EVOLUTIONARY HISTORY, GENETIC DIVERSITY AND CONSERVATION IMPLICATIONS OF SELECTED AFRO-ALPINE TAXA

BY

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EXTENDED ABSTRACT

The tropical East African and Ethiopian mountains are famous for their exceptionally unique and high biodiversity. The flora on these mountains offers good examples of distinct adaptations to different altitudes as well as evolutionary differentiation, hence an ideal natural laboratory for studies on the dynamics of biodiversity. In this study the genetic diversity, evolutionary history and conservation implications of selected taxa occurring on these mountains were assessed. The objectives were to: 1) determine the level of intraspecific and interspecific genetic diversity of selected afro-alpine plant species, 2) explore the potential of AFLP markers for delimiting species and reconstructing the evolutionary relationships among some of the selected afro-alpine plants by comparing the results with those of previous morphological and molecular studies and 3) reconstruct the phylogeographic structure of the selected afro-alpine plant species.

Data for this study were collected from 1 ha (10000 m^2) plots on 14 mountains around the region. Amplified Fragment Length Polymorphism (AFLP) markers were used to characterize the genetic patterns of the selected taxa. Six hundred eighty nine individuals from 154 populations (13 species, a total of 1168 AFLP markers with 97.9% reproducibility) of giant lobelias, 33 individuals, nine populations (two species, 172 AFLP markers, 97.86% reproducibility) of *Deschampsia*, and 153 individuals 36 populations (458 AFLP markers, 97.4% reproducibility) of *Koeleria capensis* were successfully analyzed.

Mean within-species (H_T) and within-population (H_S) genetic diversities were generally low across all species. Among the thirteen species of giant lobelias, the least diversity

was observed in the most widely distributed species, *L. giberroa* (H_T = 0.0751), followed by *L. rhynchopetalum* (H_T = 0.0832). On the contrary, most diversity was observed in the narrow endemics *L. bequaertii* (H_T = 0.2522) and *L. thuliniana* (H_T = 0.2118). The low genetic diversity among *L. giberroa* populations may be attributed to bottlenecks following reduction of its montane forest habitat by human activities, which may have been less influential in the high-alpine Ruwenzori habitat of the local endemic *L. bequaertii*. There was however no correlation between the age of mountains and levels of genetic diversity, suggesting that the current populations on the older mountains originated from colonization episodes taking place long after their formation.

Except for *Deschampsia* spp. populations, the rest of the molecular and/or morphology-based recognized species were found to be genetically distinct. In *Deschampsia*, the individuals identified as the endemic *D. angusta* were not genetically distinct from those of *D. caespitosa* sampled in the same mountain, Ruwenzori, suggesting that the characters used to distinguish these species may reflect phenotypic plasticity rather than taxonomically significant variation. For giant lobelias, the relationships among species inferred from the primarily nuclear AFLP data were, with some notable exceptions, consistent with relationships earlier inferred from morphology and/or plastid DNA restriction site polymorphisms. High-altitude-restricted *Lobelia* species were intermixed with species occurring in the forest zone in the AFLP-based tree, supporting a main scenario of initial expansions of ancestral forest populations followed by parallel high-altitude adaptation and speciation in different mountain groups. However, the results did not support the proposed instances of hybrid speciation in this group while suggesting the most distinct intermountain vicarious patterns among the giant lobelias to be primarily high-alpine. For *Koeleria capensis*, there was neither distinct geographic structuring of the genetic variation nor support for recognition of infraspecific taxa. The results

suggested that the afro-alpine populations of *Koeleria capensis* might have arisen by long-distance dispersal through Ethiopian mountains followed by intermountain dispersal into the tropical East African Mountains.

Given the current genetic structure and patterns, monitoring the most diverse and genetically most distinct populations of each species *in situ* and gemplasm tests for *ex situ* conservation are suggested in order to increase the probability for long-term survival of the studied plants and afro-alpine ecosystem at large. The study highlights that different afro-alpine species may have experienced very different phylogeographic histories and that long-distance dispersal among the isolated afro-alpine 'sky islands' can be more frequent than traditionally thought. Generally, the study demonstrates the need for further taxonomic exploration of the afro-alpine flora, in particular of taxa described as endemic.

This thesis is an outstanding contribution to knowledge as it provides for a refined evolutionary history and taxonomy of the previous morphology and molecular-based studies. For example the discovery that earlier proposed hybrid species *L. bequaertii* and *L. bambuseti* are actually not hybrids; the fact that *Deschampsia angusta* did not separate from *D. caespitosa* provides for a new idea that the previously known endemic *D. angusta* only from Ruwenzori mountains can actually be *D. caespitosa*; the knowledge about little genetic diversity within and among most of the studied species is a crucial contribution to conservationists for improved conservation strategies and; identification of areas that need further research such as the phylogenetic position of *Lobelia thuliniana* helps increase interests of natural biologists to work more on the studied taxa.

DECLARATION

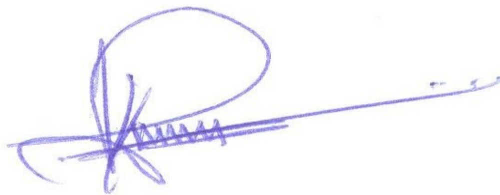
I, Catherine Aloyce Masao, do hereby declare to the Senate of the Sokoine University of Agriculture, that this thesis is my own original work and that it has neither been submitted nor concurrently being submitted in any other institution.

____________________________ ________________________

Catherine A. Masao Date

(PhD. Candidate)

The above declaration is confirmed



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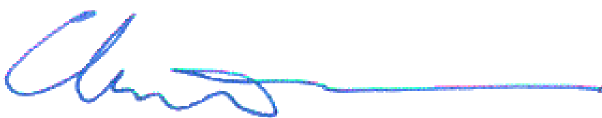

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DEDICATION

This thesis is dedicated to the Almighty God for He has been my refuge every time. It is also dedicated to my parents Aloyce and Basilisa J. Masao who, not only tirelessly endured to lay down the foundation of my education but also encouraged me to pursue my studies especially during the toughest moments of the process. My beloved husband Timothy Mmbaga and our children Brian, Bridget (Bridget unfortunately passed away during this PhD process) and Basilisa for, their patience was my strength.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
PCR	Polymerase Chain Reaction
ISSR	Inter Simple Sequence Repeats
ITS	Internal Transcribed Spacer
NHM	Natural History Museum
NUFU	Norwegian Programme for Development, Research and Higher Education
IUCN	International Union for Conservation of Nature
DNA	Deoxyribonucleic Acid
cpDNA	Chloroplast DNA
RAPD	Random Amplified Polymorphic DNA
PCoA	Principal Coordinate Analyses
MCMC	Markov Chain Monte Carlo
AMOVA	Analysis of Molecular Variance
PER	Predominantly Eastern Rift Clade
PWR	Predominantly Western Rift Clade
TANAPA	Tanzania National Parks Authority

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CHAPTER ONE

1.0 INTRODUCTION

Genetic diversity provides the building blocks of biological diversity. Thus, conserving genetic diversity within and between individual species makes an important contribution to species survival in the face of environmental change and diseases (Rauch and Bar-Yam, 2004). Although biodiversity at genetic level is important in conservation, it has always been difficult to quantify. On the other hand, evolutionary history studies help us understand how processes evolved, hence provide us with clues on the present-day biodiversity, by evaluating the past changes in biogeographic distribution and ecological habitat. As species-level phylogenies become increasingly resolved and complete, they provide opportunities to more confidently explore their tempo of diversification (Barraclough and Nee, 2001). Empirical studies in ecology and evolution often depend on accurate assessment of genetic diversity to address questions regarding genetic relatedness among individuals, population structure and phylogenetic relationships (Mueller and Wolfenbarger, 1999).

Despite the fact that much has been done on phylogenetic/taxonomic, phylogeographical and genetic diversity studies in the northern hemisphere using AFLPs (Gaudeul *et al.*, 2000; Buntjer *et al.*, 2002; Pineiro *et al.*, 2007; Koopman *et al.*, 2008; Garcia-Pereira *et al.*, 2010; Safer *et al.*, 2011), only little has been done to address similar topics in Africa and particularly on the afro-alpine flora (Koch *et al.*, 2006; Assefa *et al.*, 2007; Kebede *et al.*, 2007).

1.1 The Use of Molecular Markers in Plant Evolutionary Biology and Biogeography

In molecular systematics and evolutionary biology, one seeks to accurately reconstruct the evolutionary history of populations and species. Allozymes were the first major molecular genetic markers which were developed and used in the late 1960s. These are co-dominant protein variants (alleles) that can be visualized by appropriate staining and starch-gel electrophoresis (http://www.uwyo.edu/dbmcd/popecol/maylects/popgengloss.html).
Analyses of extensive data compilations have demonstrated that allozyme-derived population genetics parameters are comparable across studies and are closely associated with various life history traits thus they have the potential to produce information with important implications in evolutionary biology, ecology and conservation biology (Nybom, 2004). However, the problem of insufficient amount of easily accessible plant tissue as well as low levels of polymorphism brought about an increasing interest in other DNA-marker based methods (Nybom, 2004).

Among these, so far the most appreciated molecular markers are: amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and DNA sequences – either nuclear or chloroplast DNA sequences (Nybom, 2004; Sica *et al.*, 2005). The availability of these markers has enabled researchers to undertake genome mapping and to measure the rates and patterns of genetic diversity, phylogeographical structure and phylogenetic divergence (Gaudeul *et al.*, 2000; Buntjer *et al.*, 2002; Sica *et al.*, 2005; Koch *et al.*, 2006; Assefa *et al.*, 2007; Kebede *et al.*, 2007; Pineiro *et al.*, 2007).

A study comparing different nuclear markers for evaluating among and within-population diversity in wild angiosperms and gymnosperms reported that estimates derived from these markers are very similar and may be directly comparable (Nybom, 2004). However, some

markers might have advantages outweighing others justifying them being more popular to answer specific questions. For example, while sequences are very popular for phylogenetic inference, PCR-derived markers obtained with none specific primers (e.g. AFLP, RAPD and ISSR) are mostly used for population genetics based questions. For many years, DNA sequences are believed to be strong in phylogenetic inference compared to the PCR-derived markers obtained with none specific primers. However, recent AFLP based studies have successfully reconstructed evolutionary histories of some known difficult and recently diverged taxa which were difficult to resolve by using DNA sequences (Buntjer *et al.*, 2002; Koopman *et al.*, 2008; Meudt, 2009; Garcia-Pereira *et al.*, 2010; Safer *et al.*, 2011).

1.2 Use of AFLPs in Phylogeography and Phylogenetic Reconstructions

The AFLP is a fingerprinting technique based on the selective polymerase chain reaction (PCR) amplification of restriction fragments from total genomic DNA (Vos *et al.*, 1995). The technique involves four steps namely: 1) restriction ligation, 2) pre-selective amplification 3) selective amplification and 4) gel analysis (Vos *et al.*, 1995; Gaudeul *et al.*, 2000). Despite them requiring small quantities of DNA sample compared to other markers (Nybom, 2004), AFLP markers are known for their high sensitivity in determining even slight differences within populations. They produce large sets of polymorphic markers that may be used to analyze closely related taxa. This technique has aroused a lot of enthusiasm since its development in 1995. It has brought key answers to major biological issues in a wide variety of organisms like fungi, plants, birds and even humans (Bonin *et al.*, 2007). Since its development, the AFLP technique has been primarily dedicated to assessments of intraspecific genetic diversity especially in plants (Gaudeul *et al.*, 2004; Kebede *et al.*, 2007; Pineiro *et al.*, 2007). The technique has further been useful in identifying phylogeographic patterns and potential hybrids within and among taxa (Gaudeul *et al.*,

2000; Mallet, 2005; Albach *et al.*, 2006; Paun *et al.*, 2006; Kebede *et al.*, 2007; Pineiro *et al.*, 2007; Jaramillo and Atkinson, 2011).

Recent discovery that AFLP data sets may contain phylogenetic signal (Giannasi *et al.*, 2001; Buntjer *et al.*, 2002; Koopmann, 2005; Koopman *et al.*, 2008) has stimulated its use as a source of genetic information for phylogenetic inference, particularly among closely related and recently diverged taxa (Meudt *et al.*, 2009; Garcia-Pereira *et al.*, 2010). Phylogenetic relationships are usually inferred from AFLP data converting the binary matrix into a distance matrix using dissimilarity measures or using the binary matrix directly for character-based methods such as parsimony and Bayesian analysis (Koopmann, 2005; Meudt *et al.*, 2009). However, the appropriateness of AFLP data for phylogenetic reconstruction is compromised by several limitations that have been suggested to question their utility (Kosman and Leonard, 2005).

The most widely suggested drawback of the AFLP technique is that the comigrating bands of the same length may not be homologous with one another (i.e. fragment size homoplacy) (Buntjer *et al.*, 2002; Meudt *et al.*, 2009). However, a study by Garcia-Pereira *et al.* (2010) which tested when in terms of genetic divergence the quality of AFLP data becomes too low to be informative for a reliable phylogenetic reconstruction suggested that: 1) phylogenetic usefulness of AFLPs varies greatly depending on the time since divergence and the specific genomic features (e.g. G-C content) of the compared taxa 2) lack of band homology among taxa quickly increases with divergence, thus rapidly compromising the phylogenetic usefulness of AFLP data sets 3) AFLP markers are informative at somewhat lower taxonomic levels than Internal Transcribed Spacer (ITS) sequences. Thus, when necessary precautions are taken AFLPs are very useful in population genetics, phylogeographic and phylogenetic studies.

1.3 Evaluating Genetic Diversity Patterns for Conservation Purposes

Conservation managers rarely have resources required for protecting endangered and/or threatened species. Thus, they must select only a subset of the existing populations for *in situ* management. The selection is often complicated and methods vary, but the incorporation of a genetic component in this process is increasingly being accepted as essential (Ellstrand and Elam, 1993; Rivers and Brummitt, 2011). The aim of conservation, in addition to habitat preservation, is to maintain a species' existing level of genetic variation in order to maximize the chances for persistence in the face of changing environment (Simberloff, 1988; Ellstrand and Elam, 1993). The information on the genetic diversity within plant populations can be used in conservation management to determine which populations need protection and the effectiveness of existing reserves (Rieger and Sedgley, 1998). Thus, understanding the genetic diversity levels should be taken as building blocks in all conservation initiatives.

Studies on genetic diversity within and among populations of both widespread and endemic plant species using molecular markers have increased in recent years due to their central importance in planning *in situ* and *ex situ* conservation efforts (Gaudeul *et al.*, 2000; He *et al.*, 2000; Juan *et al.*, 2004; Coppi *et al.*, 2008; Makowsky *et al.*, 2009, Geleta and Bryngelsson, 2009; Yan *et al.*, 2009; Jaramillo and Atkinson, 2010; Rivers and Brummitt, 2011; Suárez-Montes *et al.*, 2011). Most studies have shown that endemics/restricted and rare taxa contain significantly less genetic diversity than widespread species (Karron *et al.*, 1988; Ellstrand and Elam, 1993; Broadhurst and Coates, 2002). This has been linked to the fact that widespread species may have a history of large, continuous populations whereas endemics might consist of smaller and more ecologically limited populations historically susceptible to loss of variation by genetic drift (i.e. random change in allelic frequencies by chance) or bottlenecks (a bottleneck occurs when a population contracts to a significantly

smaller size over a short period of time due to random environmental event). Few studies have reported similar genetic diversity between widespread and endemic species while others have found unexpectedly high genetic variation within endemic and/or restricted species compared to their widely distributed congeners (Ellstrand and Elam, 1993).

So far in the IUCN Red List Data Criteria only rare species (in the sense of low abundance, restricted range and high habitat specificity) are being given special attention world-wide. Given the differences we get from genetic diversity point of view it is important that the IUCN Red list data being prepared considering genetic diversity among important factors for decision making. This is because some widespread species might be at more risk of extinction than the restricted ones. Ellstrand and Elam (1993) argued that while rare species with large localized population sizes are expected to exhibit high levels of genetic variation, the widespread ones might have an opposite trend as a result of habitat fragmentation. Fragmentation results in smaller and more isolated populations and the expected genetic consequences of this are: a decrease in genetic diversity due to loss of rare alleles, random drift, inbreeding within the fragments and reduction in gene flow between fragments (Aquilar *et al.*, 2008).

To retain genetic diversity, emphasis should be on conserving as many populations as possible. From many studies conducted so far, those populations and/or subpopulations which show the highest genetic diversity are the recommended centres of diversity hence increased conservation efforts (Keiper and McConchie, 2000; Juan *et al.*, 2004; Kebede *et al.*, 2007; Geleta *et al.*, 2008). Genetic diversity may be partially restored to depleted populations through the introduction of individuals carrying novel genes (Butler *et al.*, 1994). However, care must be taken to avoid reduction in overall population fitness through

the introduction of genotypes that have evolved through widely different selective regimes (Hamrick *et al.*, 1991).

1.4 Study Area

This study was conducted on the afro-alpine ecosystem of the isolated high mountains in the tropical East Africa and Ethiopia (Fig. 1). The afro-alpine flora is famous for its large numbers of geographically vicariant taxa and high endemism (about 80% of its taxa are endemic) indicating that this flora has been efficiently isolated from other high mountains and temperate flora for a long time (Hedberg, 1969). The flora offers a good example of distinct adaptations to different altitudes as well as evolutionary differentiation in these highly structured 'mountain islands' (Hedberg, 1970), hence an ideal natural laboratory for studies on the dynamics of biodiversity. The ecosystem is also an essential environment as a repository of biodiversity and for water supply and agriculture in several African countries.

The alpine enclaves of the high eastern African mountains provide a number of geographically and ecologically isolated temperate islands inhabited by the afro-alpine flora, which is poor in species (approximately 280 plant species) and peculiarly adapted to the extreme climate changes (Hedberg, 1970). This vegetation can be divided into three altitudinal zones according to Hedberg (1951): the afro-montane zone, the sub-alpine ericaceous zone and the afro-alpine zone (afro-alpine zone proper). The uppermost one, the afro-alpine zone, typically occurs above 3500 m and harbours unique alpine grasslands, shrub lands and bogs interrupted by crater lakes and characterized by the famous giant lobelias and senecios (Kieffer *et al.*, 2004). The afro-alpine climate is characterized by large diurnal temperature fluctuations, which are greater than the seasonal fluctuations and thus described by Hedberg (1969) as 'summer every day and winter every night'.

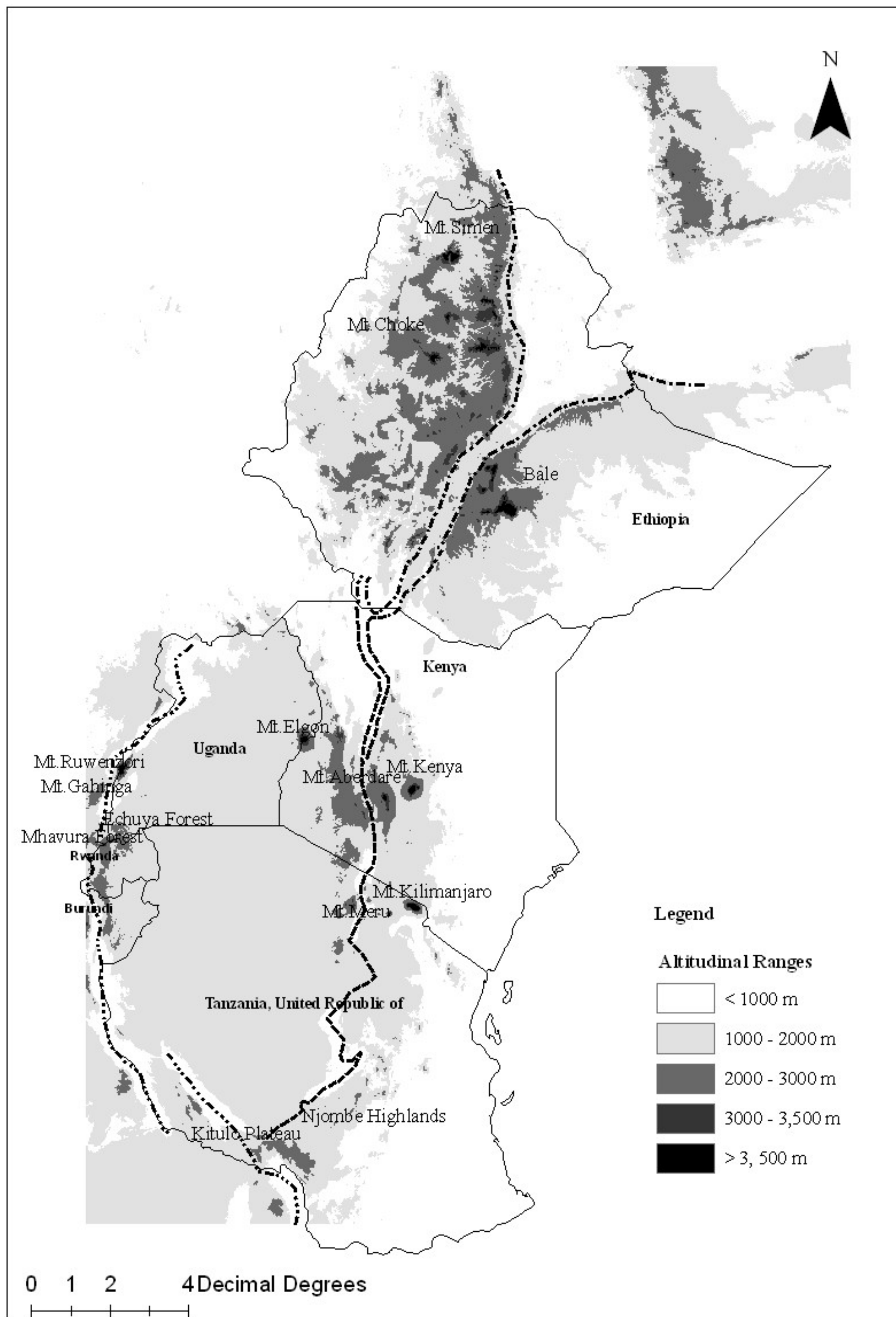

Figure 1: Map showing the study area and the sampled mountains

1.5 Study Taxa

The taxa presented in this thesis are some species and subspecies of giant lobelias in the unbranched inflorescence clade of Knox and Palmer (1998) (Fig. 2) and some grass species (*Deschampsia angusta*, *D. caespitosa* and *Koeleria capensis*). Some representative photos

for the giant lobelias and grasses are presented in plate 1. These taxa were selected due to the fact that they provide for good examples of high altitude adaptations, evolutionary differentiation and high endemism (Hedberg, 1957; 1969) while some of them are still taxonomically controversial resulting in large number of synonyms especially among the grasses (Clayton, 1970). The details of species and subspecies characterization and/or justification are presented in specific manuscripts.

Plate 1: Photographs of some of the studied taxa

1.6 The Research Problem and Justification

Although endemism of the afro-alpine flora is high indicating that the flora has long been isolated from other mountains or temperate floras (Hedberg, 1969), the time and source of immigration of these plants has always been subject to debate. While some authors have

considered these plants as Tertiary relicts with negligible possibilities for long distance dispersal (Hedberg, 1969; 1970), more recent studies have proposed some of them to result from Pleistocene long distance dispersals (Koch *et al.*, 2006), while some others are said to result from forest bridge dispersal (Kebede *et al.*, 2007) or from hybridization processes (Knox and Palmer, 1998). Thus, comparing genetic structures of different plant species from this unique ecosystem based on the same molecular technique may help provide better understanding of the biogeographical history of the flora at large. On the other hand, the degree of intra-specific genetic diversity in conservation is often neglected when developing conservation strategies because of difficulties not only in rating its significance, but also in its quantification (Till-Bottraud and Gaudeul, 2002). Following the high endemic character of the afro-alpine plants (Hedberg, 1969) and their ecological importance, there is a need to understand their spatial genetic distribution in order that necessary efforts are directed where there is a need for improvement.

1.7 Study Objectives

The study main objectives were to:

1) Determine the level and patterns of intraspecific and interspecific genetic diversity of selected afro-alpine plant species
2) Explore the potential of AFLP markers for delimiting species and reconstructing the evolutionary relationships among some of the selected afro-alpine plant species by comparing the results with those of previous morphological and molecular data
3) Reconstruct the phylogeographic structure of the selected afro-alpine plant species

Specifically, the study intended to:

A: address whether i) the species and subspecies of giant lobelias currently recognized based on morphology are genetically distinct, ii) the vicarious speciation patterns suggested by Hedberg (1957, 1969, 1997) are consistent with relationships inferred from primarily

nuclear AFLP data, and iii) the proposed hybrid origins of *L. bequaertii* and *L. bambuseti* can be corroborated (**Manuscript I**).

B: i) assess possible congruence of genetic groups with the morphology and current taxonomic treatments, ii) determine the level of genetic variation within and between populations, iii) reconstruct the phylogeographic history in the afro-alpine ecosystem, and iv) provide guidelines for conservation management (**Manuscript II**) .

C: i) determine and compare level of genetic diversity and patterns of genetic variation between and within populations of the giant lobelias with unbranched inflorescence ii) determine whether there is correlation between the age of the mountains and levels of genetic diversity therein and iii) explore implications for effective conservation of these species (**Manuscript III**).

1.8 Thesis Organization

The details of the above mentioned main objectives in specific manuscripts are organized in the form of chapters starting from chapter two. The phylogeographic structure of some selected afro-alpine plant species and the potential of AFLPs in species delimitation and reconstructing the evolutionary relationships among some of the selected afro-alpine plants by comparing the results with those of previous morphological and molecular studies objectives are addressed in chapter two and three using the giant lobelias with unbranched inflorescence, *Deschampsia angusta*, *D. caespitosa* and *Koeleria capensis* as case studies (Manuscripts I and II). Chapter four provides an analysis of the genetic diversity patterns, possible causes of such patterns and implications for the conservation of the unbranched inflorescence giant lobelias (Manuscript III).

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CHAPTER TWO

2.0 THE FAMOUS AFRO-ALPINE GIANT LOBELIAS REVISITED: TEST OF TAXA DELIMITATION AND SUGGESTED 'SKY ISLAND' SPECIATION PROCESSES USING AFLP MARKERS (Manuscript I)

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2.1 Summary

The giant lobelias are famous landmarks of the unique afro-alpine ecosystem and used as a classic example of extensive intermountain vicarious speciation among the isolated high mountains in East Africa and Ethiopia. It has however been suggested that the frequency of speciation following geographic isolation in the afro-alpine flora is overestimated and led to over-description of species, and that interspecific hybridization also played a role in speciation. Amplified fragment length polymorphism (AFLP) markers were used to test species and subspecies delimitation based on morphology as well as suggested intermountain speciation patterns and hybrid speciation in the giant lobelias. Fresh material was collected during range-wide field surveys. Six hundred and eighty nine (689) individuals from 154 populations of 13 of the 14 described species in Unbranched Inflorescence clade for 1168 AFLP markers were analyzed. It was found that all currently morphology-recognized species and several subspecies were genetically distinct. The relationships between species inferred from the primarily nuclear AFLP data were, with some notable exceptions, consistent with relationships earlier inferred from morphology and/or plastid DNA restriction site polymorphisms. High-altitude-restricted species were intermixed with species occurring in the forest zone in the AFLP-based tree, supporting a main scenario of initial expansions of ancestral forest populations followed by parallel high-altitude adaptation and speciation in different mountain groups. The results did not support the proposed instances of hybrid speciation: *L. bequaertii* grouped with the other Western Rift endemics, suggesting that its morphological and ecological similarity to the *Deckenii* group of the Eastern Rift is caused by parallel evolution rather than hybridization. *Lobelia bambuseti* formed a lineage distinct from its proposed progenitor species *L. aberdarica* and *L. giberroa*. The two most distinct intermountain vicarious patterns among the giant lobelias were primarily high-alpine and found to have formed independently in some of the same Eastern Rift mountains: 1) the classical example of the *Deckenii* group (excluding *L.*

bequaertii) with three distinct species, one of them with three distinct subspecies, and 2) *L. telekii*, with three distinct but hitherto undescribed intraspecific lineages.

2.2 Introduction

Giant lobelias together with giant senecios are emblematic of the afro-alpine flora on the isolated high mountains of eastern Africa (Fig.1a). These mountains, except for the Ruwenzori, are of Miocene to late Pleistocene volcanic origin and occur widely scattered over East Africa and Ethiopia. Several of them reach altitudes between 3500 m and 6000 m. The climate above the tree line around 3500 m is characterized by high temperature fluctuations and famously phrased as 'summer every day and winter every night' (Hedberg, 1957). The vegetation on the mountains is divided into three distinct zones: the lowermost afro-montane forest zone, the ericaceous zone and the uppermost afro-alpine proper zone (Hedberg, 1957).

During the Pleistocene glaciations in the northern hemisphere, the African tropics were cooler and drier than today (deMenocal, 1995). While the top of many mountains was covered by glaciers, both the afro-alpine and the ericaceous zones may have extended 1000-1500 m lower than today and thus occupied considerably larger areas (Gottelli *et al.*, 2004). In contrast, the extent of the afro-montane forest was reduced because it was replaced in its lower-lying parts by grasslands in response to aridification (deMenocal, 1995; Gottelli *et al.*, 2004). During warmer and more humid interglacial periods, the afro-alpine and ericaceous zones were pushed up to higher elevations while the afro-montane forest expanded upwards as well as downwards, in some cases connecting previously isolated patches via temporary forest bridges between mountains (Kebede *et al.*, 2007; Voje *et al.*, 2009). Range dynamics in the afro-alpine region during the Pleistocene interglacials was

thus characterized by increasing connectivity between forest habitats but increasing fragmentation of ericaceous and afro-alpine habitats.

It is therefore thought that species restricted to the ericaceous and afro-alpine zones in one mountain are completely isolated from those in other mountains except via very rare long-distance dispersals, for example via cyclones, whereas species occurring in the montane forest may have expanded via stepwise dispersal through interglacial forest bridges (Hedberg, 1969). Whereas the species pool restricted to altitudes above 3000 m contains 64% single-mountain endemics and 27% mountain-group endemics, the level of such narrow endemism is considerable lower among species which extend further down in the mountains (Hedberg, 1969). This pattern suggests that the ecological islands formed by the upper parts of the mountains have been effectively isolated for long time periods (Hedberg, 1969).

The isolated afro-alpine 'sky islands' became famous already among early naturalists for their remarkable vicariads or vicarious species, i.e. closely related species thought to have evolved in isolation in different mountains (or mountain groups) from a common ancestral population. The parade examples were reported among the giant lobelias and the giant senecios, which apart from gigantism also display other peculiar adaptations to the harsh high-alpine climate (e.g. Fries & Fries 1922). However, a considerable number of the described vicarious afro-alpine species, in particular those reported to be confined to a single mountain, were later reduced to synonymy in taxonomic revisions. The frequency of intermountain speciation seemed to have been overestimated by early explorers, and the herbarium material available from these difficult accessible areas at that time was too limited to allow for careful morphological comparison and assessment of variation patterns (Hedberg 1957, 1995).

This history of the exploration of vicarious taxa in the afro-alpine flora calls for renewed assessment of species numbers, delimitations and relationships using molecular markers. Here these problems are addressed in the eastern African giant lobelias, which belong to section *Rhynchopetalum* (Fresen.) Benth. & Hook. f. of subgenus *Tupa* (G. Don) E. Wimmer of the genus *Lobelia* L. (Mabberley, 1973; Thulin, 1984; Heywood, 1993). Although acknowledging that there had been a tendency for over description of vicarious species, Hedberg (1957, 1969, 1997) maintained that the giant lobelias still provide some of the most elegant examples of vicarious speciation in the afro-alpine flora. The most recent surveys enumerate a total of twenty-two species and five non-autonymous subspecies of giant lobelias in eastern Africa (Knox & Palmer, 1998; Knox *et al.*, 2004; cf. also Thulin, 1984; Knox, 1993). They are tetraploids with 2n = 4x = 28 chromosomes (Knox & Kowal, 1993), and form a monophyletic group together with one Brazilian species (Knox & Palmer, 1998). Their seeds are small and sometimes equipped with tiny wings, promoting wind dispersal (Knox & Palmer, 1998).

An early molecular-based phylogeny of all the 21 eastern African giant lobelia species known at that time was inferred from a comprehensive survey of their plastid DNA restriction-site variation and resolved two major clades, one with unbranched and one with branched inflorescences, the latter including the Brazilian species (Fig. 2; Knox & Palmer, 1998). This study suggested that a branched-inflorescence giant lobelia of Asian/Pacific origin first arrived in eastern Africa on the ancient uplands of Tanzania, before the formation of most of the current tall mountains. The Branched Inflorescence clade contained seven of the eastern African species, most of them still restricted to montane forests on the low and ancient Eastern Arc Mountains in Tanzania. The Unbranched Inflorescence clade contained the 14 remaining species, including those exhibiting the most extreme adaptations to high-alpine conditions, such as a giant rosette growth form and a

hollow, unbranched inflorescence up to 1-5 m tall with sunbird-pollinated flowers (Knox & Palmer 1998).

This study was focused on the Unbranched Inflorescence clade of the giant lobelias, which was suggested based on the plastid DNA variation (Knox & Palmer 1998) to show a primary subdivision corresponding to the Western Rift mountains *vs* the Eastern Rift mountains including Ethiopia (Figs. 1, 2). Six of its 14 species occur exclusively in the uppermost zone on the mountains, the afro-alpine zone proper (*L. bequaertii*, *L. burttii*, *L. gregoriana*, *L. rhynchopetalum*, *L. telekii* and *L. wollastonii*). The other species are either typical of the afro-montane forest zone, frequently occurring along streams or in forest openings, or they often grow in the afro-alpine or ericaceous zones but also extend down into the montane forest zone. Except for the two montane forest species *L. giberroa* (widely distributed in East Africa and Ethiopia) and *L. mildbraedii* (western East Africa and southern Tanzania), all species of the Unbranched Inflorescence clade as currently recognized are narrowly endemic (Fig. 1b). Two species are confined to a single mountain (*L. bequaertii*, *L. deckenii*), and ten species occur in a single group of neighbouring mountains (*L. aberdarica*, *L. bambuseti, L. gregoriana*, *L. telekii, L. stuhlmannii*, *L. wollastonii*, *L. burttii*, *L. thuliniana*, *L. acrochilus*, *L. rhynchopetalum*).

As the two most prominent examples of vicarious speciation patterns among the giant lobelias, Hedberg (1957, 1969) pointed to one species pair, *L. wollastonii* on the Western Rift Mountains and *L. telekii* on the Eastern Rift Mountains, as well as to the species/subspecies swarm of the *L. deckenii* group. The first example was not supported by the plastid phylogeny; *L. wollastonii* was placed in the Predominantly Western Rift clade whereas *L. telekii* was placed in the Predominantly Eastern Rift clade, suggesting that these two high-alpine species have been independently derived from divergent ancestral forest

populations rather than via direct intermountain dispersal of a common high-alpine ancestor (Fig. 2). The primarily high-alpine *L. deckenii* group has traditionally been regarded as morphologically well-defined, including one Western Rift species (*L. bequaertii*) in addition to several Eastern Rift taxa at the species and/or subspecies level (*L. deckenii, L. burtii* and *L. gregoriana*; cf. Hauman, 1934; Hedberg, 1957; 1969; Mabberley, 1973; Thulin, 1984).

Surprisingly, therefore, the uniparentally inherited plastid DNA markers turned out to be in conflict with the morphology-based delimitation of the *L. deckenii* group in placing the Western Rift endemic *L. bequaertii* together with the other Western Rift endemics (*L. stuhlmannii* and *L. wollastonii*), not with the Eastern Rift endemics of the *L. deckenii* group. Based on these results, Knox & Palmer (1998) suggested that *L. bequaertii* may have originated from a hybrid between the *L. deckenii* lineage and a *L. wollastonii*-like ancestor. As further evidence suggesting a hitherto unrecognized importance of hybrid speciation among the giant lobelias, they mentioned conflicting results from restriction site variation in plastid and nuclear ribosomal DNA for the Eastern Rift endemic *L. bambuseti*. Whereas this species grouped with another Eastern Rift endemic, *L. aberdarica*, based on its plastid DNA, its nuclear DNA pointed to a potential origin as a hybrid between *L. aberdarica* and the widespread *L. giberroa*.

Based on range-wide material collected in East Africa and Ethiopia during extensive recent field surveys, this study re-examine species delimitation and suggested speciation processes in the Unbranched Inflorescence clade of the eastern African giant lobelias using Amplified Fragment Length Polymorphism (AFLP) markers, which primarily are derived from biparentally inherited nuclear DNA (Vos *et al.*, 1995). Several studies suggest that the AFLP technique is useful to assess delimitation of and relationships among closely related

species and subspecies (e.g. Koopman *et al.*, 2008; Dasmahapatra *et al.*, 2009; Meudt *et al.*, 2009; Toyama & Yahara, 2009; Garcia-Pereira *et al.*, 2010; Safer *et al.*, 2011). In particular, the study investigated whether 1) the species and subspecies of the giant lobelias currently recognized based on morphology are genetically distinct, 2) the vicarious speciation patterns suggested by Hedberg (1957, 1969, 1997) are consistent with relationships inferred from primarily nuclear AFLP data, and 3) the proposed hybrid origins of *L. bequaertii* and *L. bambuseti* can be corroborated.

2.3 Material and Methods

2.3.1 Plant materials

Fresh young leaf samples were collected from 14 mountains in eastern Africa (Fig. 1a). Five plants of the same species found within an area of 1 hectare (100m x 100m) were considered as representing one population based on AFRO-ALP II project (2007) sampling protocol (Appendix 1). Leaf samples were dried in silica gel and voucher specimens of all five sampled individuals were pressed. The five voucher specimens from each population are deposited at the Natural History Museum, University of Oslo, Norway (1 voucher); Addis Ababa University, National Herbarium of Ethiopia (1 voucher); Sokoine University of Agriculture, Tanzania (1 voucher) and the fourth and fifth vouchers were deposited according to country of collection (East African Herbarium, Kenya, or Makerere University, Uganda). Altogether 162 populations of 14 species of the unbranched inflorescence giant lobelias were collected (in some populations, less than five individuals were found), giving a total of 894 individuals, of which 689 individuals from 13 of the species were successfully genotyped (Table 1). The 14th species, *L. acrochilus*, which was recently described as endemic to Ethiopia by Knox (1993) was not analyzed and seems to have no serious effect on the results, because this species appears to be very closely related to (and previously included in) the other Ethiopian endemic, *L. rhynchopetalum* (cf. Fig. 2).

2.3.2 DNA extraction and AFLP

Total genomic DNA was extracted from the silica-gel-dried leaves using MoleStrips™ DNA Plant kit with an automated GeneMole® robot following the manufacturer's instructions (Qiagen Nordic). Prior to loading the plant material to the GeneMole®, the following modifications were performed: leaf tissue was mechanically grounded in 2.0 mL tubes with two tungsten carbide beads for c. 2 min at 15 Hz in a mixer mill (MM301, Retsch GmbH & Co., Haan, Germany), 300 µL of lysis buffer was added to the crushed material, vortexed, spinned for 20 sec on a centrifuge at 3400 Hz, incubated on a heat block for 15 min at 65°C, and spinned on a centrifuge at 14000 Hz for 3 min. Two hundred micro-litres (200 µL) of the lysate was transferred into new tubes and loaded to the GeneMole® robot.

The AFLP protocol was optimized according to Gaudeul *et al.* (2000) except that: 1) the reaction mixture for the restriction ligation stage was incubated for 3 h; 2) the reaction volumes in the polymerase chain reaction (PCR) for this study were reduced by 50% following Kebede *et al.* (2007); 3) Thirty pre PCR cycles were used instead of 25, and 13 selective PCR cycles instead of 12 (cf. Gaudeul *et al.*, 2000); 4) for each individual, 2.0 µL 6-FAM, 2.0µL VIC and 3.0µL NED labeled selective PCR products were mixed with 11.7 µL formamide and 0.3µL GENESCAN ROX 500 size standard and run on an ABI3100 sequencer (Applied Biosystems).

Thirty primers were tested using two leaf samples of different geographic origins from each species. Twelve primers resulting in high reproducibility and many scorable polymorphic loci were then tested using eight samples of each species. The final AFLP analysis was carried out by using the three best primer combinations (*EcoRI*-AGA -(6FAM) - *Msel* – CAC, *EcoRI* - AGG - (VIC) - *Msel* – CTG and *EcoRI* -ACC - (NED) - *Msel* - CTG). An

error rate test was performed to ensure reproducibility and reliability of the results (Bonin *et al.*, 2004). Seventy-five duplicates representing about 10% of the total sample size were randomly selected (at least 10% of the total number of individuals for each species). Three quarter of the duplicates were re-extracted from new leaf material while the same DNA extracts were used for the remaining quarter.

2.3.3 Data scoring and analysis

Separate raw data for each primer combination were first visualized on Genographer (version 2.1: http://sourceforge.net/projects/genographer/files/) to evaluate the quality of the data and remove failed samples. A total of 77 individuals were removed from the dataset during this screening. This number was high because a sample was removed even if it failed only for one of the three primer combinations. Thereafter, the data were imported to GeneMapper version 4.0 (Applied Biosystems) and AFLP bands in the size range 50–500 base pairs (bp) were scored as present (1) or absent (0). A total of 689 individuals plus 75 duplicates from 13 species and three non-autonomic subspecies (16 taxa) were successfully genotyped. After error rate calculations and data cleaning (Piñeiro *et al.*, 2007), the matrices with band presence (1) or absence (0) for all the three primers were combined and the duplicates removed prior to further analyses.

Principle coordinate analyses (PCoA) were performed with NTSYS-pc V 2.1 (Rohlf, 2000) using the Dice similarity coefficient. Genetic distances were calculated according to Nei and Li (1979) as implemented in PAUP*V. 4.0 (Swofford, 2003). The genetic distances were then used to estimate a neighbor-joining tree in PAUP* V. 4.0 (Swofford, 2003) and Splits Tree4 V. 4.10 (Huson & Bryant, 2008). Because these two methods gave very similar results, only the results from PAUP* V. 4.0 are presented here. Bootstrap support for each node was estimated based on 1000 replicates using the neighbour-joining algorithm. This

approach was considered as sufficient because previous neigbour-joining, maximum parsimony and Bayesian analyses of AFLP data have yielded very similar topologies and support values in phylogenetic trees (Dasmahapatra *et al.*, 2009; Meudt *et al.*, 2009; Toyama & Yahara, 2009; Garcia-Pereira *et al.*, 2010; Mendelson & Wong, 2010).

Population structure was examined using Bayesian model-based clustering methods implemented in the software packages STRUCTURE V. 2.3.3 (Prichtchard *et al.*, 2000) and BAPS V. 5.3 (Corander *et al.*, 2008). STRUCTURE implements a model-based clustering method using Markov Chain Monte Carlo (MCMC) estimation. By comparing the likelihood of the data estimated in different runs for different numbers of groups (*K*), it is possible to identify the optimal *K*. Individuals are assigned to one of the clusters defined by allele frequencies at each locus by chance. First a no admixture model was used with correlated allele frequencies and recessive data. Ten replicate runs for each *K* ranging from 1 to 20 were carried out at the Bioportal of the University of Oslo (www.bioportal.uio.no), using a burn-in of 200 000 iterarions followed by 1 000 000 additional MCMC iterations. For comparison, an admixture model was also run with the same MCMC parameters. The mean – ln likelihood of the data vs *K*, Delta*K* and similarity coefficients for all runs were calculated and plotted by using Structure-sum 2009.R script implemented in the R program (Ehrich, (2006). BAPS is a Bayesian inference of population structure program which identifies the optimal number of clusters as well as the cluster each individual belongs to. The analysis was carried out using a maximum possible number of groups between 1 and 22 (*K*).

Analyses of molecular variance (AMOVA) were performed to investigate the partitioning of genetic variation at three different hierarchical levels using ARLEQUIN V. 3.5 (Excoffier & Lischer, 2010). First, the total variance was partitioned into 'among species'

and ‘within species’ components. Second, the total variation was partitioned into between and within the Predominantly Eastern Rift Clade (PER) and the Predominantly Western Rift Clade (PWR)’ as defined by Knox & Palmer (1998). Third, the total variance in each species that occurred in more than one mountain was partioned into among- and within-mountains components. Fourth, the total variance in each species was partitioned into among- and within-population components.

The input files for PAUP* V. 4.0, Splits Tree4 V. 4.10, ARLEQUIN V. 3.5, BAPS V. 5.3 and STRUCTURE V. 2.3.3 were prepared using R-script AFLPdat (Ehrich, 2006).

2.4 Results

A total of 689 individuals from 154 populations of the 13 species and a total of 1168 markers were retained in the final AFLP matrix (Table 1). Reproducibility of the markers was 97.9%. The principal coordinate analysis (PCoA) of the entire dataset showed quite distinct structuring corresponding well to the morphologically defined species and/or groups of species (Fig. 3). The first, second, and third axes of this PCoA explained 9.4%, 7.7%, and 6.2% of the variation, respectively. To simplify the following presentation and further analysis of the PCoA results, five ‘PCoA groups’ were tentatively delineated: 1) the *Deckenii* group *s. str*. (the eastern Rift endemics *L. deckenii*, *L. burttii*, and *L. gregoriana***)**, 2) the *Giberroa* group (the three mainly high-alpine and exclusively western Rift species *L. bequaertii*, *L. wollastonii*, and *L. stuhlmannii*; and the widespread forest zone species *L. giberroa* and the southern Tanzanian forest zone species *L. thuliniana*,), 3) the *Mildbraedii* group (the three forest zone species *L. aberdarica*, *L. bambuseti*, and *L. mildbraedii*), 4) the *L. telekii* group (the high-alpine Eastern Rift species *L. telekii*, and 5) the *L. rhynchopetalum* group (the Ethiopian high-alpine *L. rhynchopetalum*). Thus, the previously proposed hybrid

species *L. bequaertii* grouped with the other western Rift endemics and *L. bambuseti* grouped with *L. aberdarica*.

In PCoAs (Fig. 4a-c) run separately for AFLP subsets for each of the three first groups delineated above, all described species appeared as clearly distinct except that *L. thuliniana* grouped closely to, although not overlapping with, *L. giberroa* (Fig. 4b). The proposed hybrid species *L. bequaertii* and *L. bambuseti* also appeared as distinct in their respective groups (Figs. 4b, 4c).

Separate PCoAs of species for which different subspecies have been recognized and/or which had been sampled from more than one mountain were carried out. In *Lobelia deckenii*, the only species for which two subspecies have been described from a single mountain (Kilimanjaro), the genetic variation appeared to be quite continuous, although the collections referred to as subspecies *incipiens* were concentrated in the upper right part of the plot (Fig. 4d). In the Kenyan endemic *L. gregoriana*, there was distinct genetic differentiation among mountains corresponding to the three described subspecies (spp. *elgonensis* from Mt. Elgon, spp. *gregoriana* from Mt. Kenya, and spp. *sattimae* from the Aberdare Mts; Fig. 4d). In another Kenyan endemic, *L. telekii*, for which no subspecies have been described, was found a similar but even more distinct divergence among the same three mountains (Fig. 4f). There was also geographically structured, but overlapping variation in the Ethiopian endemic *L. rhynchopetalum*, with the clearest distinction across the Rift Valley (Simen/Choke vs. Bale; Fig. 4g). The separate PCoA of *L. mildbraedii* showed some distinction between the four mountains sampled, with the clearest separation corresponding to the disjunction between the Ugandan Western Rift Mts and the Southern Tanzanian Highlands (axis 1: 17.1%, axis 2: 10.0%; not shown). Analysis of *L. wollastonii* revealed no structuring between the two neighbouring Ugandan mountains sampled

(Muhavura and Ruwenzori; axis 1: 14.6%; axis 2: 8.0%; not shown). Structuring within the widespread *L. giberroa* was not assessed in more detail, since this was done based on more extensive sampling by Kebede *et al.* (2007).

In the STRUCTURE analyses, the clusters largely corresponded to individual morphologically-delimited species or species groups for $K = 9$ and $K = 10$ under the no-admixture and the admixture models, respectively (Fig. 3). For $K = 11$ to $K = 20$, the analyses did not show increased resolution of species clusters (data not shown). The mean –ln likelihood increased from $K = 1$ to a maximum value at $K = 9$ in the no-admixture model and $K = 10$ in the admixture model (Fig. 5). Under the no-admixture model, this value reached its maximum at $K = 9$ and then slightly decreased at K = 10, thereafter slightly increased and flattened out for $K > 10$. Under the admixture model, the value flattened out after $K = 10$. Notably, the clustering of individuals at $K = 9$ was identical under the two models; six of the described species each corresponded to a distinct cluster, and *L. deckenii* grouped with *L. burttii*, *L. giberroa* grouped with *L. thuliniana*, and the Western Rift species *Lobelia bequaertii*, *L. stuhlmannii* and *L. wollastonii* grouped together (Fig. 3). For $K = 10$, the only difference between the two models concerned the grouping of these three Western Rift species. Plots of ΔK vs K (Fig. 5) showed multiple peaks at $K = 9$, $K = 10$ and $K = 14$ for the admixture model and at $K = 8$ and $K = 9$ for the no admixture model.
Taking all STRUCTURE results into consideration (including ΔK and –ln likelihood of both models), it was concluded that $K = 9$ for the no-admixture model represented the most optimal number of clusters for all the values of K tested (1-20) under both models. These nine clusters revealed a structuring of the data that was very similar to that observed in the PCoA plots (Fig. 3). The no-admixture model was chosen because under the admixture model for $K > 8$, the runs were more unstable in that they had more empty groups. In the BAPS analysis, the optimal partition estimate showed ten clusters. The only difference

between these ten BAPS clusters and the nine STRUCTURE clusters from the no-admixture model was that the Western Rift species *L. wollastonii* formed its own cluster, separated from the two other Western Rift species (*L. stuhlmannii* and *L. bequaertii*; not shown).

The neighbour-joining (NJ) tree (Fig. 6) was highly consistent with the results of the PCoA and STRUCTURE analyses (Figs. 3, 4). All individuals belonging to each of the morphology-defined species formed their own group in the tree, many of them with high bootstrap support. The two species recognized as most distinct in the PCoA of the entire dataset, *L. telekii* and *L. rhynchopetalum*, formed distinct NJ groups (BS = 90% and 99%, respectively). Among the three remaining species groups tentatively delineated in the PCoA, the *Deckenii* group s.str. formed a supported NJ group (BS = 90%), the *Giberroa* group formed a separate NJ group but without BS support, and the *Mildbraedii* group did not form a separate NJ group.

The NJ tree showed a separation, although not supported, corresponding to the classification into subsections *Ruppellianae* and *Nicotianifoliae* (Fig. 6). The NJ tree was also mainly consistent with the plastid DNA phylogeny of Knox & Palmer (1998; Fig. 2): Although not supported, the NJ tree also grouped the species into a Predominantly Western Rift group (PWR, identical to the *Giberroa* group delineated in the PCoA) and a Predominantly Eastern Rift group (PER) as in the plastid DNA phylogeny, except that the Tanzanian Southern Highlands endemic *L. thuliniana* in this tree was inferred as belonging to PWR, not PER (Fig. 6). The position of *L. thuliniana* had however not been supported in the plastid phylogeny (Fig. 2). In the NJ tree, this low-altitudinal stream-side plant formed a supported group with the widespread, low-altitudinal forest plant *L. giberroa*. The remaining western Rift species (*L. bequaertii, L. wollastonii, L. stuhlmannii*), which are restricted to this area, formed a moderately supported group in the NJ tree as in the plastid

tree. Likewise, within the Predominantly Eastern Rift group, the *Deckenii* group was identical to that inferred with high support in the plastid tree. This group was however more resolved in the NJ tree, in which the two Tanzanian species (*L. deckenii* on Mt Kilimanjaro and *L. burttii* on the neighboring Mt Meru) grouped together as sister to the Kenyan species (*L. gregoriana*).

The previously proposed hybrid origins were neither supported in the NJ tree; *L. bequartii* grouped with the other Western Rift endemics in agreement with the plastid tree, and not in the *Deckenii* group of the Eastern Rift as previously suggested based on morphology; whereas *L. bambuseti* appeared as distinctly differentiated from its proposed progenitors *L. aberdarica* and *L. giberroa*.

Notably, as also seen in the plastid tree (Fig. 2), the six species which mainly are restricted to the uppermost altitudinal zones occurred scattered all over the NJ tree, five of them grouping closely with species that mainly or frequently occur in the low-altitudinal montane forest zone (Fig. 6).

In addition, the NJ tree (Fig. 6) depicted within-species differentiation similar to the PCoA plots (Fig. 4c-g). The three subspecies of the Kenyan endemic *L. gregoriana* formed separate clusters (spp. *elgonensis* from Mt. Elgon, BS = 94%; spp. *gregoriana* from Mt. Kenya, BS = 90%; spp. *sattimae* from Aberdare Mountains, BS = 67%). The subspecies described from a single mountain (Kilimanjaro) endemic *L. deckenii* were not clearly differentiated. In the Kenyan endemic *L. telekii*, most individuals grouped into three mountain-specific lineages (BS = 100%, 73%, 55%).

In the AMOVAs, the partitioning of the genetic variation was in all cases highly significant ($P < 0.001$; Table 2). In the analysis of the total dataset, nearly half (45.44%) of the AFLP variation was found among the morphology-defined species, and only 13.85% among populations within species. Only 13.96% of the variation was found between the predominantly Western Rift and Eastern Rift groups. In separate two-level analyses for each species, very high proportions of the variation were found within populations (62-91%). In the species sampled from more than one mountain, the among-mountain components were highly variable (3-33%). Highest among-mountain variation was observed in the Eastern Rift (Kenyan) endemics *L. telekii* and *L. gregoriana*, whereas very little was observed in the Western Rift endemics *L. stuhlmannii* and *L. wollastonii*.

2.5 Discussion

2.5.1 Species delimitation and number of taxa

The AFLP results demonstrate that all the 13 analyzed species of the eastern African Unbranched Inflorescence giant lobelias, as they are currently recognized based on morphology, are also distinct genetically. All of them formed separate groups in the NJ tree, in most cases with high bootstrap support (Fig. 6), and appeared as clearly distinct in the PCoAs except that *L. thuliniana* grouped closely, but not overlapping with *L. giberroa* (Figs. 4, 6). The clusters inferred from the STRUCTURE and BAPS analyses comprised of six (STRUCTURE) or seven (BAPS) individual species clusters, and the remaining three clusters each consisted of 2-3 species which formed distinct subgroups in the NJ and PCoA analyses (Figs. 3, 4). Thus, both STRUCTURE and BAPS analyses produced results that were congruent to the PCoA and NJ results, but provided less resolution in identifying distinct species and subspecies. Distinct taxonomic structuring of the AFLP variation was also evident from the AMOVA analysis, in which almost half of the total variation was

found among the morphology-defined species, and only ~14% among populations within species.

Thus, it is concluded that although there was a tendency to describe too many species by the early explorers of the fragmented afro-alpine ecosystem (Hedberg, 1957, 1969, 1997), the delimitation of species in current taxonomic treatments of the famous giant lobelias (Knox & Palmer, 1998; Knox *et al.*, 2004; cf. also Thulin, 1984; Knox, 1993) is reasonable and corresponds to genetically discernable groups of populations. This also applies to the three subspecies described of *L. gregoriana*, which occur in three different mountains, whereas the two sympatric subspecies described of the Kilimanjaro endemic *L. deckenii* are not genetically distinct and may rather reflect continuous morphological variation along altitudinal/ecological gradients (Figs. 4d-e, 6). In a single case, were discovered three very distinct, but hitherto undescribed mountain-specific genetic lineages within one described species (*L. telekii*). Whether these lineages can be recognized morphologically and described as different taxa must await further studies. In some other species occurring in more than one mountain geographically structured genetic variation were found, but most likely not warranting taxonomic recognition (Fig. 1, 4g). For one species (*L. burttii*), the previously reported morphology-based subspecies differentiation could not be assess because of lack of material.

2.5.2 Relationships among species and speciation processes

A prominent feature of the AFLP-based NJ tree is that the species which are restricted to the highest altitudes in the afro-alpine mountains, i.e. occurring in the ericaceous zone and the afro-alpine zone proper but not extending downwards into the montane forest zone, tend to be intermixed with species growing at lower altitudes (Fig. 6). Although all sister species relationships among the giant lobelias based on the AFLP data could not be confidently

inferred, this pattern suggests that the closest relatives of individual high-altitude restricted species or species pairs tend to be found among species which are typical of, or also grow in, montane forest habitats. This finding is consistent with the pattern observed in the plastid phylogeny of Knox & Palmer (1998; Fig. 2), supporting the following main evolutionary scenario for the unbranched inflorescence giant lobelias: 1) initial expansions of ancestral forest populations, 2) independent high-altitude adaptation and speciation in different mountain groups, 3) direct dispersal of high-alpine-adapted populations among mountains within mountain groups, and 4) in some cases, geographical (vicarious) speciation among mountains within mountain groups.

The inferences that can be made from this primarily nuclear AFLP data are, with some notable exceptions outlined below, consistent with relationships and speciation processes earlier inferred from morphology (Hedberg, 1957, 1969, Mabberley, 1973, Thulin, 1984, Knox, 1993) and/or plastid DNA restriction site polymorphisms (Knox & Palmer, 1998). Further detailed phylogenetic studies based on nuclear DNA sequences are however necessary to address some remaining problems, such as the exact delimitation of the Predominantly Western Rift clade (PWR) versus the Predominantly Eastern Rift clade (PER), and to confirm the phylogenetic position of *L. thuliniana*. A separation between one PWR and one PER group as indicated in the plastid phylogeny (Knox & Palmer, 1998; Fig. 2; with exception of the placement of *L. thuliniana*) also appeared in the AFLP-based NJ tree (Fig. 6), but this separation was not supported. The position of *L. thuliniana* as part of the PER was nevertheless not supported in the plastid phylogeny. In the NJ tree, this low-altitudinal stream-side plant formed a group (BS=90) with the widespread, low-altitudinal forest zone plant *L. giberroa* within the PWR.

There was no evidence supporting that hybrid speciation has played a role in the evolution of the giant lobelias. The high-alpine Western Rift endemic *L. bequaertii*, which was proposed to have originated as a hybrid between a Western Rift species and an Eastern Rift species because of conflicting morphological and plastid DNA data (Knox & Palmer, 1998), turned out to belong to the Western Rift group based on this AFLP data, in accordance with the previous plastid data. Thus, both nuclear and plastid data suggest that *L. bequaertii* originated by geographical (and/or ecological) speciation from an ancestor exclusively shared with the two other Western Rift species, and that its morphological and ecological similarity to the *Deckenii* group of the Eastern Rift therefore must be caused by parallel evolution rather than hybridization. The AFLP data did neither support the other proposed example of hybrid speciation, which involved three montane forest zone species of the giant lobelias. Whereas conflicting results from restriction site variation in plastid and nuclear ribosomal DNA in the Eastern Rift endemic *L. bambuseti* might suggest that it originated as a hybrid between the Eastern Rift endemic *L. aberdarica* and the widespread *L. giberroa* (Knox & Palmer, 1998), *L. bambuseti* formed a lineage distinct from the other two species based on the AFLP data (Fig.6).

As predicted by Hedberg (1957, 1969), it is observed that the most distinct vicarious speciation patterns to be presented by the predominantly high-alpine species groups of the giant lobelias, which probably have been efficiently isolated on their sky islands for a long time except for rare long-distance dispersals directly among mountain peaks. These findings are in concordance with the montane forest bridge hypothesis, suggesting that the montane forest species have been less isolated because of repeated formation of interglacial forest bridges between mountains.

However, contrary to Hedberg (1957, 1969), it was found that vicarious high-alpine speciation in the giant lobelias has been restricted to individual mountain groups, suggesting that high-altitude adaptation occurred independently within each mountain group followed by intermountain dispersal within each mountain group and in some cases geographical speciation. The first of the two most prominent cases described by Hedberg (1957, 1969) involved vicarious speciation between the Western Rift group of mountains (*L. wollastonii*) and the Eastern Rift group (*L. telekii*). However, these two species were inferred to be distantly related in the AFLP-based NJ tree (Fig. 6) in agreement with the plastid phylogeny (Knox & Palmer, 1998; Fig. 2), suggesting that these two high-alpine species have been independently derived from divergent ancestral forest populations rather than via direct intermountain dispersal of a common high-alpine ancestor.

In the second case, Hedberg's (1957, 1969) circumscription of the *L. deckenii* group included the Western Rift endemic *L. bequaertii* in addition to the species/subspecies swarm of the Eastern Rift mountains, implying vicarious speciation following long-distant dispersal between these two mountain groups. These taxa are morphologically quite similar, mainly differing in minute characters such as splitting of the corolla, pubescence of the bracts, corolla and anthers, and shape of the bracts (Hedberg, 1957). Most previous taxonomic studies placed *L. bequaertii* in the *Deckenii* group, and all taxa have been recognized as subspecies of *L. deckenii* (Hauman, 1934; Hedberg, 1957; 1969, Mabberley, 1973; Thulin, 1984). The alternative hypothesis of a hybrid origin of *L. bequaertii* (Knox & Palmer, 1998; cf. the discussion of hybrid speciation above) also necessitates that long-distance dispersal between the mountain groups has taken place. However, the nuclear DNA data suggest that *L. bequaertii* rather belongs to a group of Western Rift endemics.

Interestingly, the two most distinct intermountain vicarious patterns among the giant lobelias as inferred from this AFLP data were formed independently in some of the same Eastern Rift Mountains. The first corresponds to the classical example of the *L. deckenii* group of Hedberg (1957, 1969), but excluding the Western Rift species *L. bequaertii*. In the Eastern Rift Mountains, a group of three closely related but distinct species, at least one of them with distinct subspecies, have evolved among different mountains. In the analysis, *L. gregoriana*, which occurs as three distinct subspecies on three Kenyan mountains, was inferred as sister to a group of two Tanzanian species, of which *L. deckenii* is restricted to Kilimanjaro and *L. burttii* occurs on the neighbouring Mt. Meru (as well as some other Tanzanian mountains, which were not sampled; Figs.1, 4, 6). The second pattern detected from the AFLP data was particularly interesting because it involves a single described species only, *L. telekii*, which has evolved into three genetically distinct but hitherto unrecognized intraspecific lineages in the same three Kenyan mountains as *L. gregoriana* of the *L. deckenii* group, independently of that species. The results further suggest that the unbranched inflorescence giant lobelias are largely outcrossing, with most of the genetic variation in each species found within rather than among populations. This finding is in accordance with observations of sunbirds (*Nectarinia* spp.) pollinating their flowers (Knox &Palmer, 1998).

2.6 Conclusion

The AFLP results demonstrate that all the 13 analyzed species of the eastern African giant lobelias with unbranched inflorescence, as they are currently recognized based on morphology, are also distinct genetically. Thus, it is concluded that although there was a tendency to describe too many species by the early explorers of the fragmented afro-alpine ecosystem (Hedberg 1957, 1969, 1997); the delimitation of species in current taxonomic treatments of the famous giant lobelias (Knox & Palmer, 1998; Knox *et al.*, 2004; cf. also

Thulin, 1984; Knox, 1993) is reasonable and corresponds to genetically discernable groups of populations. This also applies to several of the described subspecies.

High-altitude-restricted species were intermixed with species occurring in the forest zone in the AFLP-based tree, supporting a main scenario of initial expansions of ancestral forest populations followed by parallel high-altitude adaptation and speciation in different mountain groups. The relationships among species inferred from this primarily nuclear AFLP data corroborated those earlier proposed by morphology and/or plastid DNA restriction site polymorphisms, but with some notable exceptions. There was no evidence supporting that hybrid speciation has played a role in the evolution of the giant lobelias. In particular, it was found that the supposed hybrid species *L. bequaertii* grouped with the other Western Rift endemics, suggesting that its morphological and ecological similarity to the *Deckenii* group of the Eastern Rift is caused by parallel evolution rather than hybridization. The results confirmed however that giant lobelias indeed provide elegant examples of ‘vicarious speciation’ among mountains as suggested by Hedberg (1969), although not involving direct dispersal among different groups of mountains. The two most distinct intermountain vicarious patterns identified among the giant lobelias were primarily high-alpine and found to have formed independently in some of the same Eastern Rift Mountains.

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Table 1: Material of Lobelia successfully genotyped for AFLPs, with identity numbers (DNA Bank ID in the Corema database and population ID), collection site, coordinates, and number of individuals analyzed per population (n) and total number of individuals analyzed per species (N)

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
L. aberdarica R.E.Fr. & T.C.E.Fr.	O-DP-35998 - O-DP-36002	KN_0301	Mt. Elgon	1.100667/34.6215	5
	O-DP-27246 - O-DP-27260	KN_0394	Mt.Elgon	1.093167/ 34.623667	13
	O-DP-27314	KN_0450	Mt.Elgon	1.088333/ 34.628333	1
	O-DP-27318 -O-DP-27322	KN_0453	Cherangani Hills	1.139333/ 35.341333	4
	O-DP-27325 - O-DP-27327	KN_0454	Cherangani Hills	1.117667/ 35.454	3
	O-DP-27328 - O-DP-27332	KN_0455	Cherangani Hills	1.068333/ 35.319833	4
	O-DP-27333 - O-DP-27341	KN_0456b	Cherangani Hills	-0.524333/ 36.716667	5
	O-DP-27344 - O-DP-27350	KN_0457	Aberdare Mountains	-0.524333/ 36.716667	4
	O-DP-27432 - O-DP-27435	KN_0474	Aberdare Mountains	(-)	4
	O-DP-27442 - O-DP-27445	KN_0476	Aberdare Mountains	-0.535667/36.705167	5
					N= 48
L. bambuseti R.E.Fr. & T.C.E.Fr.	O-DP-36885 - O-DP-45690	KN_1112	Mt. Kenya	-0.168833/ 37.208167	10
	O-DP-27353 - O-DP-27357	KN_0458[1]	Aberdare Mountains	-0.517667/ 36.690333	4
	O-DP-27426 - O-DP-27430	KN_0473[2]	Aberdare Mountains	-0.517667/ 36.690333	5
	O-DP-28398 - O-DP-28402	KN_0697	Aberdare Mountains	-0.339/ 36.668	5
	O-DP-28566 - O-DP-28570	KN_0742	Aberdare Mountains	-0.339/ 36.683333	5
	O-DP-36880 - O-DP-45685	KN_1111	Mt. Kenya	-0.168833/ 37.208167	9
					N= 38
L. bequaertii De Wild	O-DP-40484 - O-DP-40487	UG_2243	Ruwenzori Mountains	0.385017/ 29.9273	4
	O-DP-43051 - O-DP-43055	UG_2283	Ruwenzori Mountains	0.3852/ 29.913733	4
	O-DP-40879	UG_2347	Ruwenzori Mountains	0.392383/ 29.916983	1
	O-DP-40952 - O-DP-40956	UG_2362	Ruwenzori Mountains	0.376867/ 29.93	4
	O-DP-43648 - O-DP-43652	UG_2426	Ruwenzori Mountains	0.384883/ 29.888667	5
	O-DP-41945	UG_2591	Ruwenzori Mountains	0.376367/ 29.900533	1
					N= 19

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
L. burttii E.A. Bruce	O-DP-38624 - O-DP-38628	TZ_0408[3]	Mt. Meru	-3.218/ 36.766833	5
	O-DP-38906 - O-DP-38910	TZ_0459	Mt. Meru	-3.221667/ 36.753933	5
	O-DP-39069 - O-DP-39073	TZ_0492	Mt. Meru	(-)	5
	O-DP-39094 - O-DP-39098	TZ_0498	Mt. Meru	-3.217833/ 36.770667	5
					N= 20
Lobelia deckenii (Asch.) Hemsl. spp. *deckenii*	O-DP-37017 - O-DP-37018	TZ_0025	Mt.Kilimanjaro	-3.03425/ 37.243	2
	O-DP-37276 - O-DP-37280	TZ_0094	Mt.Kilimanjaro	-3.052333/ 37.275167	5
	O-DP-37353 - O-DP-37356	TZ_0117	Mt.Kilimanjaro	-3.062783/ 37.278167	4
	O-DP-37548 - O-DP-37552	TZ_0155	Mt.Kilimanjaro	-3.086217/ 37.3234	5
	O-DP-37614 - O-DP-37618	TZ_0173	Mt.Kilimanjaro	-3.081683/ 37.323483	4
	O-DP-37991	TZ_257	Mt.Kilimanjaro	-3.109967/ 37.421117	1
	O-DP-38106	TZ_288	Mt.Kilimanjaro	-3.109967/ 37.421117	1
	O-DP-38135	TZ_294	Mt.Kilimanjaro	-3.109967/ 37.441217	1
	O-DP-38214 - O-DP-38217	TZ_0313	Mt.Kilimanjaro	-3.109967/ 37.433833	4
	O-DP-42700 - O-DP-42704	TZ_0333	Mt.Kilimanjaro	-3.109967/ 37.433367	5
	O-DP-45591 - O-DP-45594	TZ_0542	Mt.Kilimanjaro	-3.113317/ 37.31755	5
	O-DP-45600 - O-DP-45601	TZ_0545	Mt.Kilimanjaro	-3.113317/ 37.31755	2
	O-DP-39375 - O-DP-39376	TZ_0826	Mt.Kilimanjaro	-3.153333/ 37.4855	2
	O-DP-39378 - O-DP-39381	TZ_0827	Mt.Kilimanjaro	-3.1595/ 37.493167	5
L. deckenii spp. *incipiens* E.B. Knox	O-DP-42711 - O-DP-38302	TZ_0335	Mt.Kilimanjaro	(-)	4
	O-DP-38313 - O-DP-38317	TZ_0338	Mt.Kilimanjaro	-3.1535/ 37.485	5
	O-DP-39451 - O-DP-39455	TZ_0852	Mt.Kilimanjaro	(-)	5
					N= 60
L. giberroa Hemsl.	O-DP-43834 - O-DP-43835	KN_0451	Mt.Elgon	1.176967/ 35.518383	2
	O-DP-42076	KN_0001	Mt.Elgon	1.06365/ 34.704217	1

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
	O-DP-28571 - O-DP-28575	KN_0743[4]	Aberdare Mountains	-0.320167/ 36.7685	4
	O-DP-45581 - O-DP-45584	TZ_0540	Mt.Kilimanjaro	-3.1519/ 37.291633	4
	O-DP-45586 - O-DP-45589	TZ_0541	Mt.Kilimanjaro	-3.15185/ 37.289433	3
	O-DP-45589 - O-DP-38934	TZ_0466	Mt. Meru	-3.221817/ 36.792333	3
	O-DP-38937 - O-DP-38940	TZ_0467	Mt. Meru	-3.221817/ 36.792333	4
	O-DP-38941 - O-DP-38945	TZ_0468	Mt. Meru	-3.221817/ 36.792333	5
	O-DP-38946	TZ_0469	Mt. Meru	-3.221817/ 36.792333	1
	O-DP-39228 - O-DP-39232	TZ_0703	Njombe Highlands	-9.5185/ 34.783833	5
	O-DP-39213 - O-DP-39217	TZ_0700	Njombe Highlands	-9.504833/ 34.753	5
	O-DP-39253 - O-DP-39257	TZ_0707	Kitulo Highlands	-9.076333/ 33.984167	5
	O-DP-40190 - O-DP-40194	UG_2169	Muhavura Hills	-1.367483/ 29.671317	5
	Not in DNA bank yet	TZ_864	Pare	(-)	5
	O-DP-40369 - O-DP-40371	UG_2220	Ruwenzori Mountains		3
	O-DP-40374 - O-DP-40378	UG_2221	Ruwenzori Mountains	0.354667/ 29.97	5
	O-DP-40380 - O-DP-40381	UG_2222	Gahinga Hills	-1.354667/ 29.639333	2
	O-DP-40727 - O-DP-40730	UG_2310	Ruwenzori Mountains	0.3609/ 29.9945	4
	O-DP-40578 - O-DP-40582	UG_2273	Ruwenzori Mountains	0.375/ 29.951417	4
	O-DP-40583 - O-DP-40586	UG_2274	Ruwenzori Mountains	0.36275/ 29.961583	4
	O-DP-40597	UG_2276	Ruwenzori Mountains	0.379917/ 29.944967	1
	O-DP-39235 - O-DP-39240	TZ_0704[6]	Poroto Highlands	(-)	4
	O-DP-39243 - O-DP-39247	TZ_0705[7]	Poroto Highlands	(-)	5
	O-DP-39278 - O-DP-39279	TZ_0712[8]	Poroto Highlands	(-)	2
	O-DP-39280 - O-DP-39289	TZ_0713[9]	Poroto Highlands	(-)	8
					N= 92
L. gregoriana Baker f. spp. *elgonensis* (R.E.Fr. & T.C.E.Fr.) E.B. Knox					
	O-DP-42077 - O-DP-34712	KN_0002	Mt.Elgon	1.1239/ 34.601983	5
	O-DP-34858 - O-DP-34860	KN_0036	Mt.Elgon	1.105667/ 34.601833	3

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
	O-DP-35266 - O-DP-35270	KN_0138	Mt.Elgon	1.123783/ 34.589667	5
					N= 13
L. gregoriana spp. *sattimae* (R.E.Fr. & T.C.E.Fr.) E.B. Knox					
	O-DP-27637 - O-DP-27641	KN_0525	Aberdare Mountains	-0.306167/ 36.626	5
	O-DP-27662 - O-DP-27666	KN_0530	Aberdare Mountains	-0.305333/ 36.624833	4
	O-DP-42203 - O-DP-42207	KN_0624	Aberdare Mountains	-0.333833/ 36.641667	5
	O-DP-28466 - O-DP-28469	KN_0711	Aberdare Mountains	-0.3335/ 36.643167	4
L. gregoriana spp. *gregoriana* Baker f.	O-DP-42441 - O-DP-42445	KN_0786	Mt. Kenya	-0.05625/ 37.288883	5
	O-DP-28611 - O-DP-28615	KN_0791	Mt. Kenya	-0.062983/ 37.29625	5
	O-DP-28833 - O-DP-28835	KN_0858	Mt. Kenya	-0.1392/ 37.314317	3
	O-DP-28878 - O-DP-28881	KN_0868	Mt. Kenya	-0.121383/ 37.295633	4
	O-DP-29022 - O-DP-29026	KN_0899	Mt. Kenya	-0.121417/ 37.295633	5
	O-DP-36465 - O-DP-36469	KN_0995	Mt. Kenya	-0.150333/ 37.33095	5
	O-DP-36731 - O-DP-36733	KN_1075	Mt. Kenya	-0.146117/ 37.347967	3
					N= 64
L. mildbraedii Engl.	O-DP-39250 - O-DP-39252	TZ_0706	Kitulo highlands	(-)	4
	O-DP-39218 - O-DP-39222	TZ_0701	Njombe highlands	-9.5185/ 34.783833	5
	O-DP-39223 - O-DP-39227	TZ_0702	Njombe highlands	-9.501667/ 34.7585	5
	O-DP-39258 - O-DP-39262	TZ_0708	Kitulo highlands	-9.088833/ 33.869333	4
	O-DP-39263 - O-DP-39266	TZ_0709	Kitulo highlands	-9.054167/ 33.917333	4
	O-DP-40202 - O-DP-40204	UG_2171	Muhavura Hills	-1.367483/ 29.671317	4
	O-DP-45638 - O-DP-45641	UG_2601	Echuya forest	-1.255333/ 29.809333	5
					N= 33
L. rhynchopetalum Hemsl.	O-DP-29728 - O-DP-29732	ET_0122	Simen Mountains	13.282733/ 38.110767	5
	O-DP-29850 - O-DP-29854	ET_0157	Simen Mountains	13.28525/ 38.118383	5

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
	O-DP-30493 - O-DP-30497	ET_0324	Simen Mountains	13.25135/ 38.20225	5
	O-DP-30498 - O-DP-30502	ET_0325	Simen Mountains	13.200733/ 38.26695	5
	O-DP-30907 - O-DP-30911	ET_0432	Simen Mountains	13.349067/ 38.2625	5
	O-DP-30998 - O-DP-31002	ET_0454	Simen Mountains	13.333333/ 38.233333	4
	O-DP-31032 - O-DP-31036	ET_0462	Simen Mountains	13.328467/ 38.242967	5
	O-DP-31268 - O-DP-31271	ET_0519	Bale Mountains	6.868667/ 39.882183	4
	O-DP-44380 - O-DP-45055	ET_0538	Bale Mountains	(-)	4
	O-DP-31440 - O-DP-31444	ET_0558	Simen Mountains	13.3285/ 38.240917	5
	O-DP-42130 - O-DP-42134	ET_0621	Simen Mountains	13.32705/ 38.242467	5
	O-DP-45127 - O-DP-45131	ET_0633	Bale Mountains	6.855017/ 39.878017	5
	O-DP-31849 - O-DP-31853	ET_0706	Bale Mountains	6.879267/ 39.868967	5
	O-DP-32065 - O-DP-32069	ET_0765	Bale Mountains	6.870283/ 39.8678	5
	O-DP-42114 - O-DP-32258	ET_0811	Bale Mountains	6.844833/ 39.88045	5
	O-DP-32704	ET_0912	Bale Mountains	6.86945/ 39.89465	1
	O-DP-32785 - O-DP-42029	ET_0933	Bale Mountains	6.882183/ 39.8883	5
	O-DP-33134 - O-DP-33138	ET_1029	Bale Mountains	6.8931/ 39.89735	5
	O-DP-33611 - O-DP-33615	ET_1331	Choke Hills	10.642/ 37.835667	4
	O-DP-33726 - O-DP-33730	ET_1354	Choke Hills	10.656/ 37.825667	5
	O-DP-33846 - O-DP-33850	ET_1378	Choke Hills	10.638167/ 37.839167	5
	O-DP-33866 - O-DP-33870	ET_1382	Choke Hills	10.6575/ 37.822	5
					N= 104
L. stuhlmannii Sweinf. & E.A. Bruce	O-DP-44367	UG_2004	Gahinga Hills	(-)	4
	O-DP-39770 - O-DP-39771	UG_2054	Muhavura Hills	-1.376283/ 29.671533	2
	O-DP-39782 - O-DP-39783	UG_2057	Muhavura Hills	-1.376917/ 29.67205	2
	O-DP-43040 - O-DP-43041	UG_2087	Muhavura Hills	-1.382717/ 29.677983	2
	O-DP-43042 - O-DP-39873	UG_2088	Muhavura Hills	-1.382717/ 29.677983	4
	O-DP-39894 - O-DP-39895	UG_2095	Muhavura Hills	-1.3782/ 29.673333	2
	O-DP-40230 - O-DP-42938	UG_2178	Muhavura Hills	-1.377167/ 29.672367	5

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
	O-DP-40568 - O-DP-40572	UG_2271	Ruwenzori Mountains	(-)	5
	O-DP-40598	UG_2277	Ruwenzori Mountains	0.379917/ 29.944967	1
	O-DP-40712 - O-DP-40716	UG_2307	Ruwenzori Mountains	0.387967/ 29.918333	5
	O-DP-40737 - O-DP-40741	UG_2312	Ruwenzori Mountains	0.400783/ 29.9365	5
	O-DP-40849 - O-DP-40850	UG_2340	Ruwenzori Mountains	0.392383/ 29.916983	2
	O-DP-40942 - O-DP-40946	UG_2360	Ruwenzori Mountains	0.376867/ 29.93	4
	O-DP-41845 - O-DP-41848	UG_2571	Ruwenzori Mountains	0.343967/ 29.928	4
	O-DP-41930 - O-DP-41932	UG_2588	Ruwenzori Mountains	0.381117/ 29.941217	3
					N= 50
L. telekii Schweinf.	O-DP-34713 - O-DP-34715	KN_0003	Mt.Elgon	1.1239/ 34.601983	2
	O-DP-34793 - O-DP-34796	KN_0019	Mt.Elgon	1.105667/ 34.601833	4
	O-DP-34886 - O-DP-34890	KN_0042	Mt.Elgon	1.1025/ 34.605833	5
	O-DP-35080 - O-DP-35084	KN_0092	Mt.Elgon	1.124/ 34.590333	5
	O-DP-35230 - O-DP-35234	KN_0128	Mt.Elgon	1.123167/ 34.607	5
	O-DP-35265	KN_0137	Mt.Elgon	1.123167/ 34.607	1
	O-DP-35355 - O-DP-35359	KN_0156	Mt.Elgon	1.1185/ 34.585667	5
	O-DP-36246 - O-DP-36248	KN_0355	Mt.Elgon	1.091667/ 34.617667	3
	O-DP-27436 - O-DP-27440	KN_0475	Aberdare Mountains	(-)	5
	O-DP-27578 - O-DP-27582	KN_0509	Aberdare Mountains	-0.305333/ 36.624833	5
	O-DP-27603 - O-DP-27607	KN_0514	Aberdare Mountains	-0.306167/ 36.622667	5
	O-DP-28095 - O-DP-28099	KN_0633	Aberdare Mountains	-0.334667/ 36.641333	5
	O-DP-28529 - O-DP-28533	KN_0726	Aberdare Mountains	-0.337167/ 36.650333	5
	O-DP-28591 - O-DP-28595	KN_0783	Mt. Kenya	-0.0849/ 37.28605	5
	O-DP-28624 - O-DP-28625	KN_0793	Mt. Kenya	-0.067617/ 37.2978	2
	O-DP-42280 - O-DP-28698	KN_0824	Mt. Kenya	-0.1416/ 37.313917	5
	O-DP-28922 - O-DP-28926	KN_0877	Mt. Kenya	-0.121383/ 37.295633	5
	O-DP-29164 - O-DP-45662	KN_0936	Mt. Kenya	-0.133583/ 37.2765	5
	O-DP-29247 - O-DP-29251	KN_0955	Mt. Kenya	-0.147383/ 37.331567	5

Taxon	DNA Bank ID	Population ID	Collection site	Coordinates Latitude/Longitude	n
	O-DP-36432 - O-DP-36436	KN_0981	Mt. Kenya	-0.14855/ 37.332117	5
	O-DP-36500 - O-DP-36504	KN_1002	Mt. Kenya	-0.150333/ 37.33095	5
	O-DP-36605	KN_1027	Mt. Kenya	-0.15/ 37.316667	1
	O-DP-36650 - O-DP-36654	KN_1041	Mt. Kenya	-0.146117/ 37.347967	3
	O-DP-36821 - O-DP-36825	KN_1100[5]	Mt. Kenya	-0.1693/ 37.275333	5
					N= 101
L. thuliniana E.B. Knox	O-DP-39290 - O-DP-39298	TZ_0714	Mafinga Highlands	-8.390667/ 35.184667	10
					N= 10
L. wollastonii Baker f.	O-DP-39795 - O-DP-39796	UG_2059[10]	Muhavura Hills	-1.382717/ 29.677983	2
	O-DP-40002 - O-DP-40006	UG_2125[11]	Muhavura Hills	-1.382033/ 29.676733	5
	O-DP-40210 - O-DP-40214	UG_2173	Muhavura Hills	-1.382767/ 29.677833	5
	O-DP-40752 - O-DP-40756	UG_2315	Ruwenzori Mountains	0.400783/ 29.9365	5
	O-DP-42988 - O-DP-42992	UG_2414	Ruwenzori Mountains	0.376767/ 29.901483	5
	O-DP-41200 - O-DP-41201	UG_2425	Ruwenzori Mountains	0.378767/ 29.903383	2
	O-DP-41251- O-DP-41252	UG_2436	Ruwenzori Mountains	0.384267/ 29.88875	2
	O-DP-41419 - O-DP-41423	UG_2472	Ruwenzori Mountains	0.372583/ 29.886733	4
	O-DP-41504 - O-DP-41507	UG_2495	Ruwenzori Mountains	0.391778/ 29.880389	4
	O-DP-42935 - O-DP-41552	UG_2510	Ruwenzori Mountains	0.38555/ 29.8857	4
	O-DP-41642	UG_2529	Ruwenzori Mountains	0.382283/ 29.888383	1
	O-DP-41789	UG_2559	Ruwenzori Mountains	0.375533/ 29.889233	1
	O-DP-41790 - O-DP-41794	UG_2560	Ruwenzori Mountains	0.35535/ 29.887917	5
	O-DP-41835 - O-DP-41838	UG_2569	Ruwenzori Mountains	0.3476/ 29.8878	4
	O-DP-41843 - O-DP-41844	UG_2570	Ruwenzori Mountains	0.347433/ 29.891167	2
					N= 51

(-) indicates that there are no GPS readings for these populations largely because of bad weather during field work

Table 2: AMOVAs for the unbranched inflorescence giant lobelias based on the AFLP data. A. Among and within species based on the entire dataset, B. Among and within the Predominately Eastern Rift Clade (PERC) versus the Predominantly Western Rift Clade (PWRC) based on the entire dataset, C. Among and within populations based on datasets for individual species, D. Among and within mountains based on dataset for individual species (mountains: AB = Mt. Aberdares, BL = Bale, CH = Cherangani Hills, EL = Mt.Elgon, ECH = Echuya forest, GH = Gahinga, KE = Mt. Kenya, KL = Kilimanjaro, KT = Kitulo, MH = Mhavura, MR = Mt. Meru, NJ = Njombe, PR = Pare, POR = Poroto and RW= Ruwenzori mountains. The significance of variance components and Φ-statistics was P < 0.0001 for all tests

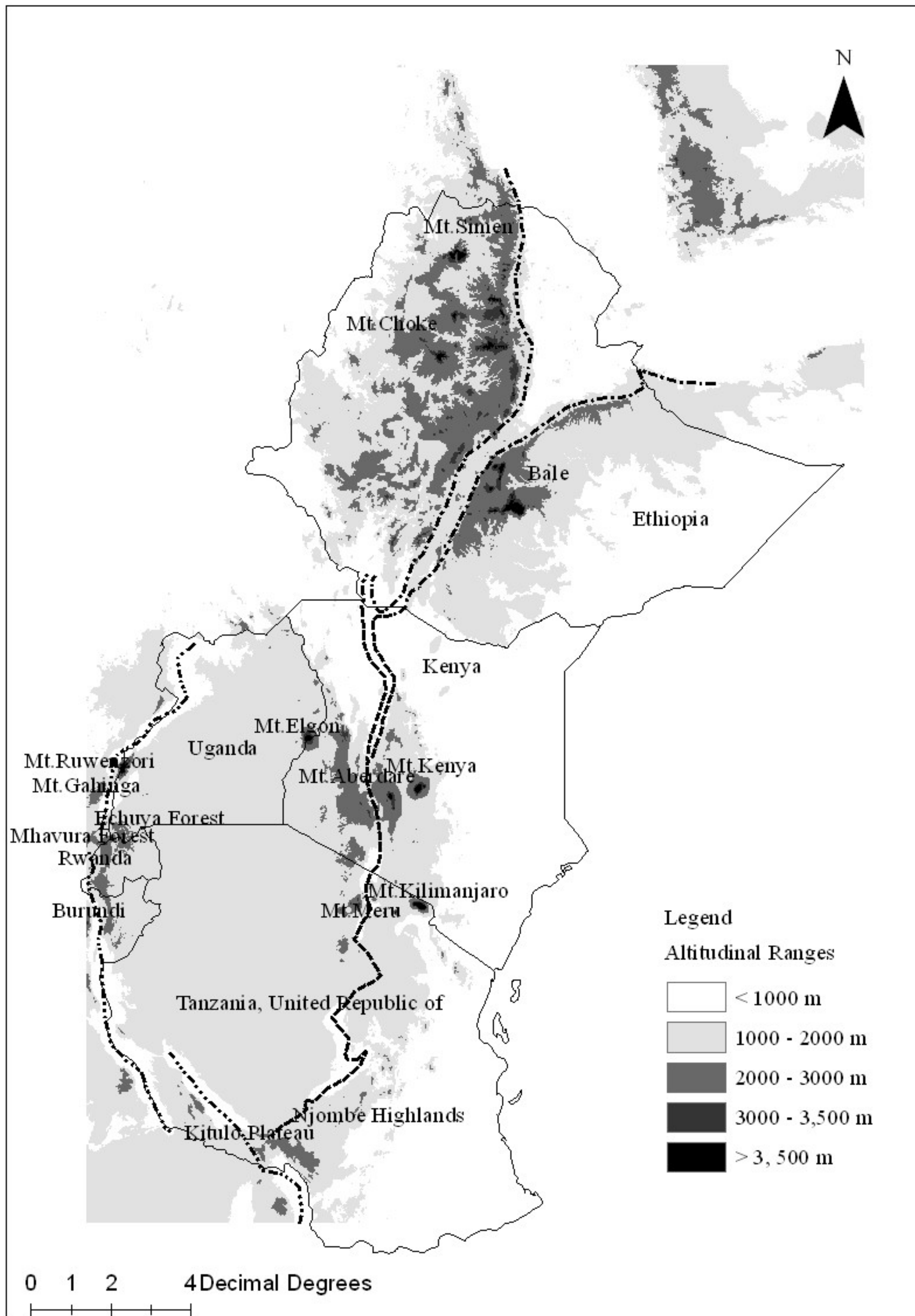
Groups	Source of variation	Degrees of freedom	% of variation	Φ statistics
A. Among and within species based on the entire dataset	Among groups	12	45.44	FCT = 0.45439
	Among populations within groups	157	13.85	FSC = 0.25383
	Within populations	516	40.71	FST = 0.59289
B. Predominately Western Rift Clade vs Predominately Eastern Rift Clade	Among groups	1	13.96	FCT = 0.13962
	Among populations within groups	168	46.82	FSC = 0.54415
	Within populations	516	39.22	FST = 0.60779
C. Among and within populations based on datasets for individual species				
L. aberdarica	Among populations	8	12.77	
	Within populations	38	87.23	FST = 0.12767
L. bambuseti	Among populations	5	9.3	
	Within populations	32	90.7	FST = 0.09303
L. bequaertii	Among populations	3	13.26	
	Within populations	13	86.74	FST = 0.13256
L. burttii	Among populations	3	13.58	
	Within populations	16	86.42	FST = 0.1358

Groups	Source of variation	Degrees of freedom	% of variation	Φ statistics
L. deckenii	Among populations	13	20.79	
	Within populations	43	79.21	FST = 0.20787
L. giberroa	Among populations	28.04		
	Within populations	71.96		FST = 0.28043
L. gregoriana	Among populations	13	28.39	
	Within populations	48	71.61	FST = 0.2839
L. mildbraedii	Among populations	6	20.74	
	Within populations	26	79.26	FST = 0.20742
L. rhynchopetalum	Among populations	17	11.9	
	Within populations	83	88.1	FST = 0.11896
L. stuhlmannii	Among populations	13	12.34	
	Within populations	36	87.66	FST = 0.12343
L. telekii	Among populations	21	37.45	
	Within populations	77	62.55	FST = 0.37446
L. wollastonii	Among populations	13	21.73	
	Within populations	37	78.27	FST = 0.21735
D. Among and within mountains based on datasets for individual species				
L. aberdarica (AB,CH and EL)	Among groups	2	9.09	FCT = 0.09087
	Among populations within groups	6	5.49	FSC = 0.06039
	Within populations	38	85.42	FST = 0.14577

Groups	Source of variation	Degrees of freedom	% of variation	Φ statistics
L. bambuseti (AB and KE)	Among groups	1	4.65	FCT = 0.04648
	Among populations within groups	4	6.27	FSC = 0.06579
	Within populations	32	89.08	FST = 0.10921
L. giberroa (AB,CH,GH, KL, KT, MR, MH, NJ, PR, and RW)	Among groups	10	17.53	FCT = 0.17527
	Among populations within groups	13	11.71	FSC = 0.14200
	Within populations	68	70.76	FST = 0.29238
L. gregoriana (AB, EL and KE)	Among groups	2	29.97	FCT = 0.29969
	Among populations within groups	11	5.47	FSC = 0.07817
	Within populations	48	64.56	FST = 0.35443
L. mildbraedii (ECH, KT, MH and NJ)	Among groups	3	24.26	FCT = 0.24256
	Among populations within groups	3	0.17	FSC = 0.00218
	Within populations	26	75.58	FST = 0.24422
L. rhynchopetalum (BL, CHK and SM)	Among groups	2	11.13	FCT = 0.11134
	Among populations within groups	18	6.89	FSC = 0.07756
	Within populations	80	81.97	FST = 0.18026
L. stuhlmannii (GH, MH RW)	Among groups	2	3.78	FCT = 0.03783
	Among populations within groups	11	9.93	FSC = 0.10316
	Within populations	36	86.29	FST = 0.13708
L. telekii (AB, EL and KE)	Among groups	2	33.29	FCT = 0.33288

Groups	Source of variation	Degrees of freedom	% of variation	Φ statistics
	Among populations within groups	19	10.9	FSC = 0.16345
	Within populations	77	55.81	FST = 0.44192
L. wollastonii (MH and RW)	Among groups	1	3.43	FCT = 0.03431
	Among populations within groups	12	19.94	FSC = 0.20647
	Within populations	37	76.63	FST = 0.23370

a)



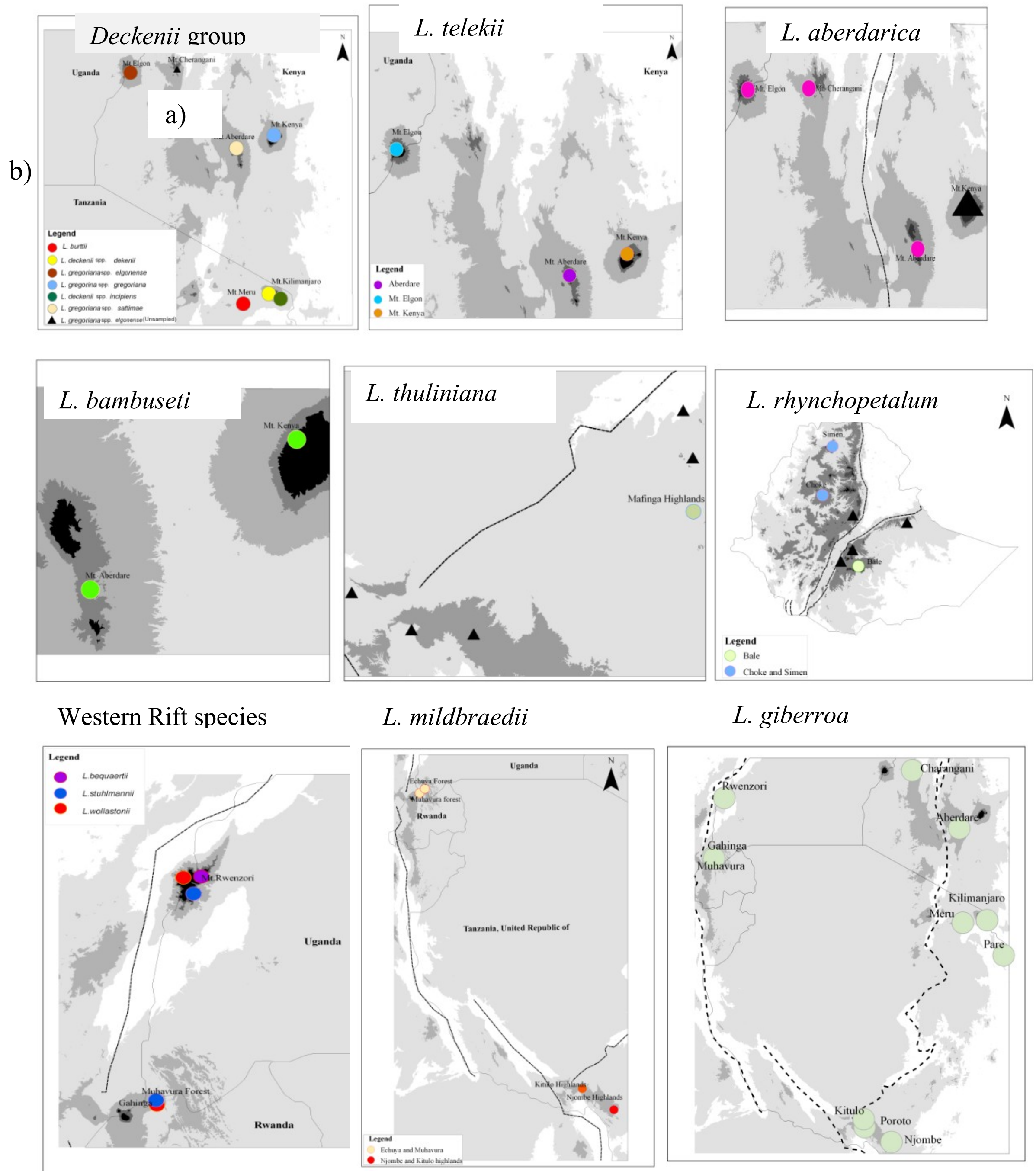


Fig. 1: Total distribution, collection sites and some of the PCoA genetic groups (the most distinct groups) inferred from AFLP analysis of the eastern African clade of unbranched-inflorescence giant lobelias. Coloured dots indicate the sampled localities while black triangles denote the un-sampled localities of the most restricted species in the area. On Fig. 1b, where a particular species for example Deckenii group species, L. telekii, L. mildbraedii and L. rhynchopetalum, indicated some strong geographical groupings on the PCoA and NJ tree, the groups are reflected by different colours on the maps

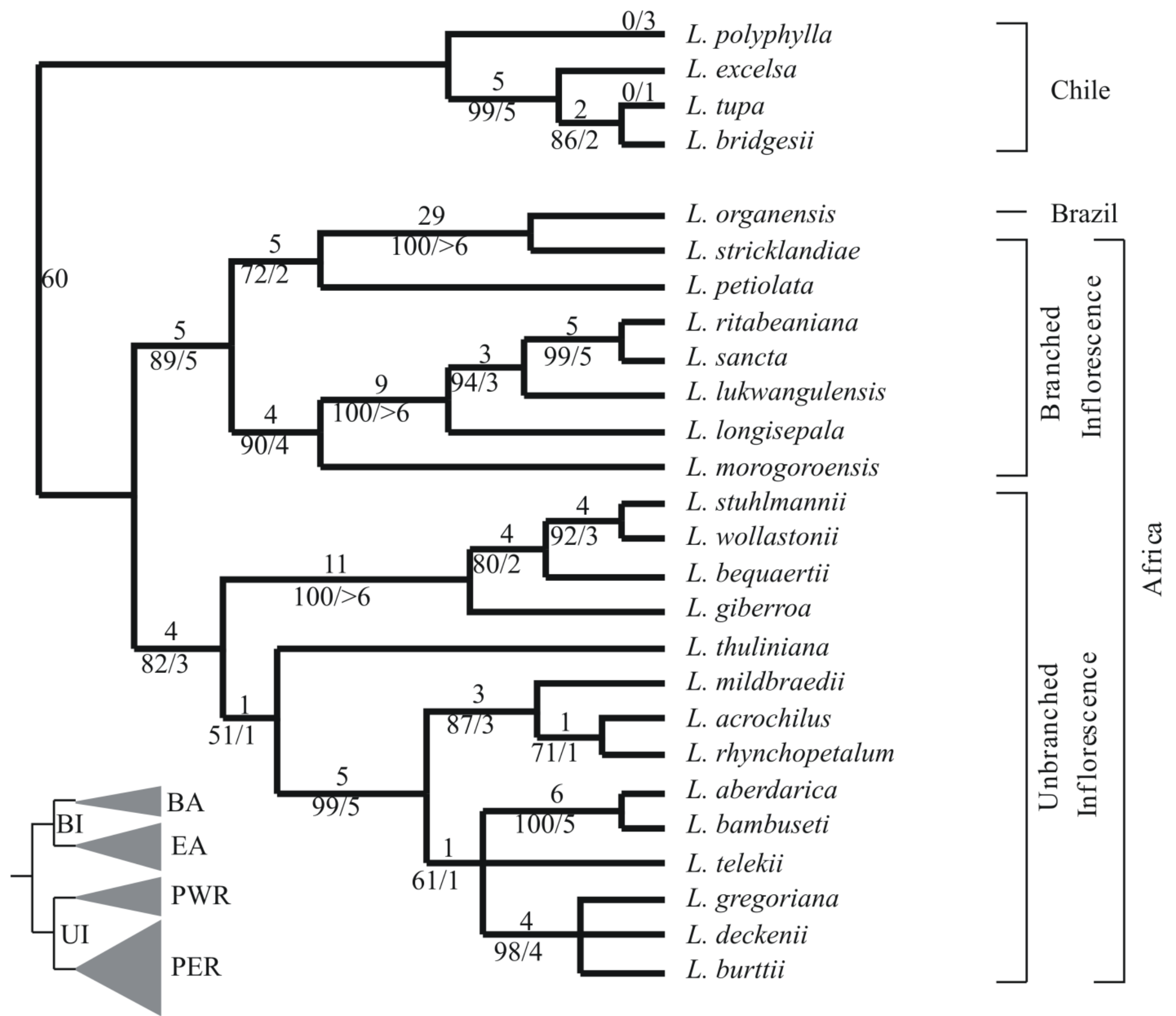


Fig. 2: Knox and Palmer's (1998) phylogeny of the eastern African giant lobelias inferred from plastid DNA restriction site variation

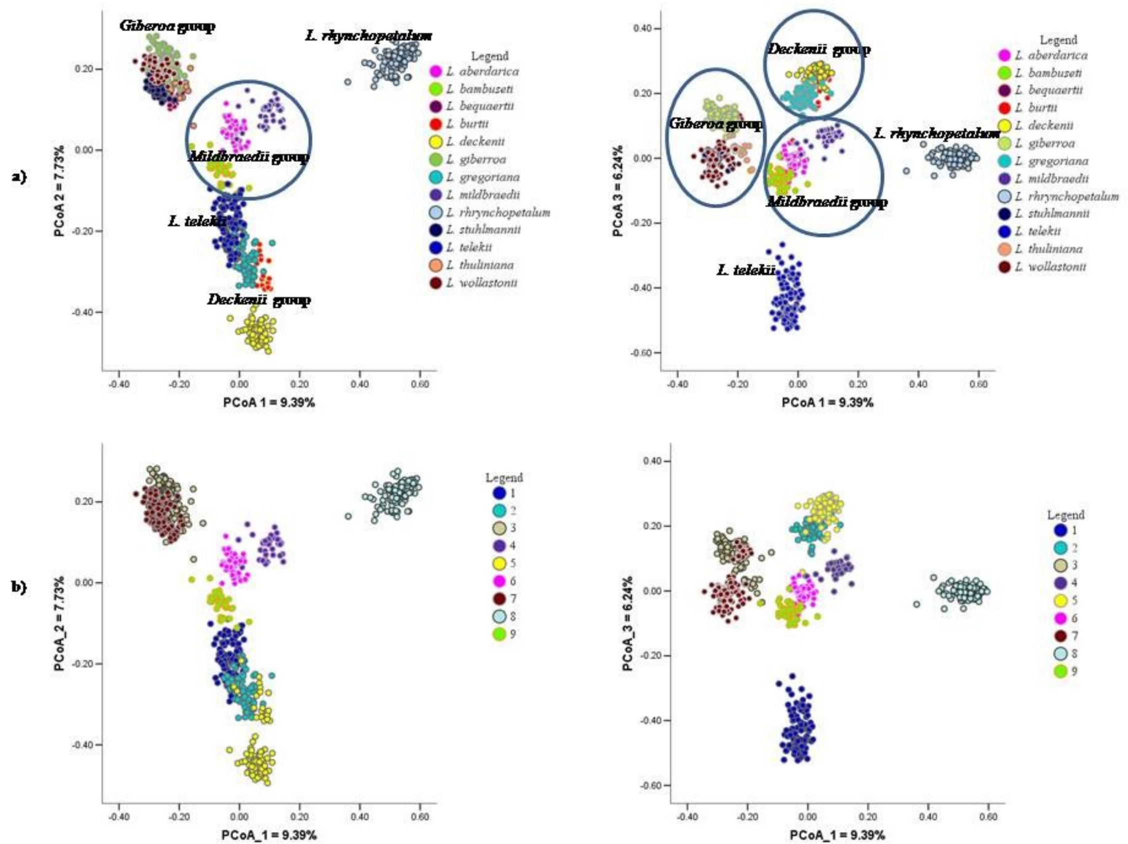
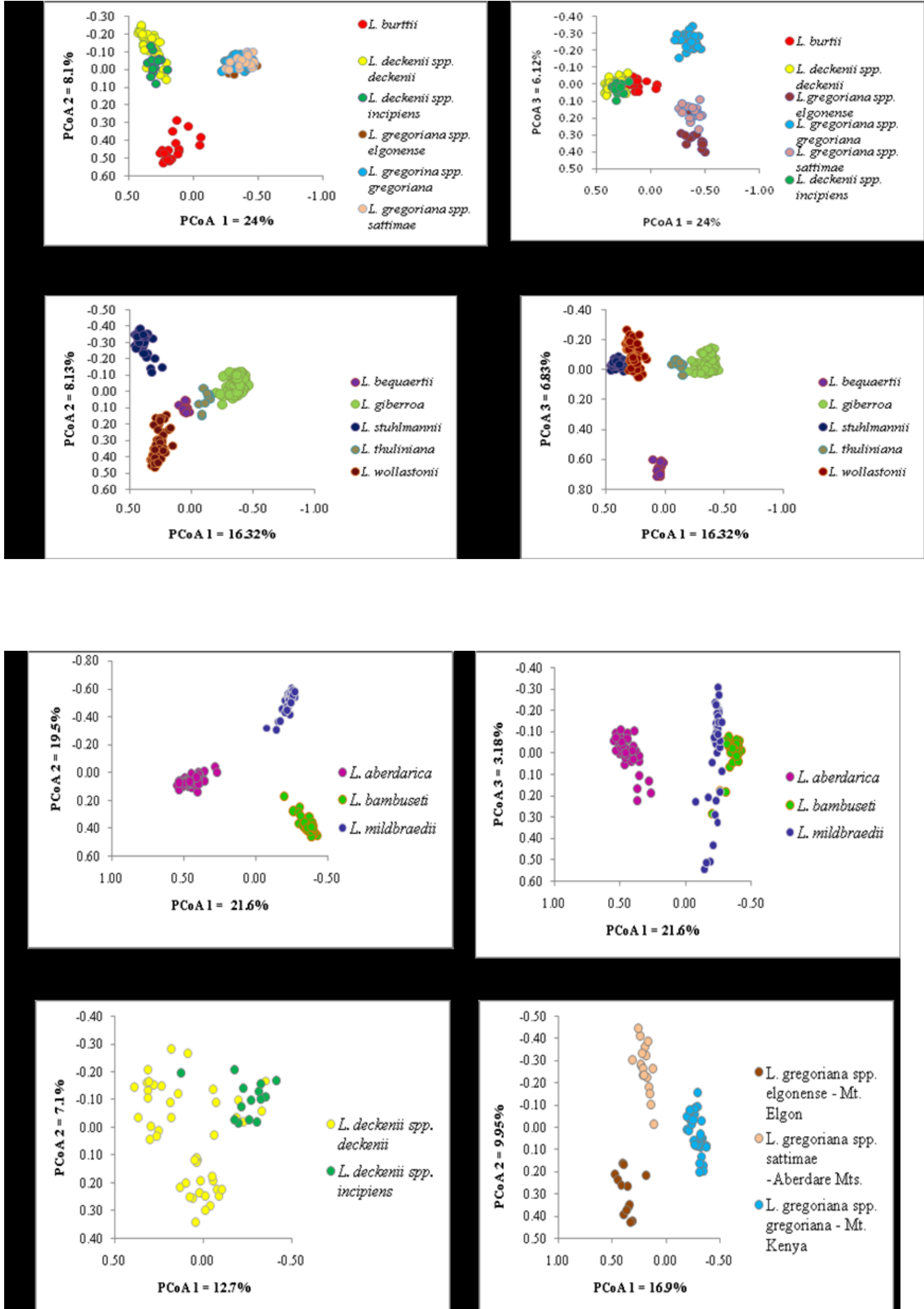


Fig. 3: PCoA of all AFLP multilocus phenotypes observed in the 13 species analyzed of the unbranched inflorescence giant lobelias superimposed with a) taxonomic designations and b) genetic groups inferred from STRUCTURE analyses. To simplify presentation and further analyses, five tentative species groups are delineated on the PCoA plot


PCoA 2 = 8.1%
PCoA 1 = 24%
L. burttii
L. deckenii spp. deckenii
L. deckenii spp. incipiens
L. gregoriana spp. elgonense
L. gregorina spp. gregoriana
L. gregoriana spp. sattimae
PCoA 3 = 6.12%
PCoA 1 = 24%
L. gregoriana spp. gregoriana
PCoA 2 = 8.13%
PCoA 1 = 16.32%
L. bequaertii
L. giberroa
L. stuhlmannii
L. thuliniana
L. wollastonii
PCoA 3 = 6.83%
PCoA 1 = 16.32%
PCoA 2 = 19.5%
PCoA 1 = 21.6%
L. aberdarica
L. bambuseti
L. mildbraedii
PCoA 3 = 3.18%
PCoA 1 = 21.6%
PCoA 2 = 7.1%
PCoA 1 = 12.7%
L. deckenii spp. deckenii
L. deckenii spp. incipiens
PCoA 2 = 9.95%
PCoA 1 = 16.9%
L. gregoriana spp. elgonense - Mt. Elgon
L. gregoriana spp. sattimae -Aberdare Mts.
L. gregoriana spp. gregoriana - Mt. Kenya

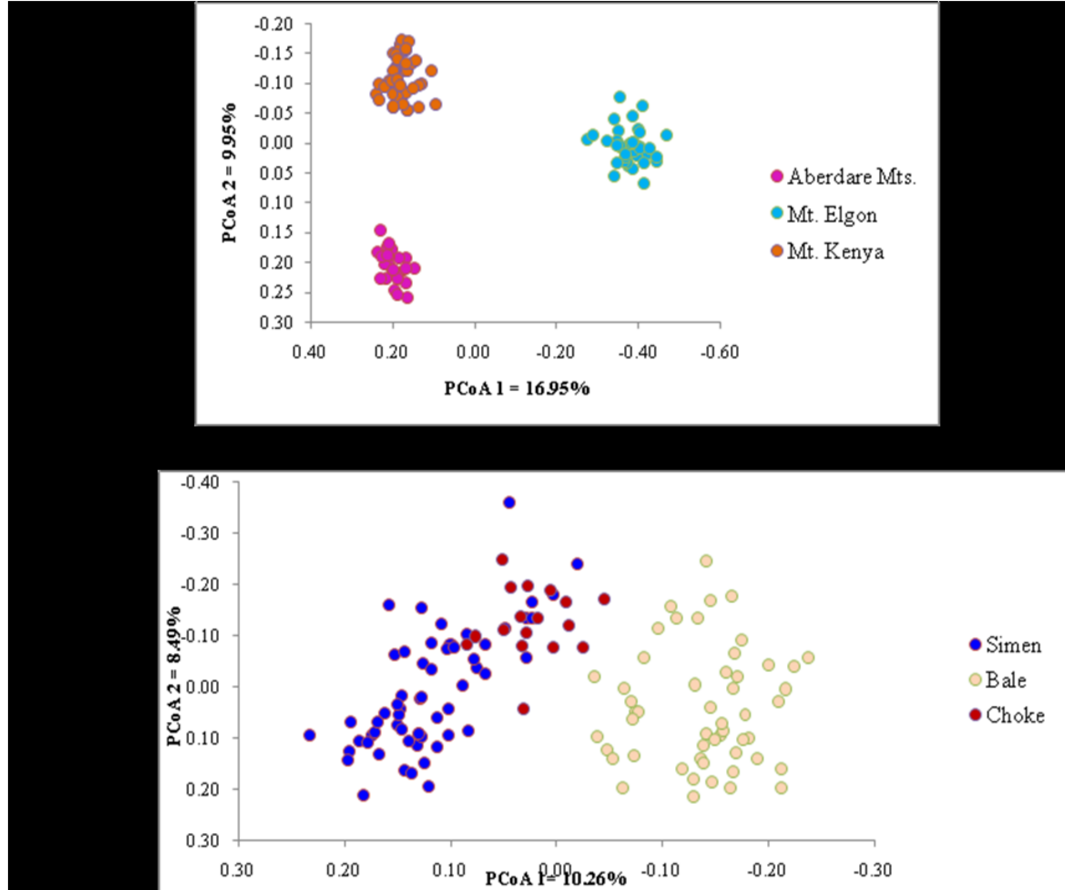


Fig. 4: Separate PCoAs of various subsets of the main AFLP data set. a) '*Deckenii*' group. b) 'Giberroa' group. c) '*Mildbraedii*' group. d) *L. deckenii*. e) *L. gregoriana*. f) L. telekii. g) *L. rhynchopetalum*.

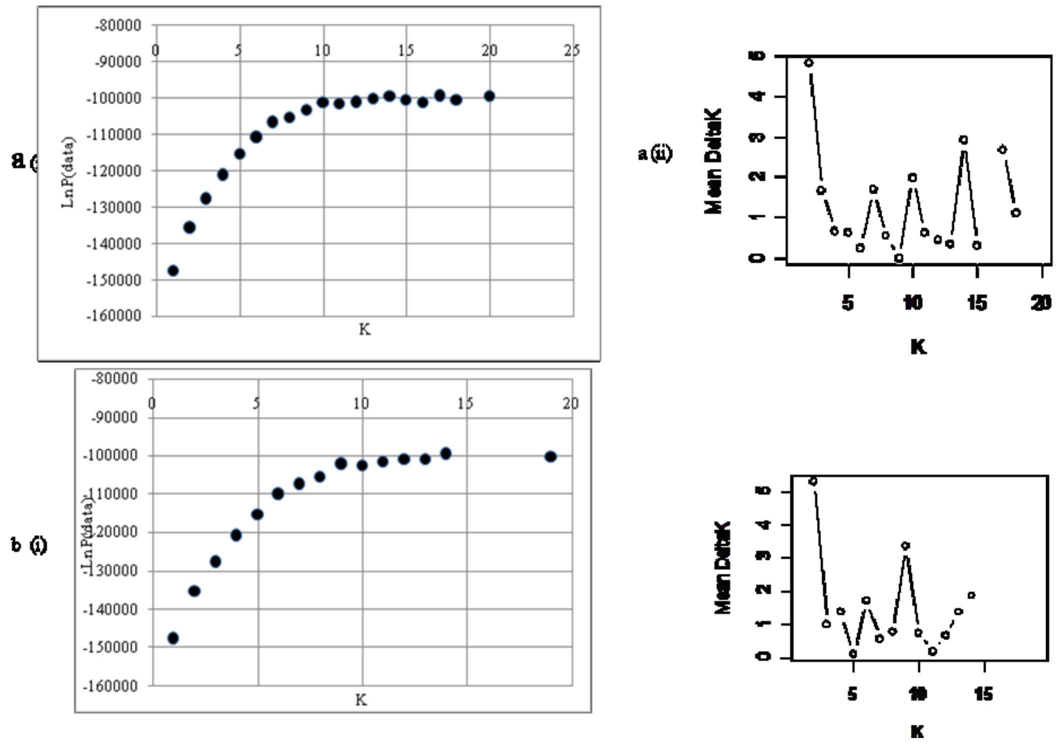


Fig. 5: Results of the STRUCTURE analyses of the AFLP data for unbranched-inflorescence clade of giant lobelias. a (i) and b (i) = K vs –ln likelihood for the admixture and no admixture models, respectively. a (ii) and b (ii) = K vs ΔK for K = 1 to K = 20 for the admixture and no admixture models, respectively

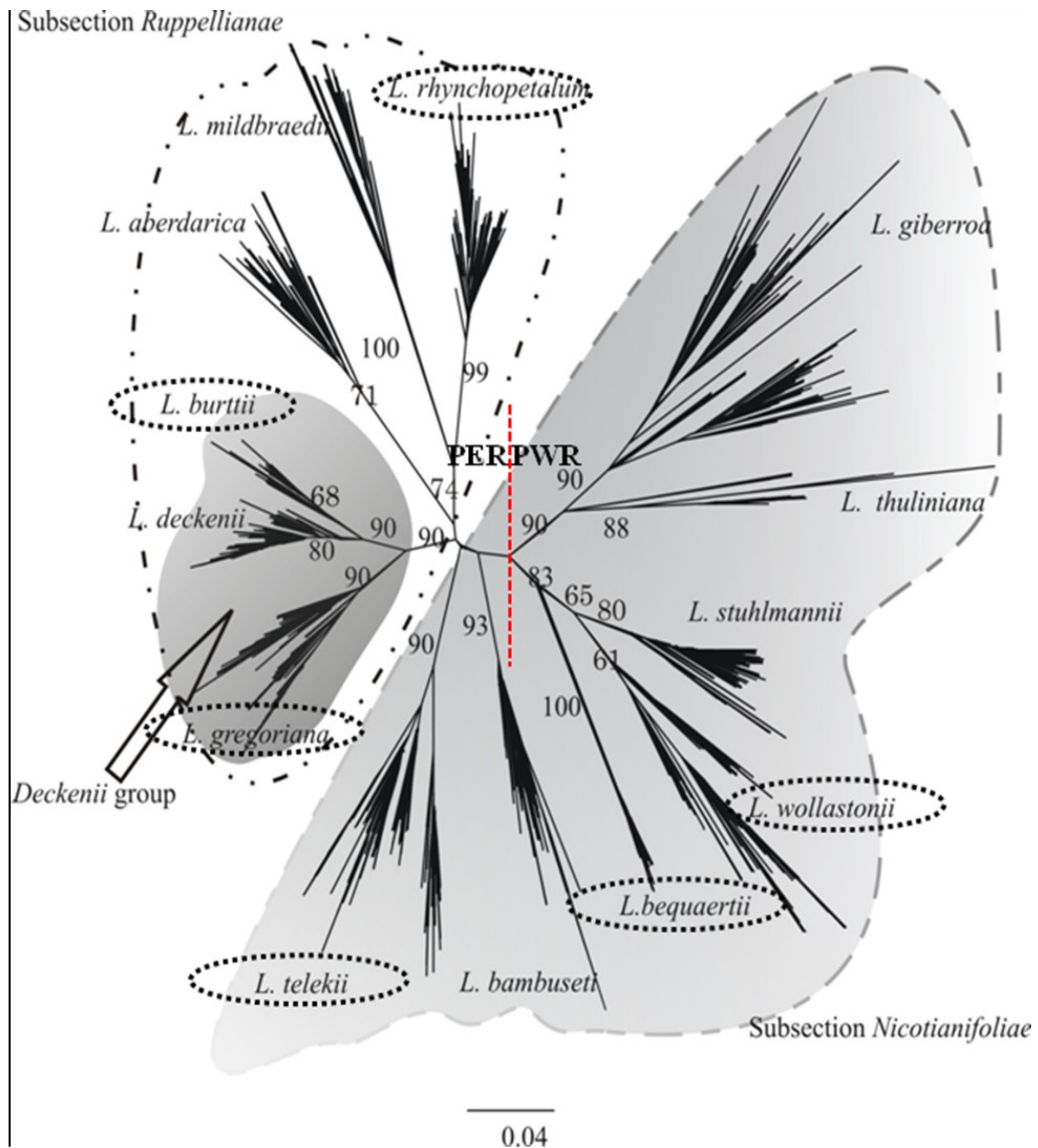


Fig. 6: Unrooted neighbour-joining tree based on the 1168 AFLP markers scored in the 689 individuals analyzed of the eastern African clade of unbranched-inflorescence giant lobelias. The tree was built from a pairwise Nei & Li (1979) distance matrix. The numbers are bootstrap values (>50%) based on 1000 replicates. The main taxonomic grouping of the species into subsections according to Mabberley (1973) are indicated, along with the division into the Predominantly Western Rift clade (PWR) and the Predominantly Eastern Rift clade (PER) according to the plastid DNA phylogeny of Knox & Palmer (1998; note however that L. thuliniana in our tree is inferred as belonging to PWR, not PER). The 'Deckenii' group, which was interpreted by Hedberg (1957) and others to provide one of the best examples of vicariant speciation in the afro-alpine region, is also indicated. The names of species which mainly are restricted to the afro-alpine zone proper are encircled; the remaining species mainly or frequently occur in the montane forest zone.

CHAPTER THREE

3.0 USE OF AFLPS TO ASSESS LOW-LEVEL TAXONOMY AND TO INFER PHYLOGEOGRAPHIC HISTORIES OF SOME AFRO-ALPINE GRASSES: *DESCHAMPSIA CAESPITOSA, D. ANGUSTA* AND *KOELERIA CAPENSIS* (Manuscript II)

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Target Journal – Taxon

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3.1 Abstract

Phylogeographic studies in the afro-alpine zones of the high mountains are hampered by lack of knowledge of taxonomic variation within and among closely related species. In this study this issue is addressed in three grass species, of which one (*Deschampsia angusta*) has been described as endemic to a single afro-alpine mountain. The infraspecific taxonomy of the afro-alpine populations of the two other species, the widely distributed *Deschampsia caespitosa* and *Koeleria capensis*, is difficult because of their complex morphological variation. Amplified fragment length polymorphism (AFLP) was used to assess genetic structuring among 33 and 153 afro-alpine individuals of *Deschampsia* spp. and *Koeleria capensis*, respectively. The plants identified as the endemic *D. angusta* were not genetically distinct from those of *D. caespitosa* sampled in the same mountain, Ruwenzori, suggesting that the characters used to distinguish these species may reflect phenotypic plasticity rather than taxonomically significant variation. Rather, two major genetic groups were identified within *D. caespitosa* s.lat. (i.e. including *D. angusta*) corresponding to the prominent geographic division between the Western Rift Mountains (represented by Ruwenzori) and the Eastern Rift Mountains including Ethiopia, represented by Kilimanjaro and Bale. The latter two mountains corresponded to a distinct genetic subdivision as well, suggesting that this species has persisted in three different and isolated afro-alpine refugia at least during the last glacial cycle. In contrast, there were no distinct geographic structuring of the genetic variation in *Koeleria capensis*, low levels of diversity, and no support for recognition of infraspecific taxa. In this species, the genetic variation within single mountains spanned much of the total variation observed in the entire afro-alpine region, suggesting recent episodes of long-distant colonization. This study demonstrates the need for further taxonomic

exploration of the afro-alpine flora, in particular of taxa described as endemic, and highlight that different afro-alpine species may have experienced very different phylogeographic histories and that long-distance dispersals among the isolated afro-alpine 'sky islands' can be more frequent than traditionally thought.

Key words: AFLP, afro-alpine grasses, infraspecific taxonomy, phylogeographic histories

3.2 Introduction

Amplified fragment length polymorphism (AFLP) is a fingerprinting technique based on selective amplification of restriction fragments from total genomic DNA (Vos & al. 1995). The technique involves four steps: 1) restriction-ligation, 2) pre-selective amplification, 3) selective amplification, and 4) fragment separation by electrophoresis (e.g. Vos & al. 1995; Gaudeul & al. 2000). This method is suitable for the study of non-model species because no pre-knowledge of the genome is required and it is superior to other fingerprinting techniques because of its high reproducibility and ability to produce a high number of unlinked loci at a time (e.g., Gaudeul & al. 2000; Garcia-Pereira & al. 2010). The limitations of the technique include their dominant nature and potential homology problems, which makes it most appropriate to use at the intraspecific level, whereas interspecific comparisons should be done with caution and only to assess recently diverged taxa (e.g., Gaudeul & al. 2000; Buntjer & al. 2002; Koopman & al. 2008; Garcia-Pereira & al. 2010; Safer & al. 2011).

AFLPs have proved as highly successful for species delimitation in plant groups where other molecular markers have failed to provide well-resolved species phylogenies (e.g., Gaudeul *&* al. 2000; Buntjer *&* al. 2002; Koopman *&* al. 2008; Meudt *&* al. 2009; Mendelson *&* Wong 2010; Wong, 2010; Olet *&* al. 2011; Safer *&* al. 2011). For instance, an AFLP-based phylogeny of the genus *Leontopodium,* highly congruent with the taxonomy of the genus, was estimated by Safer *&* al. (2011). A detailed delimitation of species of the genus *Ourisia* based on AFLPs was provided by Meudt *&* al. (2009); they identified distinct metapopulations that were consistent with morphological characters. AFLPs have also been successfully applied on some animal taxa such as snubnose daters and allies, where ALFP groups largely consistent with earlier hypotheses based on morphology or mtDNA were recovered. Here the same technique is applied to the highly variable species *Koeleria capensis* (Steud.) Nees and the species complex *Deschampsia caespitosa* (L.) P. Beauv /*D. angusta* Staff & C. E. Hubb in order to clarify their current taxonomy and reconstruct their phylogeographic history in the afro-alpine ecosystem.

Koeleria capensis is an African widely distributed species and exhibits high morphological variation. It occurs from Ethiopia, Cameroun, Kenya, Uganda, Tanzania and to the Cape in South Africa. It is commonly found in the upland grassland and moorland, sometimes on dry open ground with little vegetation at altitudes between 1800 m and 5300 m (Clayton, 1970). On Mount Kilimanjaro, there is a distinctive form of the species with small anthers, linear panicles and leaf-blades almost as long as the flowering culms, which have been separated as var. *supine* by some authors but the name has not been formally adopted (Clayton, 1970). *Koeleria capensis* appears to be closely related to the temperate European *K. cristata* as they

cannot be separated on the basis of spikelet characters, but the decaying leaf sheaths of the latter are broad, soft and papery and do not form an erect brush-like tuft (Clayton, 1970).

In *Deschampsia,* three species have been reported from the tropical East African and Ethiopian mountains (Hedberg, 1957). These are *Deschampsia angusta* Staff & C. E. Hubb., described as endemic to the Ruwenzori Mountains in Uganda; *D. caespitosa* (L.) P. Beauv., which in the afro-alpine region is found in northern Ethiopia and the East African mountains Ruwenzori, Elgon and Kilimanjaro, and *D. flexuosa* (L.) Trin. var. *afromontana* C. E. Hubb., which is widely distributed in the afro-alpine region (Hedberg, 1957). In this study, the relationship between the morphologically very similar species *D. angusta* and *D. caespitosa* will be explored.

While *D. angusta* is restricted to the upper parts of Ruwenzori, *D. caespitosa* is also widely distributed in Europe, Africa, temperate Asia, tropical Asia, Australia, North America, South America and Antarctica (Clayton, 2006). In tropical East Africa, *D. caespitosa* shows complex morphological variation and grows on moist ground along the edge of lakes and streams and in fens, mainly in the ericaceous belt and the lower part of the alpine belt between 3050 and 4000 m. In an early treatment of afro-alpine *D. caespitosa*, two varieties were recognized (Hedberg, 1957). These are var. *latifolia* (Hochst. ex A. Rich.) Hook. fil., which was reported from Ruwenzori, Mt. Elgon, Kilimanjaro and northern Ethiopia, and var. *oliveri* C. E. Hubb., which was reported as endemic to Ruwenzori. The two varieties are distinguished according to the site of insertion of the awn on the back of the lemma according to Clayton (1970), although Chrtek & Jirasek (1965, according to Clayton, 1970) had regarded this character to be of no taxonomic significance.

Deschampsia angusta is a densely tufted perennial caespitose with: culms erect 25-75 cm long, ligule an eciliate membrane; 10-18 mm long, leaf-blades flat or conduplicate; 10-30 cm long; 3-5 mm wide, leaf-blade surface ribbed; scabrous, inflorescence a panicle; 15-25 cm long, flower lodicules 2; anthers 3; 1.5-2.5 mm long, ovary glabrous. The plant grows on moist ground along streams and lakes in the alpine belt of between 3900 and 4050 m (Clayton, 2006). *Deschampsia caespitosa* is a perennial rhizomatous plant with: culms erect 20-200 cm long, ligule an eciliate membrane; 10-15 mm long, leaf-blades flat or involute 80-270(-600) cm long; 1.5-5 mm wide, inflorescence 6.5-50 cm long; 3-20 cm wide, lemma 1-awned (Clayton, 2006).

None of the few molecular studies available for *Koeleria* and *Deschampsia* (e.g., Chiapella, 2007; Quintanar *&* al. 2007; Meier, 2008; Bystrzejewska-Piotrowska & Urban, 2009; Catling, 2009) included material from the tropical East African and Ethiopian mountains. Here, the ALFP markers were used in order to contribute to a solution of the existing taxonomic uncertainties in the afro-alpine range of *Deschampsia angusta, D. caespitosa* and *Koeleria capensis* and their history in this region. Specifically, the study intended to: i) assess possible congruence of genetic groups with the morphology and current taxonomic treatments, ii) determine the level of genetic variation within and between populations, and iii) reconstruct the phylogeographic history in the afro-alpine ecosystem, and iv) provide guidelines for conservation management.

3.3 Material and Methods

3.3.1 Plant material

Fresh young leaf samples of *D. caespitosa, D. angusta,* and *K. capensis* were collected from seven mountains in tropical East Africa and Ethiopia (Fig. 1) between 2007 and 2009. Five individuals of the same species found within an area of 10000 m² were considered as representing one population. Unfortunately, only a single population of the endemic *D. angusta* was collected from the field. Altogether 49 individuals were collected for *Deschampsia* spp. (i.e. 44 individuals *D. caespitosa*, and five *D. angusta*) and 196 for *K. capensis*. Leaf samples were dried in silica gel and three voucher specimens were pressed and deposited in the following herbaria: Natural History Museum, University of Oslo, Norway (1 voucher); Addis Ababa University, National Herbarium of Ethiopia (1 voucher); the third voucher was deposited according to country of collection (East African Herbarium, Kenya; Makerere University, Uganda; or Sokoine University of Agriculture, Tanzania).

3.3.2 DNA extraction and AFLP

Total genomic DNA was extracted from the silica-gel-dried leaves using MoleStrips™ DNA Plant kit with an automated GeneMole® robot following the manufacturer's instructions (Qiagen Nordic). Prior to loading the plant material to the GeneMole®, some modifications were performed as in Masao *&* al. (2012a) unpublished. The AFLP protocol was optimized according to Gaudeul *&* al. (2000) and further modified as in Masao *&* al. (2012a) unpublished.

For each taxon, 24 primers (12 for each taxon) were tested on two samples from different geographic regions. Primers resulting in high reproducibility and many

scorable polymorphic loci were chosen. Three primer pairs (*EcoRI* - AGA -(6FAM)- *Msel* -CTG, *EcoRI* – AGG - (VIC)- *Msel* - CAT and *EcoRI* - ACC -(NED) - *Msel* - CAT) were chosen for *D. caespitosa and D. angusta* and two (*EcoRI* –ATG - (6FAM)- *Msel* - CGA, *EcoRI* – ACA- (VIC)- *Msel* - CAC) for *K. capensis*. To estimate reproducibility of the AFLP data (Bonin *&* al. 2004), approximately 10% of the samples (one individual from each population) were double-extracted and the replicated samples were independently run through the PCR process and finally scored with the whole data set into their respective taxa. Separate raw data for each primer combination were first visualized on Genographer (version 2.1: http://sourceforge.net/projects/genographer/files/) to evaluate the quality of the data and remove failed samples (Masao *&* al. 2012a). Thereafter, the data were imported to GeneMapper version 4.0 (Applied Biosystems) and AFLP bands in the size range 50-500 base pairs (bp) were scored as present (1) or absent (0). Samples of both genera were scored and analyzed in two independent datasets. A total of 33 individuals were successfully genotyped for both species of *Deschampsia* (i.e. three for *D. angusta* and thirty for *D. caespitosa*) and 153 individuals for *Koeleria capensis* (Table 1).

Principal Coordinate Analysis (PCoA) was performed with NTSYS-pc V 2.1 (Rohlf, 1990) using Dice similarity. A Neighbor-Joining (NJ) tree was built based on Nei *&* Li distance (1979), as implemented in PAUP*V. 4.0 (Swofford, 2003). Genetic structure was examined by Bayesian clustering with BAPS V. 5.3 (Corander *&* al. 2008), using a maximum possible number of groups between 1 and 10 (*K*) for both datasets. Analysis of Molecular Variance (AMOVA) was performed to investigate the partitioning of genetic variation at different hierarchical levels using ARLEQUIN V.

3.5 (Excoffier & Lischer, 2010). Genetic diversity was estimated at population, species and mountain levels based on three parameters: 1) the percentage of polymorphic loci 2) gene diversity (Hs) and Shannon information index (*I*), using POPGENE, V. 1. 32 (Yeh *&* al. 2000). The frequency-downweighted marker values (DW) were also calculated according to Schönswetter & Tribsch (2005), as implemented in AFLPdat by Ehrich (2006). The input files for ARLEQUIN V. 3.5, BAPS V. 5.3, and POPGENE V. 1. 32 were prepared using R-script AFLPdat (Ehrich, 2006).

3.4 Results

3.4.1 Deschampsia data set

The final dataset contained a total of 172 unambiguous AFLP fragments and 33 individuals (one population of *D. angusta* and eight populations of *D. caespitosa*) were successfully analyzed with a reproducibility of 97.86%. Population polymorphism ranged between 12% and 31% (Table 1), while polymorphism by mountain was as high as 62% in the Bale Mountains and as low as 15% in Mt. Kilimanjaro (Table 2).

The NJ tree (Fig. 2a) and PCoA (Fig. 2b) revealed three distinct groups corresponding to the three mountains sampled (bootstrap support was 100% for Rwenzori, 97% for Kilimanjaro, and 87% for Bale). The populations from Bale and Kilimanjaro appeared to be closest to each other and were separated from the Ruwenzori populations both in the PCoA scatterplot, mainly along axis 1 (24.5% of the variation), and in the BAPS analysis, which inferred two Bayesian clusters (Log (marginal likelihood) of optimal partition = -2023.8463). This division was also reflected by the high percentage of molecular variance found between the two BAPS

clusters in the AMOVA analysis (Table 3). Noteworthy, the individuals referred to the endemic *D. angusta* did not separate from those of *D. caespitosa* from the Ruwenzori in any of the three analyses. In the AMOVA (Table 3), while 38% of the variation was attributed to differences among populations and 62% to differences within populations only 13.42% was attributed to among populations between the two species.

Mean within-population genetic diversity ranged from 0.0482 (I = 0.0717) to 0.1197(I = 0.1766, Table 1), and was similar across all populations and mountains. The highest diversity (Hs=0.1651; I=0.1876) and frequency down weighted marker values (DW = 5.5150) were found in the Ruwenzori populations (Table 2).

3.4.2 Koeleria capensis data set

The final *K. capensis* data set contained 458 unambiguous fragments from 153 individuals (36 populations) with reproducibility of 97.4%. The percentage polymorphic loci per population ranged from 3.28% to 32.1% (Table 1). Mount Meru had the lowest polymorphism (17.25%) while Mt. Elgon had the highest (60.92%) (Table 2).

The PCoA plot for axis one and two and axis one and three (Fig. 3a, b) and the NJ tree (not shown) showed no distinct geographical groups. On the PCoAs, the genetic variation in individual mountains spanned much of the plots with the PCoA plot for axis one and three indicating some three main groups with most populations from Mt. Kilimanjaro distinct. BAPS inferred four Bayesian clusters (Log (marginal likelihood) of optimal partition = -12709.5624 which were largely mixing as in the

PCoAs. The hierarchical AMOVA indicated significant genetic differences among populations (47.06%) and all variance components were highly significant (Table 3).

Genetic diversity in most populations and mountains was very low (Table 1 and 2). The mean genetic diversity (H_S) ranged from 0.012 (I = 0.0189) in population TZ_0253 to 0.0953 (I = 0.1468) in population ET_0908. The Bale Mountains populations showed the highest mean genetic diversity (Hs = 0.1031, I = 0.1758) and genetic distinctivity (DW = 4.9819), followed by the Simen Mountains populations (Table 2).

3.5 Discussion

3.5.1 Deschampsia P. Beauv.

3.5.1.1 Taxonomy

This genetic data reflect the large morphological variation previously observed in *Deschampsia caespitosa* on the high mountains of tropical East Africa and Ethiopia (Clayton, 1970; Chiapella, 2007). Three distinct groups of populations, corresponding to the Bale, Kilimanjaro, and Ruwenzori mountains were identified. The NJ tree (Fig. 2a), PCoA (Fig. 2b) and Bayesian clustering analysis revealed that the populations from Bale and Kilimanjaro were most similar to each other and identified two main genetic groups, the Bale-Kilimanjaro group and the Ruwenzori group. Further morphological studies are needed to assess whether these two genetic lineages correspond to the two varieties described from the high mountains in tropical East Africa and Ethiopia: *D. caespitosa* (L.) P. Beauv. var. *latifolia* (Hochst. ex A. Rich.) Hook. fil. (reported from Ruwenzori, Mt. Elgon, Kilimanjaro and northern Ethiopia), and *D. caespitosa* (L.) P. Beauv. var. *oliveri* C. E. Hubb. (endemic to Ruwenzori).

Although both of these varieties have been reported from Ruwenzori, it is possible that sampling in that mountain did not represent all variation present there.

Importantly, it was not possible to separate the samples referred to *D. angusta* from those of *D. caespitosa* based on AFLP markers, despite statements on their clear morphological distinction (e.g., Hedberg, 1957; Clayton, 2006). It is therefore possible that the characters used to separate them are environmentally plastic and do not represent genetic differences. Further studies involving more populations are needed to draw firm conclusions about the taxonomic status of *D. angusta*.

3.5.1.2 Phylogeography and conservation

The two major genetic groups found in *D. caespitosa* correspond to the prominent geographic division between the Western Rift Mountains (represented by Ruwenzori) and the Eastern Rift Mountains including Ethiopia, represented by Kilimanjaro and Bale. The split between the mountains (Bale, Kilimanjaro and Ruwenzori) and between the two sides of the Great East African Rift valley explains 37.2% and 33.3% of the overall genetic variation, respectively (Table 3), whereas the populations are little differentiated within each mountain (8.68%; Table 3) suggesting that the two species could be partly outcrossing. In other studies, much lower values of variation among groups were detected (eg. Kropf & al. 2003). The distinct divergence between the populations in these three mountains indicates that they have been isolated for a long time, although AFLP data cannot be used for exact dating of their divergence (cf. Ehrich & al. 2009). Nevertheless, their clear distinction at AFLP loci suggests that the mountains were colonized before the last glacial cycle, and that the three genetic groups have persisted in different refugia since then. Paleoclimatic

studies indicate that during mid and late Pleistocene when there were large climatic oscillations, the glacial cycles affected the tropical African vegetation at a varying extent (Dupont & al. 2001). The mountains are believed to have provided relatively stable habitats for many species during this time (Fjeldså & Lovett, 1997; Fjeldså & Bowie, 2008).

The populations in the three mountains have similar levels of genetic diversity and genetic distinctivity (DW, Tables 1 and 2). This result suggests that the mountains were colonized at about the same time, because more recent colonization of a mountain should result in low diversity as well as little distinctivity. For conservation purposes, the identification of the three divergent gene pools of *Deschampsia caespitosa* on the afro-alpine range is important, and it is suggested that they are treated as distinct evolutionarily significant units (ESUs). As regards the proposed endemic *D. angusta*, there was no evidence supporting its taxonomic recognition and thus importance for conservation, but further studies to assess its status with more certainty are suggested.

3.5.2 Koeleria capensis (Steud.) Nees

3.5.2 .1 Taxonomy

Contrary to results from *Deschampsia*, there was no distinct structuring of the genetic variation in *K. capensis* that corresponded to geography. Rather, the genetic variation in individual mountains spanned much of the total variation observed across the entire afro-alpine region in the species. This result suggests that *Koeleria capensis* has experienced much more frequent and more recent long-distance dispersals among mountains compared to *Deschampsia* spp., and it was not possible to distinguish

intraspecific taxa based on the genetic data. The genetic lineages identified in *K. capensis* agreed only partially with the geography. While Bale, Elgon, and Kilimanjaro mountains exhibited distinct genetic groupings of some populations, individuals from all mountains fell into two large genetic groups in PCoA (Figs. 3a-b), NJ (not shown) and in BAPS analyses. The AMOVA results also indicated most variation within the populations than among the groups (Table 3) suggesting that the species is outcrossing.

The complex structure observed in the AFLP analyses is in agreement with the high morphological variability reported in *K. capensis*, but the lack of distinct genetic groups correlated with geography is not compatible with recognition of any intraspecific taxa in the afro-alpine region, such as var. *supine*. Notably, the Kilimanjaro populations, from where var. *supine* has been reported, spanned much of the total variation along axes 1 and 3 in the PCoA, and individuals from this mountain were found intermixed with individuals from other mountains along all three axes. This justifies the large number of intraspecific taxa and synonyms described within the species (e.g., Hedberg, 1957; Clayton, 1970).

With the current data one can be tempted to accept the previously recognized endemic Mount Kilimanjaro *K. capensis* var. *supine* which partially show some good segregation from the rest of the groups in the one versus three PCoA (Fig. 3a-b) and on the NJ tree (not shown). However, although these results show some weak groups in some populations from Bale, Elgon and Kilimanjaro, they largely support the latest morphology based classification which recognizes *K. capensis* as a single species without any subspecies (Clayton, 1970).

3.5.2.2 Phylogeographic patterns

The AFLP data suggest that recent gene flow among the populations has not been severely restricted, resulting in a widespread afro-alpine gene pool without clear internal structure. Thus, the current pattern must have been shaped by recent long-distance dispersals among the mountains. Long-distance dispersals are often associated with genetic depauperation (e.g., Kropf & al. 2006; Voje, 2008). Most populations of *K. capensis* were extremely depauperate (Table 1 and 2) suggesting very recent (postglacial) expansion into this area. Since not the whole area of the species distribution was sampled, it is impossible for us to precisely infer its source region and timing of colonization. However, the genetic patterns (Table 1 and 2) suggest that the current populations of *K. capensis* in tropical East Africa were recruited from the North (Ethiopian mountains). This is indicated by the comparatively high genetic diversities and DW values of both Bale and Simen mountains populations (Table 2). Rapid latitudinal re-colonization 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 (Leading-edge colonization) (Hewitt, 1996). Some other studies have shown that 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 allowing the persistence of areas characterized by low genetic diversity (Ehrich & al. 2007).

It should also be noted that, *K. capensis* has a wide ecological amplitude from alpine (at about 5300 m) belt descending to the montane forests to as low as 1800 m altitude (Clayton, 1970). The abundant habitats available for *K. capensis* may have increased the probability of its successful intermountain establishment after a long-distance

dispersal into the region. Hedberg (1969) argued that, while direct migration of forest elements was possible through forest bridges, the possibilities for similar dispersal on the uppermost afro-alpine flora appeared impossible. He further pointed out that the only possibility for long-distance dispersal from the afro-alpine was through strong cyclones which have only occurred twice in the history and at an interval of 80 years. However, given the most recent molecular studies in the region long-distance dispersals are reported to have occurred in the afro-alpine flora (Koch *&* al. 2006). In their study Koch *&* al. (2006) suggested that *Arabis alpina* colonized the tropical East Africa by two independent lineages originating from the Middle East, which spread south via coastal mountain ranges of the Arabian Peninsula and finally through intermountain dispersal within East Africa.

3.6 Conclusion

AFLPs remain a powerful tool for taxonomic, population genetics, phylogeography and phylogenetic studies. The samples identified as the endemic *D. angusta* are not genetically distinct from those of *D. cespitosa* sampled in the same mountain, Ruwenzori, suggesting that the characters used to distinguish these species may have reflected phenotypic plasticity rather than taxonomically significant variation. The results demonstrated two major genetic groups within *D. caespitosa s.lat.* (i.e. including *D. angusta*) corresponding to the prominent geographic division between the Western Rift Mountains (represented by Ruwenzori) and the Eastern Rift Mountains including Ethiopia, represented by Kilimanjaro and Bale. In contrast, there was no distinct geographic structuring of the genetic variation in *Koeleria capensis*, low levels of diversity, and no support for recognition of infraspecific taxa. In this species, the genetic variation within single mountains spanned much of the total

variation observed in the entire afro-alpine region, suggesting recent episodes of long-distant colonization. The lack of distinctive structure within *Koeleria capensis* was in accordance with the current species status. Since the samples for both taxa were not from the whole distribution area, it was not possible to resolve their speciation history. This first AFLP based taxonomic study for *D. angusta*, *D. caespitosa* and *Koeleria capensis* provides clear solutions to the existing controversy in the morphology based classification. It further highlights that different afro-alpine species may have experienced very different phylogeographic histories and that long-distance dispersals among the isolated afro-alpine 'sky islands' can be more frequent than traditionally thought.

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Table 1: Collections of Deschampsia and Koeleria capensis analysed using AFLP markers. Identity numbers (DNA Bank ID and population ID), Mountain, altitude, geographical coordinates (Latitude and Longitude), and number of individuals analyzed per population (n).Within population genetic diversity indices as analyzed using Popgene program are shown as follows: DW = frequency-downweighted marker values, Hs = Nei's within population genetic diversity, I = mean Shannon's Information index, Std = standard deviation, P = percentage polymorphic loci.

Population ID	n	DNA bank ID	Mountain	Altitude (m)	Latitude	Longitude	Rarity (DW)	H(Std)	*I*(Std)	(*P*)
Deschampsia angusta										
UG_2262	3	O-DP-43082	Rwenzori	3425	0.385017	29.9273	4.7694	0.1197(0.1874)	0.1766(0.2714)	30.81
D. caespitosa										
ET_0007	4	O-DP-27137	Bale	4059	6.854717	39.893317	5.8682	0.0913(0.1635)	0.1385(0.24)	26.74
ET_0687	5	O-DP-31781	Bale	3948	6.879267	39.868967	4.3816	0.1137(0.1839)	0.1696(0.264)	31.98
ET_0792	3	O-DP-32184	Bale	4017	6.868667	39.882183	5.6050	0.0482(0.1305)	0.0717(0.1911)	12.79
ET_0831	5	O-DP-32349	Bale	4019	6.855417	39.896467	4.6282	0.077(0.1479)	0.1193(0.2198)	25
TZ_0062	4	O-DP-37196	Kilimanjaro	3536	-3.0056	37.24155	4.8708	0.051(0.1311)	0.077(0.1932)	14.53
UG_2289	3	O-DP-40634	Rwenzori	3574	0.3852	29.913733	5.5171	0.0634(0.1372)	0.097(0.2067)	18.6
UG_2379	4	O-DP-42873	Rwenzori	3612	0.376867	29.93	6.2393	0.1129(0.1783)	0.1697(0.2596)	31.98
UG_2528	2	O-DP-41638	Rwenzori	3932	0.382283	29.888383	5.1818	0.0506(0.136)	0.0738(0.1986)	12.21
Koeleria capensis										
ET_0845	5	O-DP-32414	Bale	4019	6.855417	39.896467	7.8095	0.0899(0.1488)	0.1425(0.2231)	32.1
ET_0908	5	O-DP-32685	Bale	4068	6.86945	39.89465	7.2973	0.0953(0.1603)	0.1468(0.2372)	30.13
ET_1489	5	O-DP-34321	Bale	3520	6.989667	39.703	1.7898	0.0183(0.0803)	0.0283(0.1192)	5.9
ET_0011	3	O-DP-27153	Bale	4059	6.854717	39.893317	5.0652	0.0424(0.1239)	0.0629(0.1811)	11.14
ET_0743	3	O-DP-45253	Bale	4101	6.870283	39.8678	1.3628	0.0102(0.0563)	0.0161(0.0878)	3.28
ET_0789	5	O-DP-45299	Bale	4017	6.868667	39.882183	5.8070	0.0475(0.1287)	0.0711(0.1884)	13.32
ET_0824	3	O-DP-45335	Bale	4129	6.844833	39.88045	2.2769	0.0158(0.0742)	0.0241(0.1116)	4.59
KN_0029	3	O-DP-34843	Elgon	3915	1.105667	34.601833	1.0889	0.0138(0.0705)	0.021(0.1054)	3.93
KN_0054	4	O-DP-34945	Elgon	3864	1.1025	34.605833	1.1349	0.023(0.0858)	0.0361(0.1302)	7.64

Population ID	n	DNA bank ID	Mountain	Altitud e (m)	Latitude	Longitude	Rarity (DW)	H(Std)	*I*(Std)	(*P*)
KN_0201	2	O-DP-35543	Elgon	4043	1.118	34.586667	1.4344	0.0235(0.096)	0.0343(0.1401)	5.68
KN_0221	4	O-DP-35635	Elgon	3670	1.090033	34.6181	4.8805	0.0525(0.1257)	0.0813(0.1891)	16.59
KN_0234	4	O-DP-35697	Elgon	3670	1.090033	34.6181	1.1524	0.0259(0.0902)	0.0405(0.1373)	8.52
KN_0257	5	O-DP-35796	Elgon	3864	1.1083	34.606117	0.9777	0.0184(0.0742)	0.0295(0.1146)	6.77
KN_0263	5	O-DP-35826	Elgon	3979	1.102867	34.61305	8.2034	0.0683(0.1357)	0.108(0.2046)	24.02
KN_0265	5	O-DP-35835	Elgon	3979	1.102867	34.61305	1.4905	0.0281(0.0885)	0.0457(0.1375)	10.92
KN_0295	5	O-DP-35969	Elgon	3629	1.100667	34.6215	6.1073	0.0626(0.1308)	0.099(0.1977)	22.05
KN_0348	5	O-DP-36209	Elgon	3717	1.091667	34.617667	1.5585	0.0284(0.0936)	0.0449(0.142)	10.04
KN_0513	4	O-DP-27599	Aberdare	3888	-0.306167	36.622667	2.3700	0.0382(0.1012)	0.0614(0.1583)	13.76
KN_0634	5	O-DP-28101	Aberdare	3580	-0.334667	36.641333	2.1491	0.0323(0.0981)	0.0513(0.1497)	11.57
KN_0635	4	O-DP-28106	Aberdare	3582	-0.333667	36.6415	4.2841	0.0697(0.1341)	0.1103(0.2046)	24.02
KN_0723	4	O-DP-28515	Aberdare	3619	-0.3335	36.643167	1.4376	0.0228(0.087)	0.0353(0.1311)	7.21
TZ_0018	5	O-DP-36986	Kilimanjaro	3636	-3.03425	37.243	1.1948	0.0121(0.0653)	0.0185(0.0978)	3.71
TZ_0032	5	O-DP-37050	Kilimanjaro	3536	-3.0056	37.24155	1.2905	0.0118(0.0665)	0.0179(0.098)	3.49
TZ_0097	4	O-DP-42744	Kilimanjaro	3971	-3.052333	37.275167	1.9756	0.041(0.1185)	0.0619(0.175)	11.79
TZ_0143	5	O-DP-37489	Kilimanjaro	4155	-3.086217	37.3234	0.7541	0.0097(0.0573)	0.0152(0.0866)	3.28
TZ_0186	5	O-DP-37680	Kilimanjaro	4053	-3.109167	37.35495	1.1895	0.0119(0.0591)	0.0193(0.0923)	4.59
TZ_0800	4	O-DP-42720	Kilimanjaro	3242	-3.153333	37.4855	4.6686	0.0587(0.1273)	0.0922(0.1943)	19.43
TZ_0836	5	O-DP-39408	Kilimanjaro	3024	-3.1595	37.493167	5.4260	0.0819(0.1398)	0.1312(0.2126)	30.35
TZ_0253	5	O-DP-37972	Kilimanjaro	4109	-3.109967	37.421117	0.9029	0.012(0.0633)	0.0189(0.0957)	4.15
TZ_0378	3	O-DP-38487	Meru	3594	-3.217	36.769	1.6701	0.0265(0.0984)	0.0397(0.1451)	7.21
TZ_0400	5	O-DP-38592	Meru	3637	-3.218	36.766833	1.8072	0.0267(0.0907)	0.0421(0.1384)	9.17
TZ_0506	4	O-DP-39135	Meru	3589	-3.217833	36.770667	1.3608	0.0208(0.0802)	0.0327(0.1231)	6.99
ET_0141	2	O-DP-29789	Simen	3711	13.282733	38.110767	2.5711	0.0226(0.0942)	0.033(0.1375)	5.46
ET_0146	3	O-DP-29804	Simen	3718	13.28525	38.118383	2.7425	0.0318(0.1018)	0.0488(0.1538)	9.39
ET_0075	5	O-DP-29510	Simen	3574	13.2666	38.107817	2.6955	0.0363(0.1031)	0.0577(0.1576)	12.88
ET_0334	4	O-DP-30534	Simen	4035	13.25135	38.20225	5.2649	0.045(0.1191)	0.0694(0.1785)	13.97

Table 2: Genetic diversity of individuals of Deschampsia and Koeleria capensis pooled by Mountain. DW = frequency-downweighted marker values, Hs = Nei's within mountain genetic diversity, I = mean Shannon's Information index, Std = Standard deviation, (P) = percentage polymorphic loci

Mountain	N	Rarity (DW)	Hs(Std)	*I*(Std)	(*P*)
Deschampsia					
Bale	17	5.0198	0.138(0.1603)	0.224(0.2339)	62.21
Kilimanjaro	4	4.8708	0.051(0.1311)	0.077(0.1932)	14.53
Ruwenzori	12	5.5150	0.1651(0.1876)	0.2542(0.2681)	56.98
Koeleria capensis					
Aberdare	17	2.4308	0.0515(0.1021)	0.0917(0.1583)	35.59
Bale	29	4.9819	0.1031(0.1378)	0.1758(0.2035)	60.7
Elgon	43	2.9429	0.0701(0.1152)	0.1263(0.1719)	60.92
Kilimanjaro	38	2.0692	0.0468(0.0931)	0.0878(0.143)	48.25
Meru	12	1.6112	0.0309(0.0814)	0.054(0.1319)	17.25
Simen	14	3.3350	0.0711(0.1251)	0.1208(0.1884)	39.08

Table 3: Analyses of molecular variance (AMOVA) of AFLP markers for Deschampsia spp. and Koeleria capensis

Source of variation	Degrees of freedom	Variance components	% variation
Deschampsia			
A1: Grouping by field populations (both species together)			
Among populations	8	7.07222	38.22
Within populations	24	11.43056	61.78
A2: The two species as groups			
Among groups	1	2.78803	13.42
Among populations within groups	7	6.54947	31.54
Within populations	24	11.43056	55.04
B: Grouping by mountains			
Among groups	2	7.85719	37.2
Among populations within groups	6	1.8341	8.68
Within populations	24	11.43056	54.12
C: Grouping by BAPS clusters			
Among groups	1	7.32726	33.33
Among populations within groups	7	3.22541	14.67
Within populations	24	11.43056	52
Koeleria capensis			
A: Grouping by field populations			
Among populations	36	12.54075	47.06
Within populations	116	14.10876	52.94
B: Grouping by mountains			
Among groups	5	3.37187	12.38
Among populations within groups	31	9.76003	35.83
Within populations	116	14.10876	51.79

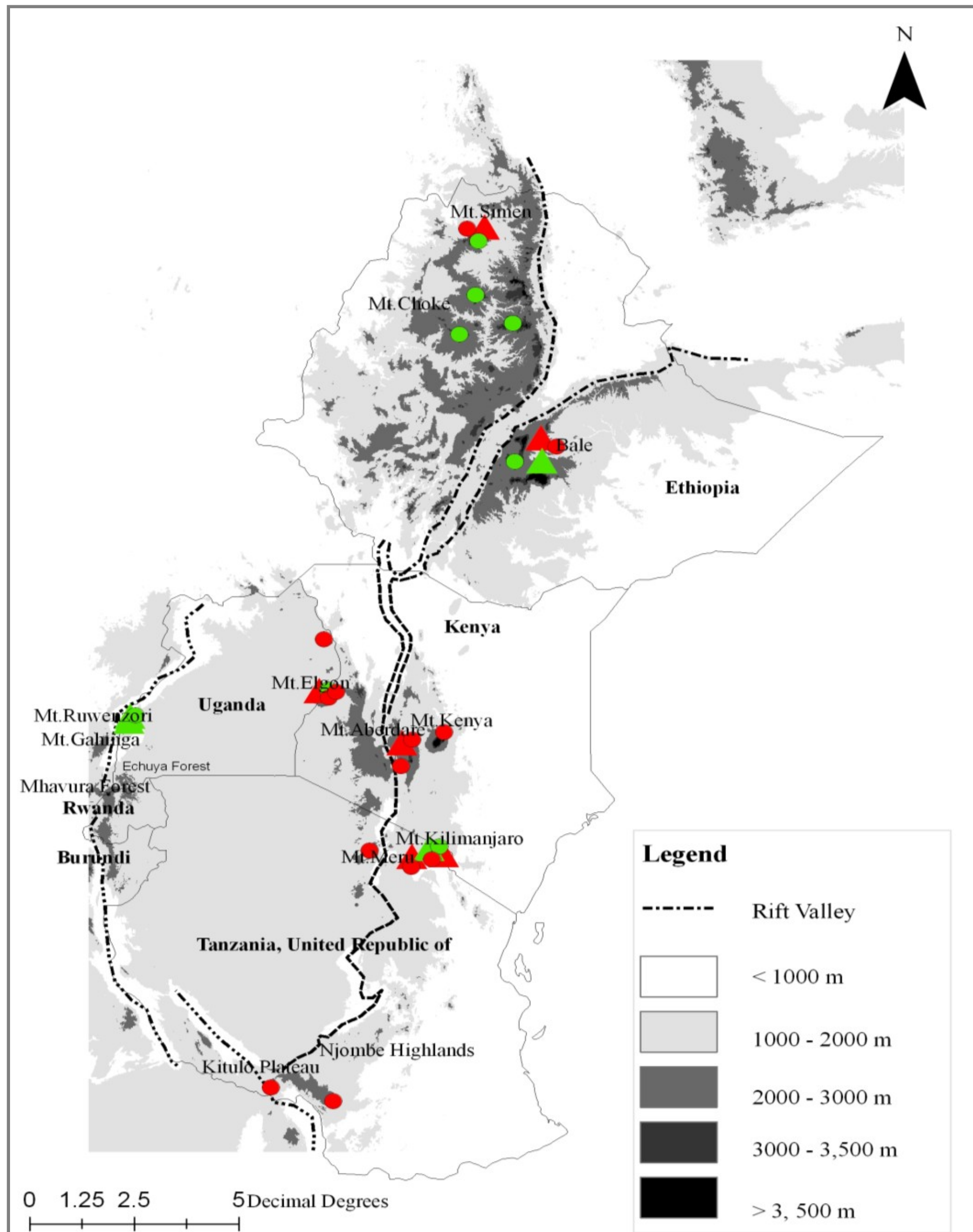

Fig. 1: Distribution (dots) and sampling localities (triangles) of Deschampsia angusta and D. caespitosa (green) and Koeleria capensis (red) in the East African and Ethiopian high mountains

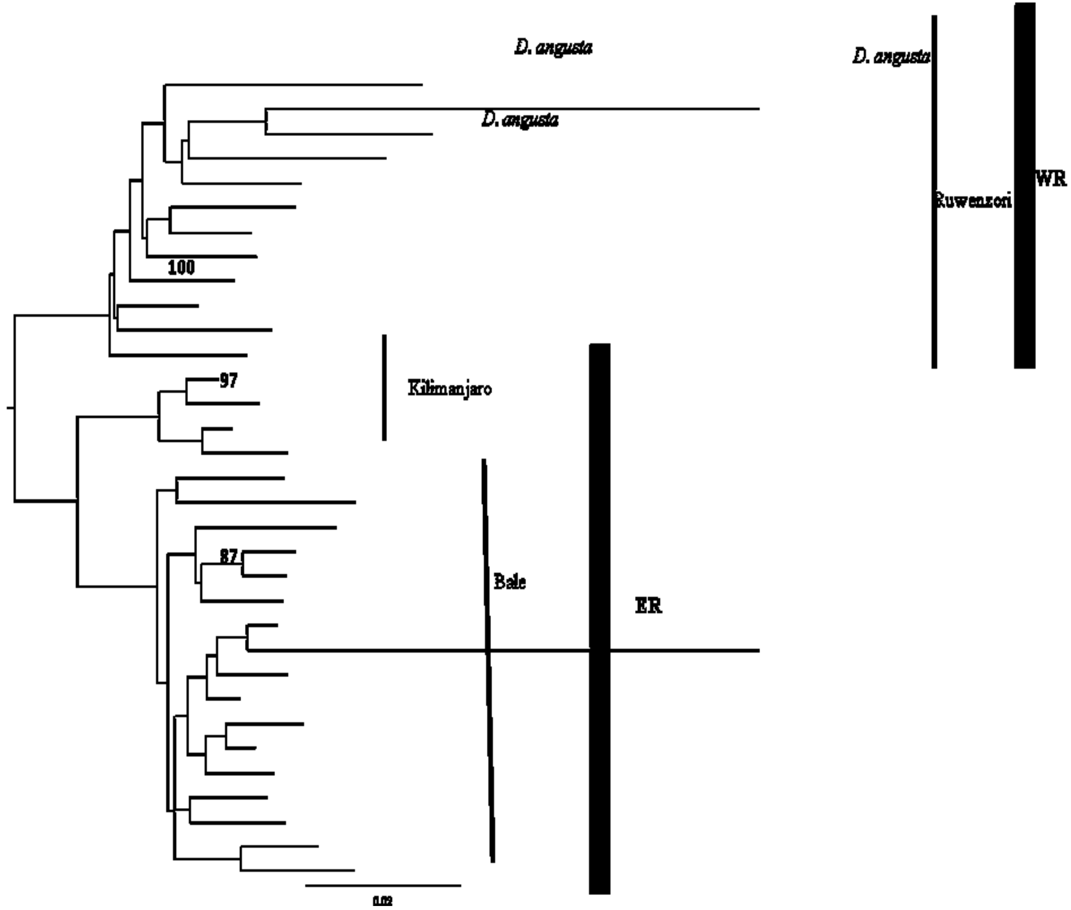

Fig. 2a: Midpoint rooted neighbor-joining tree inferred from AFLP data for 33 individuals (9 populations) of afro-alpine Deschampsia angusta and D. caespitosa. The tree was built from pairwise distance matrix based on Nei & Li distance (1979). The numbers are bootstrap values (1000 replicates) above 50%. ER = Eastern Rift, WR = Western Rift

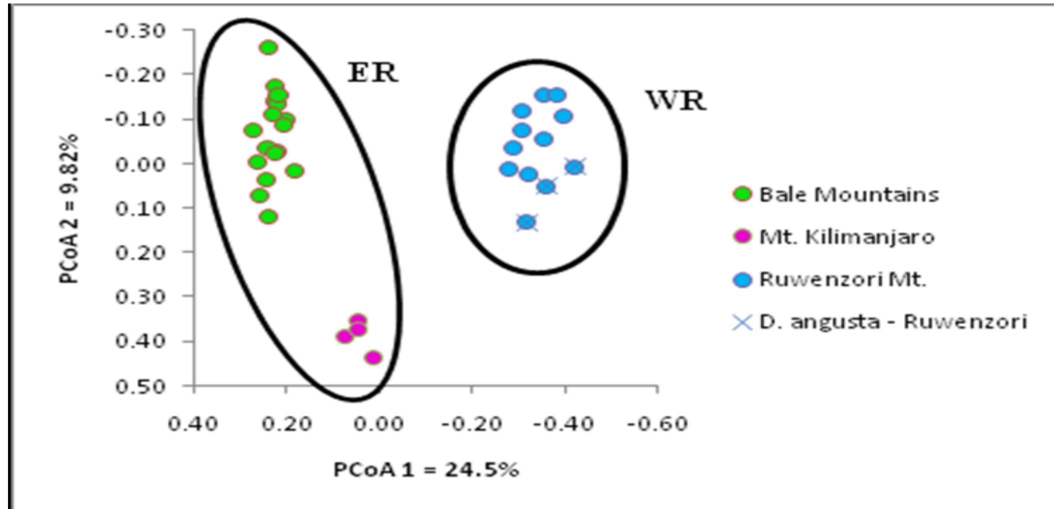

Fig. 2b: Principal coordinates analysis (PCoA) based on AFLP data for 33 individuals (9 populations) of Deschampsia spp. Colours designate the geographical origins of the accessions (see Table 1). Crossed circles represent D. angusta individuals. ER = Eastern Rift, WR = Western Rift

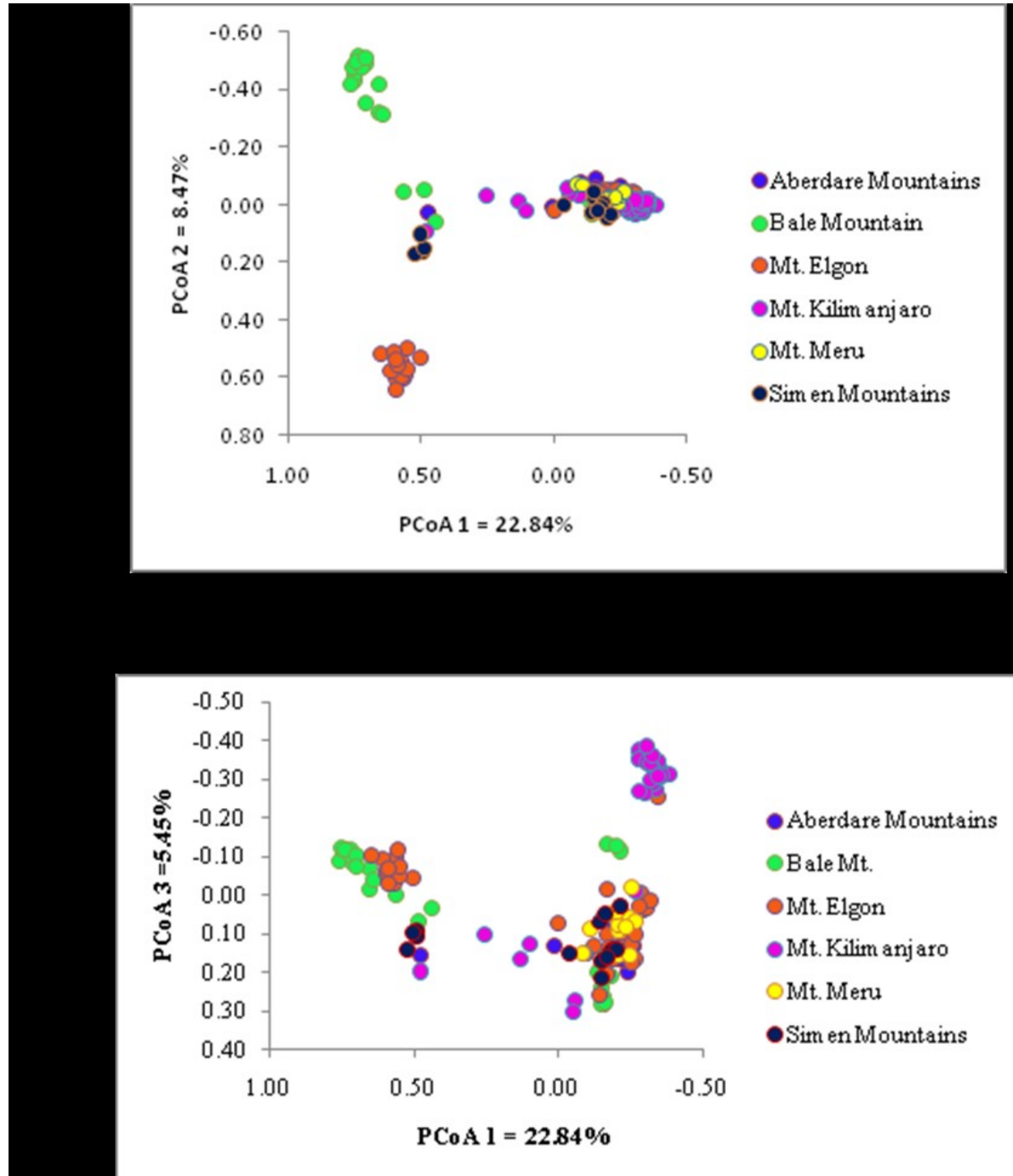

Fig. 3: Principal coordinates analysis (PCoA) based on AFLP data for 153 individuals (36 populations) of Koeleria capensis. Colours designate the geographical origins of the accessions (see Table 1). A: Axes 1 and 2, B: Axes 1 and 3.

CHAPTER FOUR

4.0 LOW GENETIC DIVERSITY IN THE ENIGMATIC AFRO-ALPINE GIANT LOBELIAS: POSSIBLE CAUSES AND IMPLICATIONS FOR THEIR CONSERVATION (Manuscript III)

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4.1 Abstract

Most giant lobelias of the Unbranched Inflorescence clade (*Lobelia* L. section *Rhynchopetalum* (Fresen.) Benth. & Hook. f.) are narrowly endemic, limited to one or a few mountains in eastern Africa. Amplified fragment length polymorphism (AFLP) markers were used to assess genetic diversity and distinctivity within 13 of the 14 known species of this clade across most of their distribution area. Within-species and within-population genetic diversities were typically low. Unexpectedly, least diversity was observed in the most widely distributed species, *L. giberroa* (H_T = 0.0751), followed by *L. rhynchopetalum* (H_T = 0.0832), which is widely distributed in Ethiopia. In contrast, highest diversity was observed in the narrow endemics *L. bequaertii* (H_T = 0.2522) and *L. thuliniana* (H_T = 0.2118). The low genetic diversity in *L. giberroa* may be caused by bottlenecks following reduction of its montane forest habitat by human activities, which may have been less influential in the high-alpine Ruwenzori habitat of the local endemic *L. bequaertii.* However, there was no correlation between the age of the mountains and levels of genetic diversity, suggesting that the current populations on the older mountains originate from colonization episodes taking place long after their formation. The generally low levels of genetic diversity observed within the unbranched inflorescence giant lobelias may be caused by genetic drift in their typically small and fragmented populations, probably most severely so during population bottlenecking in connection with the Pleistocene climatic fluctuations. In several species, fragmentation has probably been reinforced by recent expansion of agriculture, especially at low altitudes. Monitoring the most diverse and genetically most distinct populations of each species in situ and germplasm tests for ex situ conservation are suggested in

order to increase the probability for long-term survival of these highly endemic and enigmatic plants.

Key words: AFLP, eastern Africa, genetic diversity and distinctivity, giant lobelias, narrowly endemic

4.2 Introduction

Genetic variation is crucial for long-term survival of species since it provides the raw material for adaptation to environmental changes (Ellstrand and Elam 1993). Thus, an accurate estimate of the levels of genetic diversity and description of geographical patterns of the genetic variation has become an important tool in designing conservation programs of threatened and endangered plant species (Ellstrand and Elam 1993; Rivers and Brummitt 2011) along with planning for *in situ* and *ex situ* conservation (Gaudeul *et al.* 2000; He *et al.* 2000; Juan *et al.* 2004; Coppi *et al.* 2008; Makowsky *et al.* 2009, Geleta and Bryngelsson 2009; Yan *et al.* 2009; Jaramillo and Atkinson 2010; Rivers and Brummitt 2011; Suárez-Montes *et al.* 2011). Optimally, a conservation program is concerned with retention of organisms' fitness and adaptability to environmental change as well as preservation of major evolutionary lineages within taxonomic units.

Studies on genetic diversity within and among populations of both widely spread and endemic plant species using molecular markers have increased in the recent years due to their central importance in planning for *in situ* and *ex situ* conservation (Gaudeul *et al.* 2000; He *et al.* 2000; Juan *et al.* 2004; Coppi *et al.* 2008; Makowsky *et al.* 2009; Geleta and Bryngelsson 2009; Yan *et al.* 2009; Jaramillo and Atkinson 2010; Rivers and Brummitt 2011; Suárez-Montes *et al.* 2011). Among these, many have showed

that endemic and rare taxa contain significantly less genetic diversity than widespread species (Karron *et al.* 1988; Ellstrand and Elam 1993; He 2000; Broadhurst and Coates 2002; Juan *et al.* (2004); Coppi *et al.* 2008). This has been linked to the fact that widespread species may have a history of large, continuous populations whereas endemics may consist of smaller and more ecologically limited populations which may be susceptible to loss of variation by genetic drift or bottlenecks. However, some studies have reported similar genetic diversity between widespread and endemic species (Ellstrand and Elam 1993).

Except for *L. stuhlmannii*, the geographically disjunct *L. mildbraedii* and the widespread *L. giberroa* (Table 1), the eastern African giant lobelias are narrowly endemic, confined to a single mountain or single group of neighbouring mountains (Hedberg 1969). They occupy a wide diversity of habitats including montane forests, ericaceous shrublands, and alpine bogs, grasslands and rock outcrops. The high-alpine species are easily recognizable by their remarkable habit, particularly their giant rosette growth form, which is considered to represent thermal adaptations to the harsh high-alpine climate (Knox and Palmer 1998).

The afro-alpine giant lobelias are easily noticeable in their range and are highly publicized plants as tourist attractions in this unique ecosystem. The significance of giant lobelias is therefore not only ecological, but also recreational, medicinal and touristic. Fire, agricultural expansion, tourist influx and overgrazing are potential threats to long-term survival of these plants. Furthermore, island populations and narrow endemics are in general more prone to extinction than mainland and widespread ones due to genetic factors, such as loss of genetic variation via drift,

inbreeding depression, and genetic adaptations to special ecological conditions, as well as because of interactions between low genetic diversity and demographic or environmental stochasticity (Ellstrand and Elam 1993; Lomolino 2006). The high level of endemism makes these plants highly vulnerable to habitat and genetic perturbations, and thus conservation of their genetic diversity needs appropriate management of existing populations.

Two groups of eastern African giant lobelia species have long been recognized in classic taxonomic and biogeographic studies, one mainly montane group with branched inflorescences and one group mainly composed of alpine species with unbranched inflorescences (Mabberley 1975; Knox and Palmer 1998). A molecular phylogeny by Knox and Palmer (1998) based on cpDNA restriction site polymorphism confirmed the monophyly of these two groups, with the Branched Inflorescence clade also including one Brazilian species. They suggested that an ancestral lineage of giant lobelias with branched inflorescences arrived in eastern Africa from the Asian/Pacific region during the Miocene and established on the ancient uplands of Tanzania (the Eastern Arc Mountains). This lineage rapidly diversified into the two major clades recognized today, of which the unbranched inflorescence lobelias further radiated after colonization of the more recent, tall volcanic mountains which arose during the Pliocene and Pleistocene. The Unbranched Inflorescence clade contains 14 extant species showing a pronounced geographic structure of the genetic variation among the Western Rift Mountains, the Eastern Rift Mountains, and the Ethiopian Mountains (Knox and Palmer 1998). The distinctive characteristics, habitat and altitudinal ranges of these species are presented in Table 1. In this study, population genetic parameters are compared in thirteen of

the fourteen species in the Unbranched Inflorescence clade. Comparisons of the levels and patterns of genetic variation within members of the same family or genus (Petit 2005), or between pairs of rare and widespread congeners (Gitzendanner and Soltis 1999) have revealed strong effects of life history and phylogenetic position in the levels of genetic diversity (Young and Brown 1996; Rivers and Brummitt 2011). This suggests that comparing closely related congeners may be a useful strategy for conservation purposes, since it may allow measuring the levels of genetic diversity of each individual *Lobelia* species in the context of evolution and life history traits of the study group.

Changes in factors such as population size, degree of isolation, fitness and genetic variability levels are warning signs that populations may be vulnerable (Ellstrand and Elam 1993). Conservation managers may be able to use pre-existing data to determine whether such changes have occurred, but additional experimental or descriptive evidence may be necessary (Ellstrand and Elam 1993). Molecular markers provide a tool for recognition of genetic processes that are taking place at the population level for conservation purposes and identification of genetic lineages to be preserved within each species and subspecies. Amplified Fragment Length Polymorphism (AFLP) is a fingerprinting technique which has been widely used for investigating genetic variation in endemic, widespread and endangered plant populations (Gaudeul *et al.* 2000; Keiper and McConchie 2000; Juan *et al.* 2004; Nybom 2004; Kim *et al.* 2005; Kebede *et al.* 2007; Coppi *et al.* 2008; Yan *et al.* 2009; Rivers and Brummitt 2011). The AFLPs are thus used in this case to 1) determine and compare level of genetic diversity and patterns of genetic variation among and within populations of the unbranched inflorescence giant lobelias 2)

determine whether there is correlation between the age of the mountains and levels of genetic diversity therein and 3) explore implications for effective conservation of these species.

4.3 Material and Methods

4.3.1 Sampling and genetic analysis

The AFLP data were taken from Masao *et al.* (2012a unpublished) in which Fresh young leaf samples were collected from 14 mountains in eastern Africa between 2007 and 2009. Five individuals of the same species found within an area of 10000 m^2 were considered as representing one population. Leaf samples were dried in silica gel and voucher specimens of all five sampled individuals were pressed. Altogether 162 populations of 14 species of the unbranched inflorescence giant lobelias were collected (in some populations, less than five individuals were found), giving a total of 894 individuals, of which 689 individuals from 13 of the species were successfully genotyped (Masao *et al.* 2012a unpublished). Total genomic DNA was extracted from the silica-gel-dried leaves using MoleStrips™ DNA Plant kit with an automated GeneMole® robot following the manufacturer's instructions (Qiagen Nordic). Prior to loading the plant material to the GeneMole®, some modifications were performed and the AFLP protocol was optimized according to Gaudeul *et al.* (2000) and further modified (Masao *et al.* 2012a unpublished). The data were then scored and cleaned before further analyses (Masao *et al.* 2012a unpublished).

For the purposes of this paper, all populations with only one individual were removed from Masao *et al.* (2012a unpublished) and the data were further analyzed statistically. Genetic diversity of the 13 species of eastern African Unbranched

Inflorescence giant lobelias was estimated at the total within-species level (H_T) and at the within-population/mountain level (H_S) for all the retained AFLP loci based on three parameters: 1) allele richness, estimated as the percentage of polymorphic loci (*P*) (Yeh and Boyle 1997), 2) average gene diversity using Nei's unbiased diversity estimator for each locus and computed over all loci (H_S/H_T; Nei 1978), and allele similarity using Shannon information index (*I*; Lewontin 1972). The average within-population diversity (H_S) was calculated from the within-population estimates while the total within-species (H_T) and within-mountain diversity was calculated by pooling all samples from populations of each species and mountains separately. These genetic parameters were calculated using the software package POPGENE, V. 1. 32 (Yeh *et al.* 2000). Genetic distinctivity of each species, population and mountain was estimated as frequency-downweighted marker values (DW) according to Schönswetter and Tribsch (2005) with some modifications as implemented in AFLPdat by Ehrich (2006). A regression analysis between genetic diversity of populations, altitude and the age of a mountain was conducted for all the species using the regression model included in GraphPad InStat version 3.00 for Windows 95 (www.graphpad.com).

4.4 Results

A total of 672 individuals from 150 populations of the 16 taxa (13 species and 3 non-autonymous subspecies) were analyzed in this study. The number of markers per species ranged between 90 in *L. bequaertii* to 720 in *L. giberroa* (Table 2). Reproducibility of the markers for all the species together was 97.9%. The number of polymorphic loci ranged between 98.01% and 100% within species (Table 2), and between 5.97% and 63.23% within populations (Table 3).

Nei's gene diversity (within-species diversity = H_T, within-population/mountain diversity = H_s) and Shannon information index (*I*) were relatively low in all species (Tables 2, 3). Concerning total species diversity, the widespread *L. giberroa* had the lowest diversity (H_T = 0.0751, *I* = 0.1422, Std = 0.1075) followed by *L. rhynchopetalum* (H_T = 0.0832, *I* = 0.1479, Std = 0.1315). In contrast, *L. bequaertii* had relatively high mean diversity (H_T = 0.2522, I_T = 0.3954, Std = 0.17) followed by *L. thuliniana* (H_T = 0.2118, *I* = 0.3515, Std = 0.139). In agreement with the values observed at the species level, within-population diversity was also found to be low in all species (Table 3). Nei's gene diversity ranged from Hs=0.0204 in *L. telekii* to 0.2132 in *L. bequaertii*; while *I* ranged from 0.0298 in *L. telekii* to 0.32 in *L. bequaertii*. Taking the two parameters into consideration, all populations of *L. bequaertii* showed relatively high gene diversity. There was strong correlation between H_T/H_s and *I* (H_T versus *I*: r = 0.6076, R^2 = 0.3691, P = 0.0010; H_s versus *I*: r = 0.8207, R^2 = 0.6735, P < 0.0001). The frequency down-weighted marker value (DW) was highest in the only population analyzed of *L. thuliniana* from the Mafinga Highlands (Table 2). Notably, many populations of *L. aberdarica*, *L. burttii*, *L. giberroa*, *L. mildbraedii* and *L. wollastonii* had high DW values compared to others (Fig. 1).

The regression analyses demonstrated some weak positive correlation between genetic diversity and altitude for most populations, except for those of *L. bequaertii*, *L. burttii*, *L. rhynchopetalum* and *L. wollastonii*, which showed some weak negative correlation to altitude change. Some mountains harboured genetically more diverse populations than others, in particular Mt. Kenya, Muhavura and Ruwenzori (Fig. 1).

Notably, there was no significant correlation between mountain age and genetic diversity (Fig. 3). The oldest of the high mountains, Mt. Elgon, actually harboured lowest diversity for most species, and the ancient southern highlands of Tanzania harboured intermediate diversities (Fig. 1, Table 4).

4.5 Discussion

The results indicate general low levels of genetic diversity in the unbranched inflorescence giant lobelias both at the species (H_T 0.0751-0.2522) and at the population level (H_S 0.0204-0.2132). Demographic factors, life history traits such as breeding system or growth form, and phylogenetic position are important in explaining genetic diversity within species and the apportioning of the diversity among their populations (Ellstrand and Elam 1993, Hamrick and Godt 1996). This study group, which is largely composed of outcrossing species (Masao *et al.*, 2012a unpublished), the gene diversity levels detected are lower than average values estimated for outcrossing species using other markers e.g. RAPD H_{pop} was 0.27 while STMS-derived H_E was 0.65 (Nybom 2004). A number of studies providing genetic diversity estimates for endemic outcrossing and other endangered species also provide low to intermediate values of AFLP diversity. For instance, Gaudeul *et al.* (2000), He (2000), Juan *et al.* (2004), Piñeiro *et al.* (2007) and Coppi *et al.* (2008) found Hs-values varying between 0.035 and 0.391. The low genetic diversity observed seems thus to be better explained by: 1) genetic drift in their typically small and fragmented populations, probably most severely so during population bottlenecking in connection with the Pleistocene climatic fluctuations, 2) in several species, fragmentation has probably been reinforced by recent expansion of

agriculture, especially at low altitudes 3) the recent evolutionary history and rapid diversification of the group as proposed by Knox and Palmer (1998).

A study by Lawton-Rauh (2007) on the recently derived species of the Hawaiian silversword alliance based on nucleotide sequences and floral regulatory genes showed low estimates of nucleotide diversity (ranging from 0.0005 to 0075) across all the studied species suggesting that the species had undergone recent population expansion. Broadhurst and Coates (2002) also suggested that low genetic variation in rare and/or geographically restricted species may be due to founder events associated with recent speciation.

Contrary to expectations, the lowest mean genetic diversity ($H_T = 0.0751$) was found within the most widely distributed species, *L. giberroa* (Table 2). Many studies comparing genetic diversity levels report higher diversity in widespread than in rare/restricted species (Karron *et al.* 1988; Ellstrand and Elam 1993; Gitzendanner and Soltis; He 2000; Juan *et al.* 2004). A few studies have also reported endemic species to have similar or higher levels of genetic diversities as do widespread species (Ellstrand and Elam 1993). *Lobelia giberroa* exhibits a patchy distribution and grows in relatively disturbed habitats compared to the remaining *Lobelia* species in eastern Africa. It is seriously affected by agriculture expansion and other human activities. Fragmentation results in smaller and more isolated populations which may in turn lead to decreased genetic diversity due to loss of rare alleles via genetic drift, inbreeding within the habitat fragments and reduction in gene flow among habitat fragments (Aquilar *et al.* 2008). A previous detailed phylogeographical study of *L. giberroa* (Kebede *et al.* 2007) also reported low genetic diversity within the species,

and a structuring of the genetic diversity suggesting recent contact among forest patches early in the present interglacial, before agricultural expansion reduced the extension of the montane forest.

The high-alpine *Lobelia rhynchopetalum,* which had the second lowest within-species diversity (H_T = 0.0832) among the 13 studied species, is endemic to Ethiopia but widespread among the mountains on the elevated plateaus on both sides of the Rift Valley. Its genetic structure is affected by the Rift Valley acting as a barrier to gene flow; it comprises two genetic lineages which are restricted to different sides of this valley (Masao *et al.* 2012a unpublished). A previous study on this species based on Inter Simple Sequence Repeats (ISSRs) reported higher genetic diversity than found in this study (H_T = 0.37, Hs = 0.13) (Geleta and Bryngelson 2009), in agreement with the tendency of the co-dominant ISSR markers to yield higher diversity estimates than dominant markers (Nybom 2004). However, both ISSR and the AFLP estimates revealed relatively higher diversity levels in the Simen Mountains as compared to the rest of populations of *L. rhynchopetalum*. The general low level of genetic diversity in this species can be attributed to human disturbance particularly overgrazing which is very common on the mountains in Ethiopia.

Lobelia bequaertii, despite being among the most narrowly distributed species in the group, occurring only on the Ruwenzori Mts., revealed relatively high within-species (H_T = 0.2522) and within-population (H_S = 0.2132) diversity compared to the other species. This can be due to the fact that despite the local distribution of this species; the populations of *L. bequaertii* are relatively large and are less affected by human disturbance compared to others. Ellstrand and Elam (1993) pointed out that rare

species with large localized populations are expected to exhibit high levels of genetic variation.

The analyses further indicated that, the most diverse populations corresponded to a particular altitudinal range (Fig. 2). The species growing at intermediate altitudes between 3100 m and 3800 m had relatively higher genetic diversity compared to those growing below or above this range (Table 2 and 3). For instance, *Lobelia bequaertii*, *L. burttii* and *L. mildbraedii*, which are among the most common species at intermediate altitudes, showed relatively high gene diversities. A study by Yan *et al.* (2009) reported a considerable correlation between genetic diversity of the *Elymus* populations and their altitudinal distribution on the Quinghai-Tibetan Plateau. They found significantly high levels of genetic diversity at medium altitudes, although their study system had wide altitudinal range compared to the eastern African giant lobelias. Another study by Geleta and Bryngelsson (2009) reported that the mid-altitude *L. rhynchopetalum* populations were more diverse compared to others at higher or low altitudinal ranges. The low genetic diversities at low or high altitudes among giant lobelias seem to be best explained by marginal ecological conditions at the lower and upper extreme of their range or could be a result of temperature fluctuations during major episodes of climate change when populations moved up or down in response to climate changes. During such periods, it is possible that the top populations could only experience gene flow from below; the bottom populations only from above, whereas the middle populations might have enjoyed genetic input from both above and below hence the observed structure.

Notably, the regression analyses revealed that there is no association between age of a mountain and genetic diversity therein (Fig. 3), suggesting that the contemporary populations of a certain giant lobelia species colonized (or re-colonized) the mountain long after its formation. In some instances, it is possible that the species occurring on more than one mountain may have gone extinct or nearly extinct on some of the mountains during Pleistocene climate change, and then re-colonized the mountains as climate improved thus the current structure. This result is in support of the study by Knox and Palmer (1998) who inferred the origin of different species of giant lobelias on different mountains irrespective of their age.

From conservation point of view, all giant lobelias of eastern Africa have been thought to be safe because they grow inside protected areas (Knox 1993). However, some of these areas, such as Kilimanjaro National Park in Tanzania, Ruwenzori National Park and Mt. Kenya National Park, are highly affected by tourism influx and possible introduction of invasive species. Despite the fact that conservation managers may be able to use pre-existing data to determine whether ecological changes have occurred, additional experimental or descriptive evidence may be necessary to make conclusions regarding conservation of a particular area. Given the genetic status revealed by this study and the current human disturbance experienced by giant lobelias and other plants in the National Parks, it is important that improved conservation strategies are considered.

Following the example of recent conservation programs, a conservation strategy that targets each taxon (i.e. species and subspecies) proposed as an independent entity of which the most diverse populations are prioritized for protection (He *et al.* 2000;

Makowsky *et al.* 2009, Geleta and Bryngelsson 2009; Yan *et al.* 2009). It is hereby proposed that this strategy be applied to all sixteen taxa in all populations with highest genetic diversities and those with the highest DW values. *Ex situ* conservation is another potential strategy. For this purpose, one need to design and maintain a gemplasm bank for the eastern African giant lobelias that may prioritize the populations with the highest genetic variability and those with high genetic distinctivity (DW), in order to conserve both allelic richness and allelic uniqueness.

4.6 Acknowledgements

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Table 1: Distinctive features, common habitat, altitudinal range, and known localities for unbranched inflorescence eastern African giant lobelias. The table is modified from Thulin, (1984); Knox, (1993); and Knox & Palmer, (1998)

Species/subspecies and author	Distinctive characteristics	Altitude range (m)	Most common Habitat	Known Locations
L. aberdarica R.E.Fr. & T.C.E.Fr.	Bracts shorter than flowers	1800-3450	Wet meadow	Kenya (Elgon, Cherangani, Aberdares)
L. bambuseti R.E.Fr. & T.C.E. Fr	Plant 3 - 8 m tall in flower; bracteoles inserted at the base of pedicel; calyx lobes 20 - 30mm long, seeds 0.7-0.8 mm long, not winged	2700 -3480	Mist forest	Kenya (Aberdares, Mt. Kenya)
L. bequaertii De Wild	Bracts 3.5-6cm wide, acuminate	3000 - 4000	Alpine bogs	Uganda (Ruwenzori)
L. burttii E.A. Bruce subsp. *burtii*	Bracts 2.5-3cm wide, acute	3250- 3360	Alpine bogs	Tanzania (Hanang)
subsp. *meruensis* E.B. Knox	Bracts 6.5-8 cm long, 2.4-3.2 cm wide, ovate, apex acute; corolla 27-37 mm long	3000 - 3970	Alpine bogs	Tanzania (Meru)
subsp. *telmaticola E.B. Knox*	Bracts 8 cm long, 3.5 cm wide, ovate; apex acuminate; corolla 33-42 mm long	3325 - 3350	Alpine bogs	Tanzania (Loolmalassin)
Lobelia deckenii (Asch.) Hemsl subsp. *deckenii*	Corolla glabrous within, bracts longer than flowers	2900 - 4300	Alpine bogs	Tanzania (Kilimanjaro)
L. deckenii subsp. *incipiens* E.B. Knox	Corolla 36 mm long, white	2500 - 3150	Mist forest	Tanzania (Kilimanjaro)
L. giberroa Hemsl.	Leaves 4-15 cm wide; corolla 25-35 mm long, greenish or greenish white, often tinged with blue or purple	1260 - 3350	Woodland forest	All eastern African mountains and highlands to Burundi, Congo, Malawi, and Zambia
L. gregoriana Baker f. subsp. *elgonensis* (R.E.Fr. & T.C.E.Fr.) E.B. Knox	Bracts glabrous	3660 - 4050	Alpine bogs	Kenya (Elgon, Cherangani)
L. gregoriana spp. *sattimae* (R.E.Fr. & T.C.E.Fr.) E.B. Knox	Bracts pubescent both surfaces	3360 - 3970	Alpine bogs	Kenya (Aberdares)
L. gregoriana subsp. *gregoriana* Baker f.	Anther-tube hairy on the back	3050 - 4350	Alpine bogs	Kenya (Mt. Kenya)
L. mildbraedii Engl.	Plant 1-3.5 m tall in flower; bracteoles inserted near middle of pedicel; calyx lobes 8-15 mm long, seeds 1.2-1.5 mm long, winged	1950 - 3050	Wet meadow	Tanzania (Nyungwe, Muhinga, Karisoke, Kitulo, Nyika, Dabaga highlands), Burundi, Congo, Rwanda, and Uganda.
L. rhynchopetalum Hemsl.		3150 - 4375	Drained alpine	Ethiopia (Arsi, Bale, Boralucu, Choke, Gonder, Shewa, Simen, and Wef Washa)

Species/subspecies and author	Distinctive characteristics	Altitude range (m)	Most common Habitat	Known Locations
L. stuhlmannii Sweinf. & E.A. Bruce	Leaves 1.5-5(-5) cm wide; corolla 40-50mm long, dark mauve or purplish	2900 - 3900	Mist forest	Uganda (Ruwenzori, Sabinyo, Muhavura), Congo, Rwanda, Tanzania
L. telekii Schweinf.	Corolla 14-18 mm long; bracts 10-20 (-25) cm long	2960 - 4650	Drained alpine	Kenya (Elgon, Aberdares, Mt. Kenya)
L. thuliniana E.B. Knox	Leaves 25-45 cm long, 2.5-4 cm wide, acute at the apex; lowermost bracts 45-65 mm long, 2-4 mm wide; coolla28-35 mm long; seeds ovate in outline, 0.8 mm long, orange brown	1800 - 1900	Stream side	Tanzania (Kibidula, Ndumbera, Dabaga highlands)
L. wollastonii Baker f.	Bracts, calyx and pedicels densely shaggy pubescent	3500 - 4400	Drained alpine	Uganda (Karisimbi, Muhavura, Ruwenzori)

Table 2: Pooled within species genetic diversity patterns as analyzed using Popgene program. N= total number of individuals analysed per each species, DW = frequency-downweighted marker values HT = average within species genetic diversity, I = mean Shannon's Information index, Std = Standard deviation, (P) = Percentage polymorphic loci

Species	N	DW	H_T(Std)	I_T(Std)	No. of markers	No. of polymorphic loci	(*P*)
L. aberdarica	47	8.3617	0.1193(0.1458)	0.208(0.1996)	393	393	100
L. bambuseti	38	8.1579	0.1063(0.1339)	0.1913(0.1835)	310	310	100
L. bequaertii	17	5.2941	0.2522(0.17)	0.3954(0.2179)	90	90	100
L. burttii	20	8.6	0.1815(0.1698)	0.2988(0.2202)	172	172	100
L. deckenii	57	3.5965	0.1399(0.1626)	0.2341(0.2225)	205	205	100
L. giberroa	91	7.8132	0.0751(0.1075)	0.1422(0.1572)	720	711	98.75
L. gregoriana	61	4.8525	0.1258(0.1495)	0.2148(0.2097)	312	296	98.01
L. mildbraedii	33	10.0606	0.148(0.1573)	0.2501(0.2128)	332	332	99.7
L. rhynchopetalum	101	3.0099	0.0832(0.1315)	0.1479(0.188)	304	304	99.67
L. stuhlmannii	50	5.0000	0.1308(0.1503)	0.224(0.2078)	250	250	100
L. telekii	99	3.8889	0.1(0.1308)	0.1782(0.1871)	385	385	99.74
L. thuliniana	10	26.3000	0.2118(0.139)	0.3515(0.1745)	264	263	99.62
L. wollastonii	49	8.0612	0.1276(0.1451)	0.2208(0.2012)	396	395	99.75

Table 3: Within-population genetic diversity from 150 populations of 13 species of Lobelia. DW = frequency-downweighted marker values, Hs = average within-population genetic diversity, I = mean Shannon's Information index, Std = Standard deviation, (P) = percentage of polymorphic loci, n = number of individuals analyzed per population and N = total number of individuals analyzed per species

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
L. aberdarica	KN_0301	O-DP-35998 - O-DP-36002	Elgon	5	3629	7.6463	0.0844(0.1483)	0.1324(0.2221)	28.75
	KN_0394	O-DP-27246 - O-DP-27260	Elgon	13	3557	6.8318	0.0935(0.1489)	0.1527(0.2177)	44.27
	KN_0453	O-DP-27318 -O-DP-27322	Cherangani	4	2832	9.3347	0.0763(0.1434)	0.1186(0.2166)	24.43
	KN_0454	O-DP-27325 - O-DP-27327	Cherangani	3	2892	10.7674	0.0754(0.1487)	0.1145(0.2223)	21.63
	KN_0455	O-DP-27328 - O-DP-27332	Cherangani	4	2359	9.6932	0.0831(0.1534)	0.1276(0.2279)	25.45
	KN_0456b	O-DP-27333 - O-DP-27341	Cherangani	5	2954	11.4200	0.1006(0.1529)	0.1596(0.2296)	35.88
	KN_0457	O-DP-27344 - O-DP-27350	Aberdare	4	2954	5.8505	0.0652(0.1446)	0.0986(0.2126)	18.83
	KN_0474	O-DP-27432 - O-DP-27435	Aberdare	4	-	9.9870	0.0881(0.1481)	0.1383(0.2243)	29.26
	KN_0476	O-DP-27442 - O-DP-27445	Aberdare	5	3015	7.4182	0.0825(0.149)	0.1288(0.222)	27.74
Total/average				N=47		8.7721	0.0832(0.1486)	0.1301(0.2217)	28.5
L. bambuseti	KN_1112	O-DP-36885 - O-DP-45690	Kenya	10	3053	13.6116	0.1195(0.1398)	0.2034(0.2049)	63.23
	KN_0458	O-DP-27353 - O-DP-27357	Aberdare	4	3117	7.4686	0.075(0.1368)	0.1189(0.2096)	25.81
	KN_0473	O-DP-27426 - O-DP-27430	Aberdare	5	3117	12.3185	0.105(0.1555)	0.1662(0.2331)	37.1
	KN_0697	O-DP-28398 - O-DP-28402	Aberdare	5	3466	3.8993	0.0586(0.1346)	0.0906(0.1991)	19.03
	KN_0742	O-DP-28566 - O-DP-28570	Aberdare	5	3270	3.5141	0.0604(0.1383)	0.0921(0.2041)	18.39
	KN_1111	O-DP-36880 - O-DP-45685	Kenya	9	3326	5.0389	0.0754(0.1372)	0.1226(0.2047)	32.58
Total/average				N =38		7.6418	0.0823(0.1404)	0.1323(0.2093)	32.7
L. bequaertii	UG_2243	O-DP-40484 - O-DP-40487	Ruwenzori	4	3425	4.4652	0.1519(0.1822)	0.2322(0.2683)	45.56
	UG_2283	O-DP-43051 - O-DP-43055	Ruwenzori	4	3574	3.7829	0.1631(0.2037)	0.2404(0.293)	42.22
	UG_2362	O-DP-40952 - O-DP-40956	Ruwenzori	4	3612	6.4097	0.2132(0.1979)	0.32(0.2825)	60
	UG_2426	O-DP-43648 - O-DP-43652	Ruwenzori	5	3968	6.2737	0.1927(0.1999)	0.2896(0.2847)	55.56
Total/average				N =17		5.2329	0.1802(0.1959)	0.2706(0.2821)	50.84

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
L. burttii	TZ_0408	O-DP-38624 - O-DP-38628	Meru	5	3637	8.3986	0.1225(0.1726)	0.1894(0.2532)	39.53
	TZ_0459	O-DP-38906 - O-DP-38910	Meru	5	3936	8.0846	0.1216(0.1748)	0.1873(0.2548)	38.95
	TZ_0492	O-DP-39069 - O-DP-39073	Meru	5	3608	5.6519	0.0877(0.1632)	0.1324(0.2384)	25.58
	TZ_0498	O-DP-39094 - O-DP-39098	Meru	5	3589	12.2650	0.1746(0.177)	0.2725(0.2562)	58.72
Total/average				N =20		8.6000	0.1266(0.1719)	0.1954(0.2507)	40.7
L. deckenii spp. deckenii	TZ_0025	O-DP-37017 - O-DP-37018	Kilimanjaro	2	3636	2.1758	0.0328(0.112)	0.0478(0.1636)	7.91
	TZ_0094	O-DP-37276 - O-DP-37280	Kilimanjaro	5	3971	3.4846	0.0924(0.1638)	0.1403(0.2405)	27.44
	TZ_0117	O-DP-37353 - O-DP-37356	Kilimanjaro	4	3900	2.5338	0.064(0.1385)	0.0986(0.2062)	20
	TZ_0155	O-DP-37548 - O-DP-37552	Kilimanjaro	5	4155	3.5136	0.079(0.1494)	0.1221(0.2224)	25.12
	TZ_0173	O-DP-37614 - O-DP-37618	Kilimanjaro	4	4390	2.6801	0.0913(0.1717)	0.135(0.248)	24.19
	TZ_0313	O-DP-38214 - O-DP-38217	Kilimanjaro	4	3808	6.1293	0.1172(0.1729)	0.1792(0.2547)	35.35
	TZ_0333	O-DP-42700 - O-DP-42704	Kilimanjaro	5	3861	2.9660	0.0566(0.1325)	0.0873(0.1966)	18.14
	TZ_0542	O-DP-45591 - O-DP-45594	Kilimanjaro	5	3484	4.1370	0.0958(0.1638)	0.1467(0.2404)	29.77
	TZ_0545	O-DP-45600 - O-DP-45601	Kilimanjaro	2	3484	3.0212	0.0405(0.1233)	0.0591(0.1799)	9.77
	TZ_0826	O-DP-39375 - O-DP-39376	Kilimanjaro	2	3242	5.0718	0.052(0.1376)	0.0759(0.2009)	12.56
	TZ_0827	O-DP-39378 - O-DP-39381		5	3024	2.7001	0.0659(0.139)	0.1021(0.2072)	21.4
L. deckenii spp. incipiens	TZ_0335	O-DP-42711 - O-DP-38302	Kilimanjaro	4		2.7785	0.0598(0.1419)	0.0897(0.2075)	16.74
	TZ_0338	O-DP-38313 - O-DP-38317	Kilimanjaro	5	3228	4.4551	0.0829(0.1489)	0.1296(0.2223)	27.91
	TZ_0852	O-DP-39451 - O-DP-39455	Kilimanjaro	5		4.3386	0.0836(0.1502)	0.1303(0.2237)	27.91
Total/average				N =57		3.5704	0.0724(0.1461)	0.1103(0.2153)	21.7
L. giberroa									
	KN_0451	O-DP-43834 - O-DP-43835	Cherangani	2	3100	4.8504	0.0247(0.0982)	0.0361(0.1434)	5.97
	KN_0743	O-DP-28571 - O-DP-28575	Aberdare	4	-		0.0443(0.1185)	0.0684(0.1771)	13.89
	TZ_0540	O-DP-45581 - O-DP-45584	Kilimanjaro	3	2978	15.4363	0.0711(0.1314)	0.1134(0.2033)	25
	TZ_0541	O-DP-45586 - O-DP-45589	Kilimanjaro	3	2250	13.3739	0.0644(0.1393)	0.0981(0.2087)	18.61
	TZ_0466	O-DP-45589 - O-DP-38934	Meru	3	2572	6.4362	0.0425(0.1211)	0.0638(0.1789)	11.67

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
	TZ_0467	O-DP-38937 - O-DP-38940	Meru	4	2572	6.2036	0.0424(0.1122)	0.0665(0.1706)	14.03
	TZ_0468	O-DP-38941 - O-DP-38945	Meru	5	2572	7.2846	0.0466(0.1173)	0.0735(0.1769)	16.25
	TZ_0703	O-DP-39228 - O-DP-39232	Njombe	5	2102	4.7396	0.0325(0.1007)	0.0511(0.1518)	11.25
	TZ_0704	O-DP-39235 - O-DP-39240	Poroto	4	-	11.5551	0.0677(0.1347)	0.1063(0.2046)	22.64
	TZ_0705	O-DP-39243 - O-DP-39247	Poroto	5		9.8227	0.0633(0.1347)	0.0989(0.2018)	21.25
	TZ_0700	O-DP-39213 - O-DP-39217	Njombe	5	2173	3.4183	0.0302(0.104)	0.0458(0.1527)	9.03
	TZ_0707	O-DP-39253 - O-DP-39257	Kitulo	5	2598	7.8519	0.0554(0.1254)	0.0874(0.1891)	19.44
	TZ_0712	O-DP-39278 - O-DP-39279	Poroto	2	-	5.8336	0.0236(0.0961)	0.0344(0.1402)	5.69
	TZ_0713	O-DP-39280 - O-DP-39289	Poroto	8	-	7.4100	0.0572(0.1219)	0.0935(0.1841)	24.86
	UG_2169	O-DP-40190 - O-DP-40194	Mhavura	5	3058	8.0572	0.0494(0.1206)	0.0775(0.1819)	16.81
	TZ_864	Not in DNA bank yet	Pare	5	-	5.0366	0.0318(0.1009)	0.0497(0.1515)	10.69
	UG_2220	O-DP-40369 - O-DP-40371	Ruwenzori	3	-	7.7276	0.0563(0.1306)	0.0862(0.1968)	16.53
	UG_2221	O-DP-40374 - O-DP-40378	Ruwenzori	5	2597	5.4616	0.0419(0.1106)	0.0663(0.1679)	14.86
	UG_2222	O-DP-40380 - O-DP-40381	Gahinga	2	3397	12.7372	0.0639(0.1497)	0.0932(0.2185)	15.42
	UG_2310	O-DP-40727 - O-DP-40730	Ruwenzori	4	2069	12.8499	0.0593(0.1299)	0.0926(0.1965)	19.31
	UG_2273	O-DP-40578 - O-DP-40582	Ruwenzori	4	3111	4.5508	0.034(0.1067)	0.0522(0.1589)	10.42
	UG_2274	O-DP-40583 - O-DP-40586	Ruwenzori	4	3111	8.7324	0.0488(0.1198)	0.0763(0.1815)	15.97
Total/average				N =90		8.0652	0.0478(0.1193)	0.0741(0.1789)	15.44
L. gregoriana spp. elgonensis	KN_0002	O-DP-42077 - O-DP-34712	Elgon	5	4224	3.0999	0.0552(0.1349)	0.0836(0.1986)	16.23
	KN_0036	O-DP-34858 - O-DP-34860	Elgon	3	3915	2.9717	0.0381(0.1208)	0.0559(0.1748)	9.6
	KN_0138	O-DP-35266 - O-DP-35270	Elgon	5	4224	3.9988	0.0742(0.1542)	0.1118(0.2249)	21.52
L. gregoriana spp. sattimae	KN_0525	O-DP-27637 - O-DP-27641	Aberdare	5	3588	4.9076	0.0797(0.155)	0.1213(0.2275)	24.17
	KN_0530	O-DP-27662 - O-DP-27666	Aberdare	4	3806	5.7730	0.0895(0.1663)	0.134(0.2423)	24.83
	KN_0624	O-DP-42203 - O-DP-42207	Aberdare	5	3590	5.2299	0.0938(0.1637)	0.1432(0.2398)	28.81
	KN_0711	O-DP-28466 - O-DP-28469	Aberdare	4	3619	4.7353	0.0697(0.1488)	0.1051(0.2188)	19.87
L. gregoriana spp.	KN_0786	O-DP-42441 - O-DP-42445	Kenya	5	3472	4.1614	0.0737(0.1472)	0.1137(0.2178)	23.51

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
gregoriana									
	KN_0791	O-DP-28611 - O-DP-28615	Kenya	5	3652	4.4220	0.0717(0.1418)	0.1117(0.212)	23.84
	KN_0858	O-DP-28833 - O-DP-28835	Kenya	3	4230	3.8879	0.0716(0.151)	0.1073(0.2225)	19.54
	KN_0868	O-DP-28878 - O-DP-28881	Kenya	4	4047	6.8594	0.0637(0.1307)	0.1004(0.1993)	21.52
	KN_0899	O-DP-29022 - O-DP-29026	Kenya	5	4044	4.7936	0.0797(0.1531)	0.1222(0.2256)	24.83
	KN_0995	O-DP-36465 - O-DP-36469	Kenya	5	4379	6.4579	0.1034(0.1592)	0.1617(0.2372)	34.77
	KN_1075	O-DP-36731 - O-DP-36733	Kenya	3	4019	6.8650	0.0906(0.1642)	0.136(0.2419)	24.83
Total/average				N =61		4.8688	0.0753(0.1494)	0.1149(0.2202)	22.7
L. mildbraedii									
	TZ_0706	O-DP-39250 - O-DP-39252	Kitulo	6	-	9.2733	0.1177(0.1693)	0.1826(0.249)	39.04
	TZ_0701	O-DP-39218 - O-DP-39222	Njombe	5	2102	7.4422	0.0845(0.1599)	0.1281(0.2336)	25.23
	TZ_0702	O-DP-39223 - O-DP-39227	Njombe	5	2168	9.7116	0.0909(0.156)	0.1413(0.2311)	30.03
	TZ_0708	O-DP-39258 - O-DP-39262	Kitulo	4	2816	5.2927	0.0834(0.1646)	0.1242(0.2381)	22.82
	TZ_0709	O-DP-39263 - O-DP-39266	Kitulo	4	2816	5.5842	0.0686(0.1445)	0.1049(0.2139)	20.72
	UG_2171	O-DP-40202 - O-DP-40204	Mhavura	4	3058	22.5018	0.1453(0.1693)	0.227(0.2528)	47.45
	UG_2601	O-DP-45638 - O-DP-45641	Echuya forest	5	2300	11.4153	0.102(0.1605)	0.1589(0.238)	33.93
Total/average				N =33		10.1744	0.0989(0.1606)	0.1524(0.2366)	31.03
L. rhynchopetalum	ET_0122	O-DP-29728 - O-DP-29732	Simen	5	3711	3.5258	0.0426(0.1106)	0.0676(0.1689)	15.08
	ET_0157	O-DP-29850 - O-DP-29854	Simen	5	3718	2.1856	0.0401(0.1166)	0.0614(0.1719)	12.46
	ET_0324	O-DP-30493 - O-DP-30497	Simen	5	4035	2.9142	0.0566(0.1303)	0.0885(0.1937)	19.34
	ET_0325	O-DP-30498 - O-DP-30502	Simen	5	3748	1.9104	0.0446(0.1227)	0.0681(0.1804)	13.77
	ET_0432	O-DP-30907 - O-DP-30911	Simen	5	3912	1.8842	0.0421(0.1203)	0.0638(0.1771)	12.46
	ET_0454	O-DP-30998 - O-DP-31002	Simen	4	3760	2.1672	0.0541(0.1354)	0.0817(0.1978)	15.74
	ET_0462	O-DP-31032 - O-DP-31036	Simen	5	3681	2.9325	0.0567(0.1286)	0.089(0.1924)	19.67
	ET_0519	O-DP-31268 - O-DP-31271	Bale	4	4017	2.8435	0.0536(0.1353)	0.0805(0.198)	15.08
	ET_0538	O-DP-44380 - O-DP-45055	Bale	4		2.7476	0.0424(0.1184)	0.0647(0.1757)	12.79

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
	ET_0558	O-DP-31440 - O-DP-31444	Simen	5	3643	4.0376	0.0478(0.1184)	0.0755(0.1787)	16.72
	ET_0621	O-DP-42130 - O-DP-42134	Simen	5	3682	5.8224	0.0677(0.135)	0.1071(0.2036)	23.93
	ET_0633	O-DP-45127 - O-DP-45131	Bale	5	4143	2.2251	0.0393(0.1136)	0.0608(0.1685)	12.79
	ET_0706	O-DP-31849 - O-DP-31853	Bale	5	3948	4.2144	0.0508(0.1211)	0.0802(0.1831)	17.7
	ET_0765	O-DP-32065 - O-DP-32069	Bale	5	4101	2.9468	0.0408(0.1197)	0.0617(0.1751)	12.13
	ET_0811	O-DP-42114 - O-DP-32258	Bale	5	4129	2.1782	0.0421(0.1198)	0.0641(0.1762)	12.79
	ET_0933	O-DP-32785 - O-DP-42029	Bale	5	3986	3.4749	0.0587(0.1347)	0.0904(0.1996)	18.69
	ET_1029	O-DP-33134 - O-DP-33138	Bale	5	3875	2.0940	0.0502(0.1264)	0.0771(0.1878)	15.74
	ET_1331	O-DP-33611 - O-DP-33615	Choke	4	3960	3.3548	0.047(0.123)	0.0722(0.183)	14.43
	ET_1354	O-DP-33726 - O-DP-33730	Choke	5	3943	3.8022	0.0483(0.1177)	0.0765(0.1785)	17.05
	ET_1378	O-DP-33846 - O-DP-33850	Choke	5	3908	2.7607	0.0498(0.1279)	0.0764(0.1881)	15.74
	ET_1382	O-DP-33866 - O-DP-33870	Choke	5	3919	3.0004	0.0512(0.1267)	0.0793(0.1881)	16.72
Total/average				N=101		3.0011	0.0489(0.1239)	0.0756(0.1841)	15.8
L. stuhlmannii	UG_2004	O-DP-44367	Gahinga	4	-	3.9079	0.0776(0.1567)	0.1163(0.2295)	21.6
	UG_2054	O-DP-39770 - O-DP-39771	Mhavura	2	3550	4.9538	0.0712(0.1566)	0.104(0.2287)	17.2
	UG_2057	O-DP-39782 - O-DP-39783	Mhavura	2	3713	3.1502	0.0563(0.1423)	0.0822(0.2077)	13.6
	UG_2087	O-DP-43040 - O-DP-43041	Mhavura	2	3450	5.2290	0.0746(0.1595)	0.1088(0.2328)	18
	UG_2088	O-DP-43042 - O-DP-39873	Mhavura	4	3600	4.2289	0.0889(0.1581)	0.1359(0.2344)	26.8
	UG_2095	O-DP-39894 - O-DP-39895	Mhavura	2	3700	3.3884	0.0514(0.1368)	0.075(0.1997)	12.4
	UG_2178	O-DP-40230 - O-DP-42938	Mhavura	5	3593	3.6395	0.0834(0.1546)	0.1284(0.2281)	26.4
	UG_2271	O-DP-40568 - O-DP-40572	Ruwenzori	5	3425	3.4304	0.0765(0.1484)	0.1181(0.2202)	24.4
	UG_2307	O-DP-40712 - O-DP-40716	Ruwenzori	5	3533	2.6632	0.0772(0.146)	0.1202(0.2179)	25.6
	UG_2312	O-DP-40737 - O-DP-40741	Ruwenzori	5	3795	6.6120	0.1044(0.1629)	0.1622(0.2408)	34.4
	UG_2340	O-DP-40849 - O-DP-40850	Ruwenzori	3	3768	12.8728	0.1119(0.1665)	0.1711(0.2501)	32.8
	UG_2360	O-DP-40942 - O-DP-40946	Ruwenzori	4	3612	4.9722	0.0864(0.1594)	0.1313(0.2344)	25.6
	UG_2571	O-DP-41845 - O-DP-41848	Ruwenzori	4	3473	7.8595	0.1191(0.1699)	0.1836(0.2516)	37.2
	UG_2588	O-DP-41930 - O-DP-41932	Ruwenzori	3	3286	4.1129	0.0696(0.1579)	0.1016(0.2273)	17.2
Total/average				N=50		5.0729	0.0820(0.1554)	0.1242(0.2288)	23.8

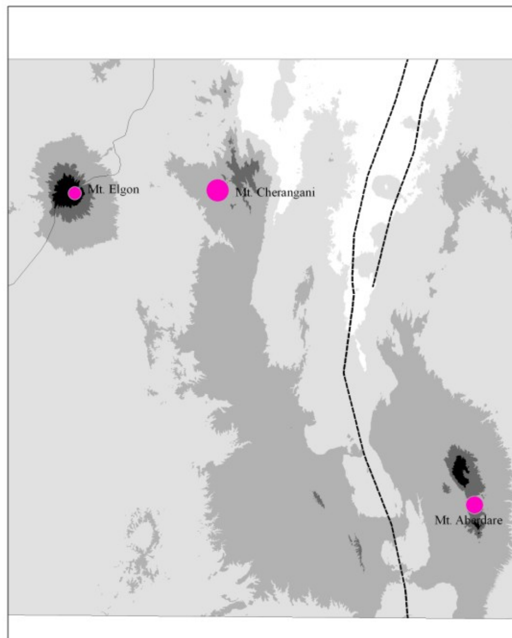
Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
L. telekii	KN_0003	O-DP-34713 - O-DP-34715	Elgon	2	4224	4.7796	0.0204(0.0897)	0.0298(0.131)	4.92
	KN_0019	O-DP-34793 - O-DP-34796	Elgon	4	3915	6.1092	0.0532(0.1332)	0.0803(0.196)	15.28
	KN_0042	O-DP-34886 - O-DP-34890	Elgon	5	3864	4.1542	0.0627(0.1375)	0.097(0.2041)	20.21
	KN_0092	O-DP-35080 - O-DP-35084	Elgon	5	3864	3.5774	0.0492(0.127)	0.0755(0.187)	15.54
	KN_0128	O-DP-35230 - O-DP-35234	Elgon	5	3995	4.0823	0.0571(0.1321)	0.088(0.1968)	18.13
	KN_0156	O-DP-35355 - O-DP-35359	Elgon	5	4029	3.8323	0.0567(0.1244)	0.0902(0.1889)	20.47
	KN_0355	O-DP-36246 - O-DP-36248	Elgon	3	3717	3.3397	0.055(0.1369)	0.082(0.201)	14.77
	KN_0475	O-DP-27436 - O-DP-27440	Aberdare	5	-	3.9110	0.0396(0.1116)	0.0616(0.1676)	12.95
	KN_0509	O-DP-27578 - O-DP-27582	Aberdare	5	3906	4.0438	0.0584(0.1304)	0.0909(0.1962)	19.17
	KN_0514	O-DP-27603 - O-DP-27607	Aberdare	5	3580	4.5360	0.0595(0.1302)	0.0937(0.1954)	20.73
	KN_0633	O-DP-28095 - O-DP-28099	Aberdare	5	3580	3.9378	0.0453(0.1181)	0.0708(0.1768)	15.28
	KN_0726	O-DP-28529 - O-DP-28533	Aberdare	5	3655	1.8737	0.0413(0.1181)	0.063(0.1742)	12.69
	KN_0783	O-DP-28591 - O-DP-28595	Kenya	5	3719	2.9593	0.0494(0.1271)	0.0754(0.1879)	15.03
	KN_0793	O-DP-28624 - O-DP-28625	Kenya	2	3696	2.0973	0.0236(0.0962)	0.0345(0.1404)	5.7
	KN_0824	O-DP-42280 - O-DP-28698	Kenya	5	4271	3.2527	0.0461(0.1188)	0.0721(0.178)	15.54
	KN_0877	O-DP-28922 - O-DP-28926	Kenya	5	4047	4.3882	0.0659(0.1412)	0.1016(0.2086)	21.24
	KN_0936	O-DP-29164 - O-DP-45662	Kenya	5	4193	5.2049	0.0583(0.1229)	0.0938(0.188)	22.02
	KN_0955	O-DP-29247 - O-DP-29251	Kenya	5	4372	2.7436	0.0473(0.1226)	0.0731(0.1822)	15.28
	KN_0981	O-DP-36432 - O-DP-36436	Kenya	5	4386	2.9001	0.0644(0.1394)	0.0997(0.2062)	20.98
	KN_1002	O-DP-36500 - O-DP-36504	Kenya	5	4379	2.4093	0.051(0.1269)	0.0785(0.1888)	16.06
	KN_1041	O-DP-36650 - O-DP-36654	Kenya	3	4019	2.3443	0.0529(0.1346)	0.079(0.1978)	14.25
	KN_1100	O-DP-36821 - O-DP-36825	Kenya	5	4149	8.1449	0.0679(0.1431)	0.1042(0.2123)	20.98
Total/average				N=99		8.8464	0.0511(0.1255)	0.0789(0.1866)	16.2
L. thuliniana	TZ_0714	O-DP-39290 - O-DP-39298	Mafinga	10	1851	26.3000	0.2118(0.139)	0.3515(0.1745)	99.6
Total/average				N=10		26.3000	0.2118(0.139)	0.3515(0.1745)	99.6
L. wollastonii	UG_2059	O-DP-39795 - O-DP-39796	Mhavura	2	4136	8.7866	0.0523(0.1378)	0.0764(0.2011)	12.63
	UG_2125	O-DP-40002 - O-DP-40006	Mhavura	5	4019	13.1130	0.0913(0.1491)	0.1446(0.2243)	32.32
	UG_2173	O-DP-40210 - O-DP-40214	Mhavura	5	4139	9.4245	0.1117(0.1754)	0.1694(0.2553)	33.33

Species name	Population ID	DNA bank ID	Locality/ Mountain	n	Altitude (m)	Rarity (DW)	Hs (Hs std)	*I*(*I* Std)	(*P*)
	UG_2315	O-DP-40752 - O-DP-40756	Ruwenzori	5	3795	6.8309	0.0985(0.16)	0.1531(0.2366)	32.58
	UG_2414	O-DP-42988 - O-DP-42992	Ruwenzori	5	3868	6.2670	0.0936(0.1606)	0.144(0.2368)	29.55
	UG_2425	O-DP-41200 - O-DP-41201	Ruwenzori	2	3769	7.4678	0.0669(0.1527)	0.0977(0.2229)	16.16
	UG_2436	O-DP-41251- O-DP-41252	Ruwenzori	2	3953	6.4386	0.0722(0.1573)	0.1054(0.2297)	17.42
	UG_2472	O-DP-41419 - O-DP-41423	Ruwenzori	4	4045	10.3328	0.089(0.1522)	0.1383(0.2285)	28.54
	UG_2495	O-DP-41504 - O-DP-41507	Ruwenzori	4	4153	5.9687	0.0708(0.1488)	0.1072(0.2188)	20.71
	UG_2510	O-DP-42935 - O-DP-41552	Ruwenzori	4	4070	8.1379	0.0684(0.1426)	0.1051(0.2123)	20.96
	UG_2560	O-DP-41790 - O-DP-41794	Ruwenzori	5	4064	5.6064	0.0797(0.1532)	0.1219(0.226)	24.49
	UG_2569	O-DP-41835 - O-DP-41838	Ruwenzori	4	4046	6.5151	0.0701(0.1468)	0.1067(0.2169)	20.71
	UG_2570	O-DP-41843 - O-DP-41844	Ruwenzori	2	4215	9.7933	0.0533(0.1389)	0.0779(0.2028)	12.88
Total/average				N=49		8.0525	0.0783(0.1520)	0.1191(0.2240)	23.3

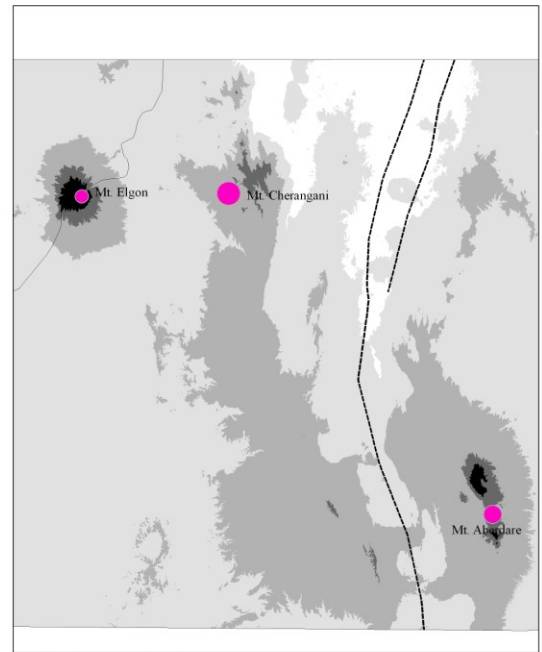
Table 4: Within mountains genetic diversity patterns as analyzed using Popgene program. Mya (million years ago), HS = average within population genetic diversity, I = mean Shannon's Information index, Std = Standard deviation, (P) = percentage polymorphic loci, (-) = Not known. On this table the populations of each mountain are compared in relation to the age of a mountain system. The ages of the mountains are adopted from Knox and Palmer, (1998).

Species	Mountain	Age of mountains (Mya)	H_S(Std)	*I*(Std)	No. of polymorphic loci	*(P)*
L. aberdarica	Aberdare	6.5 - 5.0	0.1012(0.1432)	0.17(0.21)	215	54.71
	Cherangani	3.0 - 2.0	0.1136(0.1434)	0.193(0.2079)	255	64.89
	Elgon	23 - 15	0.0977(0.148)	0.1615(0.2157)	199	50.64
L. bambuseti	Aberdare	6.5 - 5.0	0.0929(0.1398)	0.1579(0.2035)	173	55.81
	Kenya	3.5 - 2.0	0.107(0.1368)	0.1875(0.1938)	232	74.84
L. bequaertii	Ruwenzori	12.0 - 1	0.2522(0.17)	0.3954(0.2179)	90	100
L. burttii	Meru	0.19 - 0.09	0.1815(0.1698)	0.2988(0.2988)	172	100
L. deckenii	Kilimanjaro	1.1 -0.23	0.1399(0.1626)	0.2341(0.2225)	205	100
L. giberroa	Aberdare	6.5 - 5.0	0.0443(0.1185)	0.0684(0.1771)	100	13.89
	Cherangani	3.0 - 2.0	0.0247(0.0982)	0.0361(0.1434)	43	5.97
	Gahinga	0.26	0.0639(0.1497)	0.0932(0.2185)	111	15.42
	Kilimanjaro	1.1 -0.23	0.0818(0.1214)	0.1401(0.1895)	299	41.53
	Kitulo	(-)	0.0554(0.1254)	0.0874(0.1891)	140	19.44
	Meru	0.19 - 0.09	0.0557(0.1184)	0.0928(0.1787)	201	27.92
	Muhavura	0.20	0.0535(0.1228)	0.0848(0.1856)	140	19.44
	Njombe	(-)	0.0486(0.1191)	0.0775(0.1787)	138	19.17
	Pare	(-)	0.0318(0.1009)	0.0497(0.1515)	77	10.69
	Poroto	(-)	0.0706(0.1207)	0.123(0.1813)	338	46.94
	Rwenzori	12.0 - 1.0	0.0635(0.1163)	0.1116(0.1742)	317	44.03

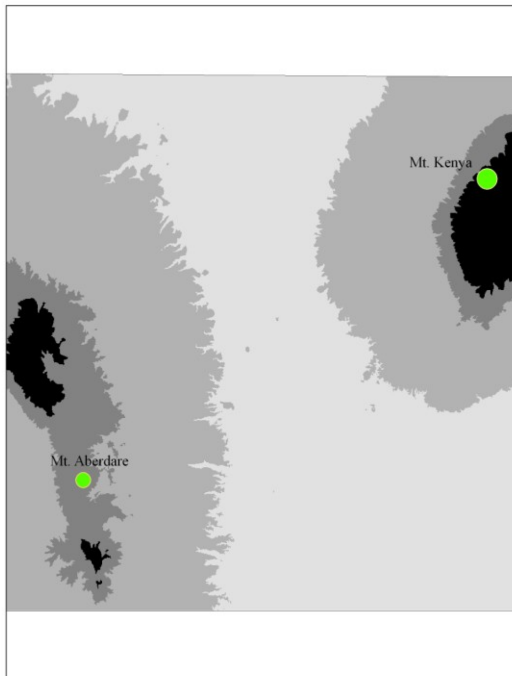
Species	Mountain	Age of mountains (Mya)	H_S(Std)	*I*(Std)	No. of polymorphic loci	*(P)*
L. gregoriana	Aberdare	6.5 - 5.0	0.1033(0.157)	0.1663(0.2289)	148	47.44
	Elgon	23.0 - 15.0	0.0714(0.1464)	0.1107(0.2148)	79	25.32
	Kenya	3.5 - 2.0	0.1071(0.1449)	0.1825(0.208)	228	73.08
L. mildbraedii	Echuya	(-)	0.1023(0.1606)	0.1593(0.2382)	113	34.04
	Kitulo	(-)	0.1184(0.165)	0.1886(0.2395)	163	49.1
	Muhavura	0.20	0.1458(0.1694)	0.2277(0.2529)	158	47.59
	Njombe	(-)	0.0992(0.1565)	0.1587(0.2285)	136	40.96
L. rhynchopetalum	Bale	(-)	0.0758(0.1335)	0.129(0.1952)	167	54.93
	Choke	(-)	0.0708(0.131)	0.1181(0.195)	119	39.14
	Simen	(-)	0.0766(0.1333)	0.1313(0.1946)	187	61.51
L. stuhlmannii	Gahinga	0.26	0.0776(0.1567)	0.1163(0.2295)	54	21.6
	Mhavura	0.20	0.121(0.1648)	0.1949(0.2366)	138	55.2
	Ruwenzori	12.0 - 1.0	0.1234(0.1464)	0.2117(0.2056)	210	84
L. telekii	Aberdare	6.5 - 5.0	0.0636(0.1198)	0.1089(0.1808)	154	40
	Elgon	23.0 - 15.0	0.0792(0.1405)	0.1317(0.2045)	182	47.27
	Kenya	3.5 - 2.0	0.0911(0.1392)	0.1555(0.2023)	248	64.42
L. thuliniana	Mafinga highlands	(-)	0.2118(0.139)	0.3515(0.1745)	263	99.62
L. wollastonii	Mhavura	0.20	0.1271(0.1488)	0.2112(0.2186)	243	61.36
	Ruwenzori	12.0 - 1.0	0.1227(0.1511)	0.2075(0.2141)	324	81.82



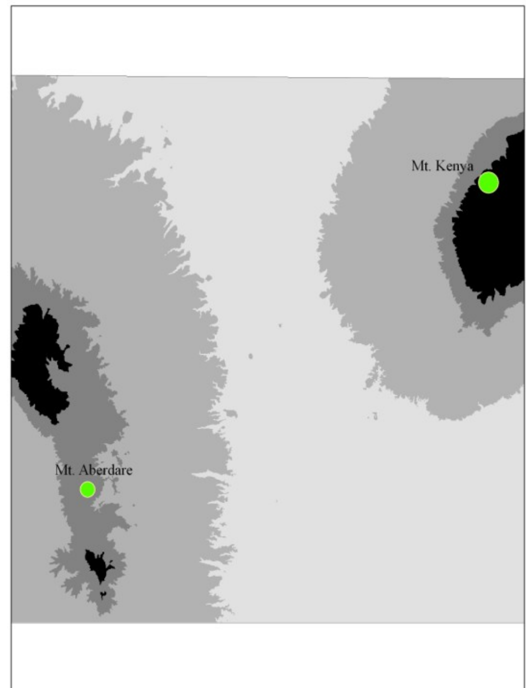
L. aberdarica, gene diversity
- 0.000 - 7.1155
- 7.1156 - 7.5758
- 7.5759 - 10.3446



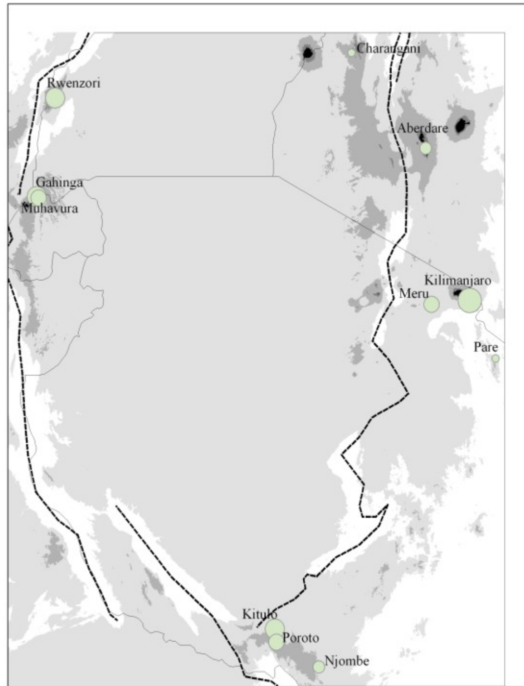
L. aberdarica, gene diversity
- 0.0000 - 0.0977
- 0.0978 - 0.1012
- 0.1013 - 0.1136



L. bambuseti, gene diversity
- 0.000 - 0.0929
- 0.0930 - 0.1070

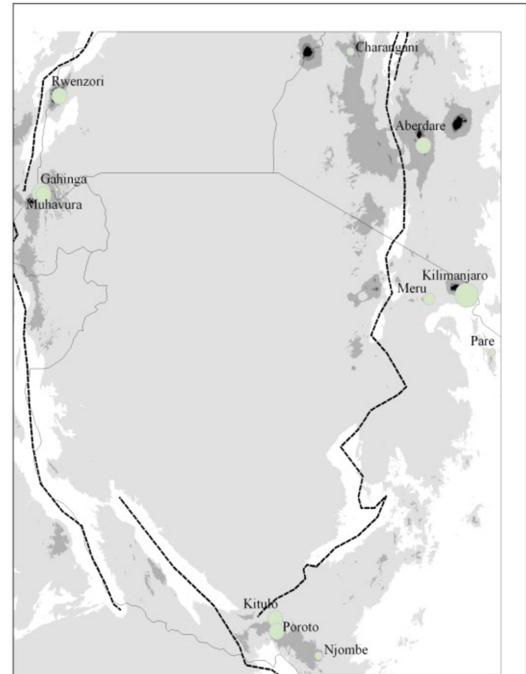


L. bambuseti, genetic distinctiveness
- 0.000 - 6.7649
- 6.7650 - 9.5509



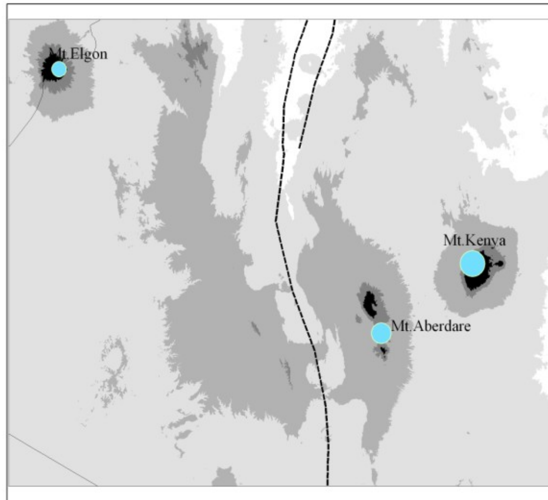
L. giberroa, gene diversity

- 0.0247 - 0.0318
- 0.0319 - 0.0486
- 0.0487 - 0.0557
- 0.0558 - 0.0706
- 0.0707 - 0.0818



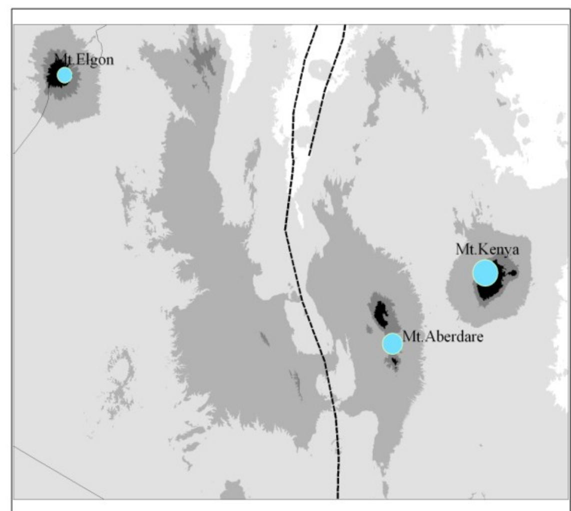
L. giberroa, genetic distinctiveness

- 4.0081 - 4.8509
- 4.8510 - 6.4590
- 6.4591 - 8.6644
- 8.6645 - 11.3602
- 11.3603 - 15.1225



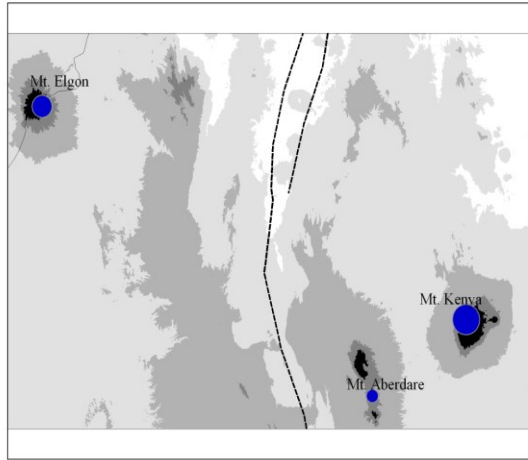
L. gregoriana, gene diversity

- 0.0000 - 0.0714
- 0.0715 - 0.1033
- 0.1034 - 0.1071

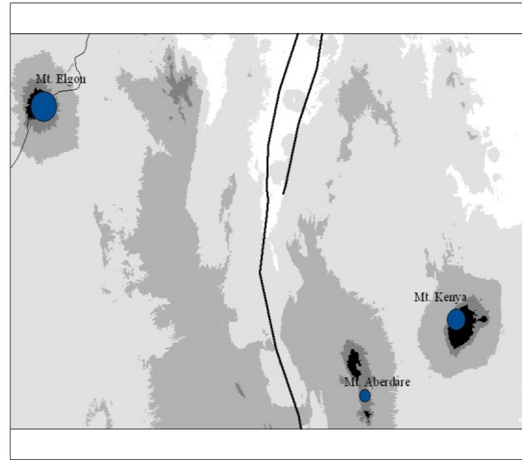


L. gregoriana, genetic distinctiveness

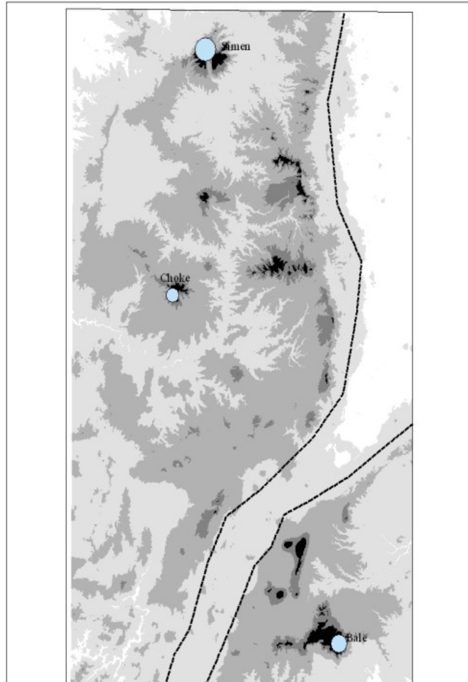
- 0.0000 - 3.3949
- 3.3950 - 5.0136
- 5.0137 - 5.7297



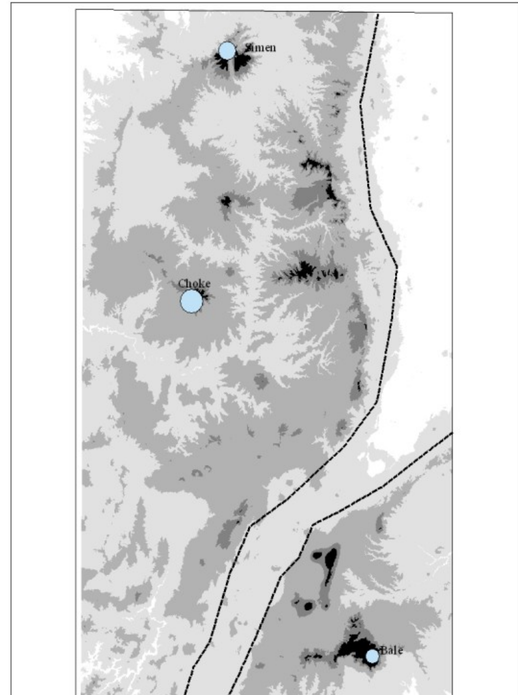
L. telekii, gene diversity
- 0.0000 - 0.0636
- 0.0637 - 0.0792
- 0.0793 - 0.0911



***L. telekii*, genetic distinctiveness**
- 0.0000 - 3.6605
- 3.6606 - 3.8054
- 3.8055 - 4.2154



L. rhynchopetalum, gene diversity
- 0.000 - 0.0708
- 0.0709 - 0.0758
- 0.0759 - 0.0766



L. rhynchopetalum, genetic distinctiveness
- 0.0000 - 2.8429
- 2.8430 - 3.0621
- 3.0622 - 3.2229

***L. Mildbraedii*, gene diversity**

***L. Mildbraedii*, genetic distinctiveness**

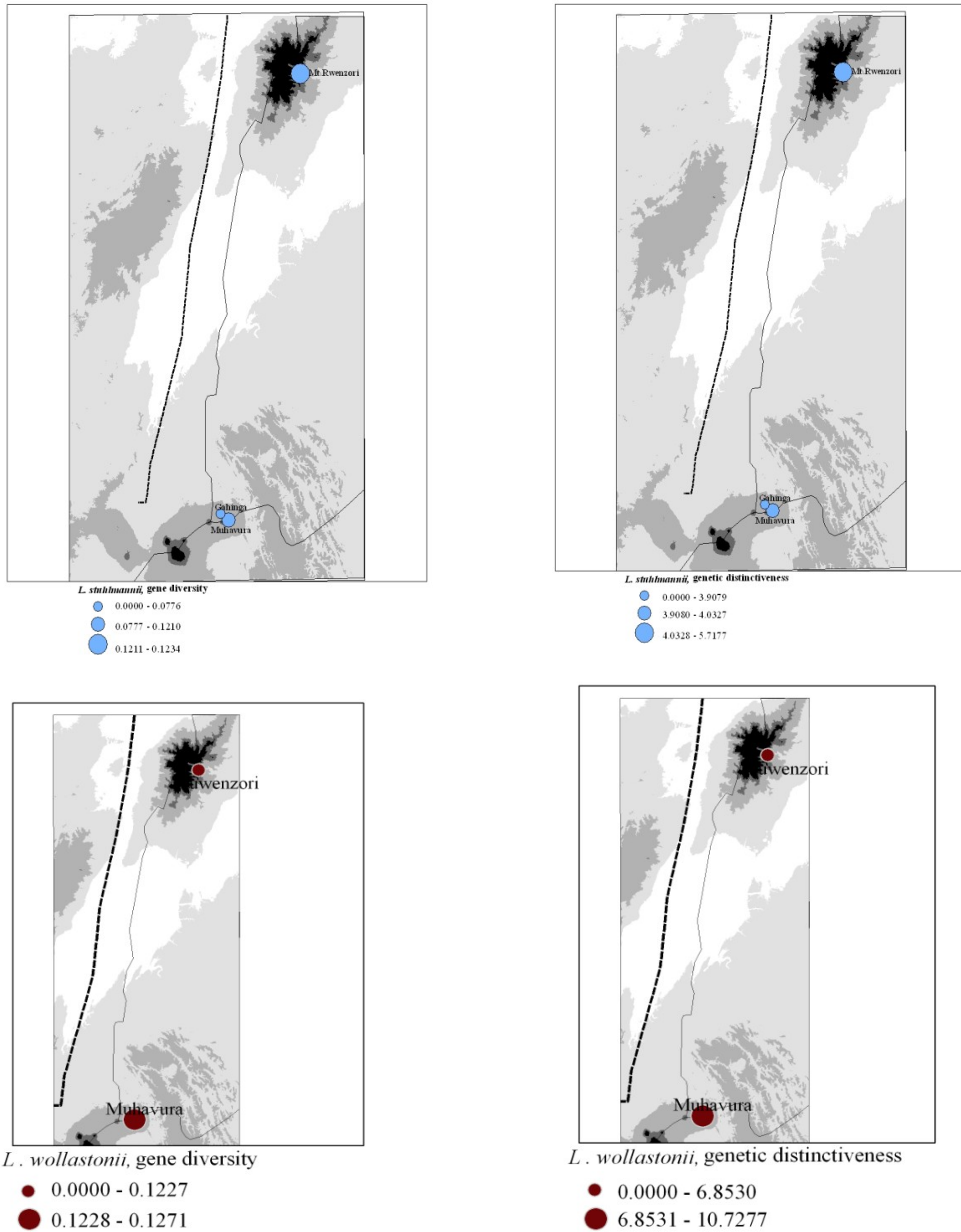


Fig. 1: **Map showing the genetic diversities and distinctiveness of the studied plants which occur on more than one mountain**

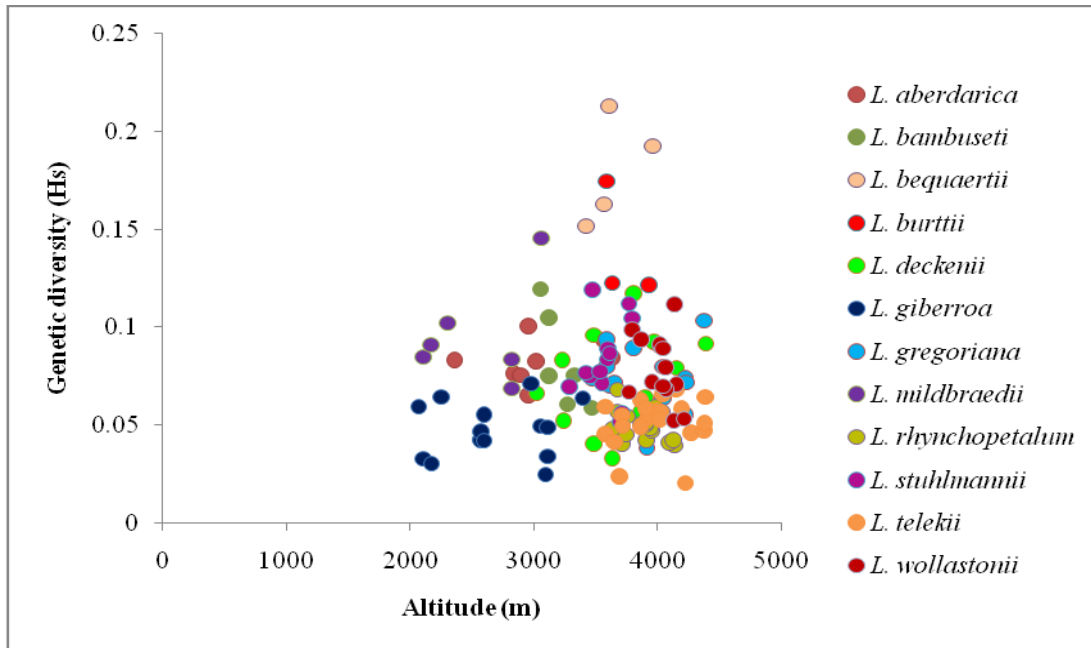


Fig. 2: **A scartter plot indicating the genetic diversity distribution across the sampled altitudinal ranges. Note the highest diversities between 3100 and 3800 m.**

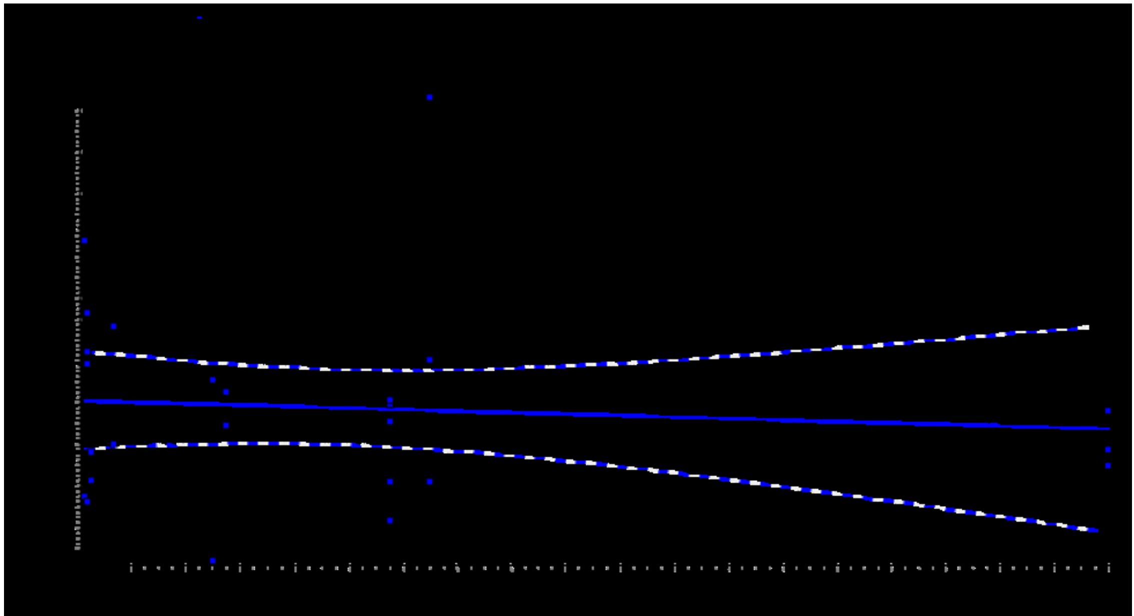


Fig. 3: Genetic diversity of the studied species versus age of the sampled mountains

5.0 GENERAL CONCLUSION

The findings of this study are consistent with earlier studies supporting the applicability of AFLP markers for delimiting species boundaries and testing of the evolutionary history of closely related and recently radiated taxa (Kropf *et al.*, 2006; Koopman *et al.*, 2008; Meudt *et al.*, 2009; Garcia-Pereira *et al.*, 2010; Safer *et al.*, 2011). The PCoA, Bayesian assignment (STRUCTURE and/or BAPS) and NJ tree analyses of the AFLP data together (although with differing resolution capacity) identified the species and several subspecies previously inferred from morphology and plastid DNA studies. Except for *Deschampsia caespitosa* and *D. angusta* (Chapter three) which showed no genetic distinction between the two as suggested by morphology-based taxonomists, the relationships and status among the rest of the species inferred from this primarily nuclear AFLP data corroborated those earlier proposed by morphology and/or plastid DNA restriction site polymorphisms with some notable exceptions. This intensive AFLP analysis provides for better understanding of the species status and/or relationships and gave insight into the origin and diversification process for the studied afro-alpine plant species. The study highlights that different afro-alpine species may have experienced very different phylogeographic histories and that long-distance dispersals among the isolated afro-alpine 'sky islands' can be more frequent than traditionally thought. Generally, the study demonstrates the need for further taxonomic exploration of the afro-alpine flora, in particular of taxa described as endemic.

6.0 APPENDIX

Appendix 1: PLOT Protocol for data sampling (AFROALP II 2008)

Country	**Mountain**	**Locality**	**Habitat**	**Plot No**	**Altitude**	**Aspect**
Slope	**Photo refs.**	**Date**	**Coordinates**		**Investigator(s)**	

Description of plot:

Subplot no:	**1**	**2**	**3**	**4**	**5**
Coordinates (NS):					
Coordinates (EW):					
Altitude:					
Investigator of subplot:					
Photo references					
Naked soil (%)					
Rocks/stone (%)					
Total vegetation cover (%)					
Cover of vascular plants (%)					
Cover of bryophytes (%)					
Cover of lichens (%)					

Taxon list for vascular plants (including sample number for blind sample X) and estimated cover of indiv ual species in each subplot:

	X	**1**	**2**	**3**	**4**	**5**	**Out**	**Tot**	**Close**