced to leave their destroyed and degraded habitats and move to neighboring farms in search of food and water. It is during such movements that many animals are heavily hunted to death either at the hand of poachers or people defending themselves and their livelihoods. Yet wildlife is essential for the tourism industry in most of the countries in Africa, particularly tropical East Africa. Therefore, destroying habitats that support wildlife undermines the capacity of the region to generate income from tourism and support projects, which could contribute to the effort of eradicating poverty.

In some cases, conservation activities seem to be in conflict with the short-term interest of economic development or the subsistence needs of a growing rural population. At several sites in Ethiopia, *e.g.* Mt Chilallo, Kaka, Bale and Simen Mts, and East Africa, such as the Cherangani Hills in Kenya and in Tanzania the east Usambara Mountains (see Knox 1993), immediate needs make conservation very challenging. While the growing ecotourism movement seems to promises to be important economic force favoring conservation, long-term strategies need to consider the importance of a direct participation of and tangible benefits to the local population.

Information about the amount and distribution of genetic variation in plant populations is important for efficient management and for wise utilization of the enormous resources.

Moreover, the conservation importance of an area is determined by assessing its diversity using species as the basic units of biodiversity. Lack of the will to prioritize biodiversity conservation continues to be the problem in the East African regions. This is, in part, due to the preoccupation of governments with other issues that require immediate attention including conflicts, drought, the occasional famine and extreme poverty in many parts of the region. In addition, no target-oriented and systematic studies have been carried out on mountains' biodiversity (based on complementary molecular techniques).

For long lasting solution, it is important to understanding strategies to increase the awareness of the local, regional and national governments as well as the population regarding the wider importance of protecting and preserving natural biodiversity. In particular, it should be possible to reverse the worrying trend of the expansion of the monocultures of commercial plantations in many parts of the region. Ways and means should be designed to encouraging local communities to plant indigenous tree seedlings in the degraded parts of forests under professional guidance and supervision.

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APPENDICES

Appendix 4.1 Population numbers, name of the species, study areas, altitudes above sea level, geographical coordinates, dates of collection, control samples or blind-lists and selected individuals of three populations per mountain system for extraction of *A. alpina*, *S. merjamie* and *C. afromontanum* in Ethiopian and East African mountain systems

Country	**Locality**	**Collection number**	**Species name**	**Altitude (m)**	**Coordinate (°)**	**Date of collection**	**X-List**	**Individuals selected for extraction**
Ethiopia	Bale Mts	AFR 001	*Arabis alpina*	4377	N-6.82684 E-039.819	30-09-2003	10	2, 4, 6, 8, 10, X
Ethiopia		AFR 006	*A. alpina*	4374	N-6.82683 E-039.8194	25-10-2003	2	All
Ethiopia		AFR 131	*A. alpina*	3609	N-7.06159 E-39.65286	24-8-2004	9	All
Ethiopia	Mt Kaka	AFR 036	*A. alpina*	4183	N-7.36379 E-039.15764	30-10-2003	2	2, 4, 6, 8, 10, X
Ethiopia		AFR 037	*A. alpina*	4097	N-7.36840 E-039.1637	30-10-2003	1	1, 3, 5, 7, 9, X
Ethiopia		AFR 038	*A. alpina*	4010	N-7.37134 E-039.17085	30-10-2003	2	All
Ethiopia	Simen Mts	AFR 060	*A. alpina*	3755	N-3.26187 E-038.20103	02-10-2004	6	2, 4, 6, 8, 10, X
Ethiopia		AFR 062	*A. alpina*	3897	N-3.28567 E-038.14255	02-11-2004	5	3, 5, 7, 9, 11, X

Ethiopia		AFR 071	*A. alpina*	3220	N-3.23302 E-038.03877	02-12-2004	1	All
Ethiopia	Mt Choke	AFR 259	*A. alpina*	4063	N-10.42570 E-037.50649	19-11-2004	1	All
Ethiopia		AFR 262	*A. alpina*	4250	N-0.426830 E-037.50688	19-11-2004	1	1, 3, 5, 7, 9, X
Ethiopia		AFR 263	*A. alpina*	4100	N-0.426760 E-037.50592	19-11-2004	1	1, 3, 5, 7, 9, X
Tanzania	Mt Kilimanjaro	AFR 084	*A. alpina*	4072	S-3.120920 E-037.42672	22-04-2004	1	1, 3, 5, 7, 9, X
Tanzania		AFR 085	*A. alpina*	4145	S-03.11900 E-037.43028	22-04-2004	10	2, 4, 6, 8, 10, X
Tanzania		AFR 086	*A. alpina*	4175	S-03.11843 E-037.43172	22-04-2004	9	All
Tanzania	Mt Meru	AFR 095	*A. alpina*	3611	S-03. 21762 E-036.77106	29-04-2004	2	2, 4, 6, 8, 10, X
Tanzania		AFR 096	*A. alpina*	3672	S-03.22088 E-036.76878	30-04-2004	1	1, 3, 5, 7, 9, X
Tanzania		AFR 097	*A. alpina*	3670	S-03.21909 E-036.76751	30-04-2004	5	All
Ethiopia	Gara Muleta	AFR 106	*A. alpina*	2600	N-09.20797 E-041.79826	13-05-2004	3	All
Ethiopia		AFR 109	*A. alpina*	3020	N-09.22818 E-041.79341	14-08-2004	1	1, 3, 5, 7, 9, X
Ethiopia		AFR 115	*A. alpina*	2768	N-09.20987 E-041.79477	15-08-2004	1	1, 3, 5, 7, 9, X
Kenya	Aberdares	AFR 144	*A. alpina*	3669	S-00.33389 E-036.65130	26-09-2004	8	2, 4, 6, 8, 10, X
Kenya		AFR 145	*A. alpina*	3726	S-00.34304 E-036.65324	26-09-2004	5	3, 5, 7, 9, 11, X

Kenya		AFR 151	*A. alpina*	3096	S-00.45448 E-036.71400	27-09-2004	7	All
Kenya	Mt Kenya	AFR 164	*A. alpina*	3895	S-00.16790 E-037.25100	10-03-2004	5	1, 3, 5, 7, 9, X
Kenya		AFR 165	*A. alpina*	3931	S-00.16791 E-037.25395	10-03-2004	3	1, 3, 5, 7, 9, X
Kenya		AFR 180	*A. alpina*	3742	S-00.08402 E-037.28631	10-06-2004	1	All
Kenya	Mt Elgon	AFR 194	*A. alpina*	4070	N-01.12122 E-034.59727	15-10-2004	1	1, 3, 5, 7, 9, X
Kenya		AFR 195	*A. alpina*	4067	N-01.12142 E-034.59716	15-10-2004	5	3, 5, 7, 9, 11, X
Kenya		AFR 196	*A. alpina*	4050	N-01.12095 E-034.58623	15-10-2004	1	All
Ethiopia	Mt Chilallo	AFR 307	*A. alpina*	3695	N-07.92831 E-039.21788	08-11-2004	4	2, 4, 6, 8, 10, X
Ethiopia		AFR 300	*A. alpina*	3602	N-07.82916 E-039.35724	09-11-2004	11	3, 5, 7, 9, 11, X
Ethiopia		AFR 347	*A. alpina*	3615	N-06.93948 E-039.21801	20-11-2004	5	All
Ethiopia	Bale Mts	AFR 010	*Salvia. merjamie*	3514	N-06.92256 E-039.93095	26-10-2003	6	All
Ethiopia		AFR 014	*S. merjamie*	3200	N-07.09363 E-039.78915	27-10-2003	1	1, 3, 5, 7, 9, X
Ethiopia		AFR 023	*S. merjamie*	3560	N-07.07025 E-039.65493	28-10-2003	3	3, 5, 7, 9, 11, X
Ethiopia	Simen Mts	AFR 064	*S. merjamie*	3258	N-13.23045 E-038.04062	02-12-2004	3	All
Ethiopia		AFR 230	*S. merjamie*	3560	N-13.16050 E-038.06604	11-11-2004	7	1, 3, 5, 7, 9, X
Ethiopia		AFR 236	*S. merjamie*	3242	N-13.13 919 E-038.02419	12-11-2004	10	2, 4, 6, 8, 10, X

Tanzania	Mt Kilimanjaro	AFR 079	*S. merjamie*	3027	S-03.16600 E-037.49874	20-04-2004	1	All
Tanzania	Mt Meru	AFR 100	*S. merjamie*	3312	S-03.22042 E-036.78055	05-01-2004	3	3, 5, 7, 9, 11, X
Tanzania		AFR 101	*S. merjamie*	3101	S-03.22502 E-036.78611	05-01-2004	10	All
Tanzania		AFR 103	*S. merjamie*	2625	S-03.22517 E-036.79781	05-01-2004	1	1, 3, 5, 7, 9, X
Ethiopia	Gara Muleta	AFR 107	*S. merjamie*	2509	N-09.20769 E-041.80115	13-08-2004	8	All
Ethiopia		AFR 118	*S. merjamie*	2462	N-09.22629 E-041.80959	16-08-2004	7	1, 3, 5, 7, 9, X
Ethiopia		AFR 123	*S. merjamie*	2585	N-09.23278 E-041.74273	16-08-2004	1	1, 3, 5, 7, 9, X
Kenya	Aberdares	AFR 156	*S. merjamie*	2821	S--00.72276 E-036.67835	28-09-2004	7	All
Kenya		AFR 157	*S. merjamie*	2821	S-00.72286 E-036.67835	28-09-2004	6	2, 4, 6, 8, 10, X
Kenya		AFR 162	*S. merjamie*	2712	S-00.70272 E-036.68669	29-09-2004	8	2, 4, 6, 8, 10, X
Kenya	Mt Kenya	AFR 172	*S. merjamie*	3051	S-00.17063 E-037.21357	10-04-2004	7	All
Kenya		AFR 174	*S. merjamie*	2575	S-00.17496 E-037.16957	10-05-2004	8	2, 4, 6, 8, 10, X
Kenya		AFR 175	*S. merjamie*	2666	S-00.18008 E-037.18518	10-05-2004	3	1, 3, 5, 7, 9, X
Kenya	Mt Elgon	AFR 190	*S. merjamie*	2517	N-01.06544 E-034.76694	14-10-2004	3	All
Kenya		AFR191	*S. merjamie*	2215	N-01.04695 E-034.77478	14-10-2004	5	3, 5, 7, 9, 11, X
Kenya		AFR 193	*S. merjamie*	2835	N-01.06397 E-034.71920	14-10-2004	4	2, 4, 6, 8, 10, X
Kenya	Cherangani Hills	AFR 219	*S. merjamie*	2575	N-01.20582 E-035.28007	17-10-2004	7	All
Kenya		AFR 220	*S. merjamie*	2384	N-01.13236 E-035.16069	17-10-2004	5	1, 3, 5, 7, 9, X

Kenya		AFR 222	*S. merjamie*	2690	N-01.17177 E-035.33190	17-10-2004	1	1, 3, 5, 7, 9, X
Ethiopia	Mt Chilallo	AFR 334	*S. merjamie*	3122	N-07.91820 E-039.18941	08-11-2004	1	All
Ethiopia		AFR 335	*S. merjamie*	3150	N-07.91843 E-039.18941	08-11-2004	11	3, 5, 7, 9, 11, X
Ethiopia		AFR 306	*S. merjamie*	3290	N-07.92043 E-039.19639	08-11-2004	11	3, 5, 7, 9, 11, X
Ethiopia	Mt Kaka	AFR 314	*S. merjamie*	3218	N-07.363225 E-039.21011	11-11-2004	10	All
Ethiopia		AFR 317	*S. merjamie*	3163	N-07.36382 E-039.21296	11-11-2004	8	2, 4, 6, 8, 10, X
Ethiopia		AFR 338	*S. merjamie*	3440	N-07.36421 E-039.19853	12-11-2004	4	2, 4, 6, 8, 10, X
Ethiopia	Bale Mts	AFR 017	*Cerastium afromontanum*	3332	N-07.04575 E-39.75152	27-10-2003	3	3, 5, 7, 9, 11, X
Ethiopia		AFR 024	*C. afromontanum*	3560	N-07.07025 E-39.65493	28-10-2003	2	All
Ethiopia		AFR 346	*C. afromontanum*	3139	N-07.10225 E-39.79129	19-11-2004	5	1, 3, 5, 7, 9, X
Ethiopia	Mt Kaka	AFR 044	*C. afromontanum*	3426	N-07.36520 E-39.19740	30-10-2003	1	1, 3, 5, 7, 9, X
Ethiopia		AFR 313	*C. afromontanum*	3186	N-07.36343 E-39.21233	11-11-2004	11	All
Ethiopia		AFR 315	*C. afromontanum*	3297	N-07.38332 E-39.21188	11-11-2004	7	3, 5, 7, 9, 11, X
Ethiopia	Simen	AFR 243	*C. afromontanum*	3286	N-13.14119 E-3802.919	13-11-2004	1	All
Ethiopia		AFR 245	*C. afromontanum*	3238	N-13.13834 E-3802.354	14-11-2004	1	All
Tanzania	Mt Meru	AFR 091	*C. afromontanum*	2733	S-03.22540 E-36.79434	29-04-2004	6	2, 4, 6, 8, 10, X
Tanzania		AFR 092	*C. afromontanum*	3229	S-03.22216 E-36.78234	29-04-2004	3	All
Tanzania		AFR 102	*C. afromontanum*	3090	S-03.22413 E-36.78650	05-01-2004	3	1, 3, 5, 7, 9, X

Ethiopia	Gara Muleta	AFR 110	*C. afromontanum*	3035	N-09.22722 E-41.79249	14-08-2004	2	All
Kenya	Aberdares	AFR 141	*C. afromontanum*	3647	S-00.33497 E-36.65145	26-09-2004	4	2, 4, 6, 8, 10, X
Kenya		AFR 147	*C. afromontanum*	3494	S-00.34076 E-36.66951	26-09-2004	9	All
Kenya		AFR 153	*C. afromontanum*	2927	S-00.52575 E-36.72239	28-09-2004	9	3, 5, 7, 9, 11, X
Kenya	Mt Kenya	AFR 159	*C. afromontanum*	3051	S-00.17046 E-37.21450	10-02-2004	9	3, 5, 7, 9, 11, X
Kenya		AFR 163	*C. afromontanum*	3337	S-00.16743 E-37.22628	10-03-2004	4	All
Kenya		AFR 171	*C. afromontanum*	3137	S-00.16856 E-37.21842	10-04-2004	7	1, 3, 5, 7, 9, X
Kenya	Mt Elgon	AFR 192	*C. afromontanum*	2515	N-01.07395 E-34.74686	14-10-2004	4	2, 4, 6, 8, 10, X
Kenya		AFR 197	*C. afromontanum*	2999	N-01.06134 E-34.68774	15-10-2004	11	All
Kenya		AFR 199	*C. afromontanum*	3329	N-01.07896 E-34.64173	15-10-2004	3	1, 3, 5, 7, 9, X
Kenya	Cherangani Hills	AFR 221	*C. afromontanum*	2575	N-01.20582 E-34.28007	17-10-2004	2	All
Kenya		AFR 223	*C. afromontanum*	2909	N-01.07174 E-35.41853	17-10-2004	5	All
Ethiopia	Mt Chilallo	AFR 301	*C. afromontanum*	3602	N-07.82916 E-39.35724	09-11-2004	11	All
Ethiopia		AFR 303	*C. afromontanum*	3047	N-07.92096 E-39.18377	10-11-2004	8	2, 4, 6, 8, 10, X
Ethiopia		AFR 308	*C. afromontanum*	3695	N-07.92831 E-39.21788	08-11-2004	8	2, 4, 6, 8, 10, X

Appendix 4.2 Collection localities, population number, altitude, geographical coordinates, and date of collection, individual number and haplotype for the individuals of *A. alpina* sequenced for the cpDNA *trn*L intron and *trn*L-*trn*F intergenic spacer

Locality	Pop	Altitude (m)	Coordinates (°)	Date	Ind	Haplotype
Bale Mts	AFR 001	4380	N-06.82684 E-039.81903	30-9-2003	2	B
					4	B
	AFR 006	4370	N-06.82683 E-039.81938	25-10-2003	2	B
					4	B
	AFR 131	3610	N-07.06159 E-039.65286	24/8/2004	10	D
					11	D
Mt Kaka	AFR 036	4180	N-07.36379 E-039.15764	30-10-2003	2	C
					4	F
	AFR 037	4100	N-07.36840 E-039.16370	30-10-2003	3	C
					5	C
	AFR 038	4010	N-07.37134 E-039.17085	30-10-2003	11	A
					X	A
Simen Mts	AFR 060	3760	N-13.26187 E-038.20103	10-2-2004	2	4
					4	4
	AFR 062	3900	N-13.28567 E-038.14255	11-2-2004	3	C
					5	4
	AFR 071	3220	N-13.23302 E-038.03877	12-2-2004	2	4
					4	4
Mt Choke	AFR 263	4100	N 10 42.676 E-037.50592	11-19-2004	3	19
					5	19
	AFR 259	4060	N-10. 42.570 E-037.50649	11-19-2004	10	19
					11	19
	AFR 262	4250	N-10 42.683 E-037.50.688	11-19-2004	3	19
					5	19
Mt Kilimanjaro	AFR 084	4070	S-03.12092 E-037.42672	22-4-2004	3	19
					5	19
	AFR 085	4150	S-03.11900 E-037.43028	22-4-2004	2	19
					4	19
	AFR 086	4180	S-03.11843 E-037.43172	22-4-2004	2	19
					4	E

Mt Meru	AFR 095	3610	S-03. 21762 E-036.77106	29-4-2004	2	19
					4	19
	AFR 096	3670	S-03.22088 E-036.76878	30-4-2004	3	19
					5	19
	AFR 097	3670	S-03.21909 E-036.76751	30-4-2004	3	19
					5	E
Gara Muleta	AFR 106	2600	N-09.20797 E-041.79826	13-5-2004	3	4
					5	4
	AFR 109	3020	N-09.22818 E-41.79341	14-8-2004	3	4
					5	4
	AFR 115	2770	N-09.20987 E-041.79477	15-8-2004	3	G
					5	G
Aberdares	AFR 144	3670	S-00.33389E-036.65130	26-9-2004	2	A
					4	A
	AFR 145	3730	S-00.34304 E-036. 65324	26-9-2004	3	A
					5	B
	AFR 151	3100	S-00.45448 E036.71400	27-9-2004	10	A
					11	A
Mt Kenya	AFR 164	3890	S-00.16790 E-037. 25100	3-10-2004	3	A
					5	A
	AFR 165	3930	S-00.16791 E-037.25395	3-10-2004	3	A
					5	A
	AFR 180	3740	S-00.08402 E-037.28631	6-10-2004	11	A
					X	A
Mt Elgon	AFR 194	4070	N-01.12122 E-034.59727	15-10-2004	3	19
					5	19
	AFR 195	4070	N-01.12142 E-034.59716	15-10-2004	3	19
					5	19
	AFR 196	4050	N-01.12095 E-034.58623	15-10-2004	10	19
					11	19
Mt Chilallo	AFR 307	3700	N-07.92831 E-039.21788	11-8-2004	2	A
					4	A
	AFR 300	3600	N-07.82916 E-039.35724	11-9-2004	9	A
					11	A
	AFR 347	3620	N-06.93948 E-039.21801	11-20-2004	2	A
					4	A

DECLARATON

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.

Name: Adane Assefa Terefe
Signature: ___________________

Date: August, 2007

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

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