Molecular Ecology (2007) **16**, 1233–1243 doi: 10.1111/j.1365-294X.2007.03232.x

Phylogeography and conservation genetics of a giant lobelia (*Lobelia giberroa*) in Ethiopian and Tropical East African mountains

MULUGETA KEBEDE,*† DOROTHEE EHRICH,† PIERRE TABERLET,‡ SILESHI NEMOMISSA* and CHRISTIAN BROCHMANN†

**Department of Biology, Addis Ababa University, P.O. Box 3434, Addis Ababa, Ethiopia, †National Centre for Biosystematics, Natural History Museum, University of Oslo, P.O. Box 1172, Blindern, NO-0318 Oslo, Norway, ‡Laboratoire d'Ecologie Alpine, CNRS UMR 5553, Université Joseph Fourier, BP 53, F-38041 Grenoble Cedex 09, France*

Abstract

***Lobelia giberroa* is a giant rosette plant growing in the afro-montane belt of the afro-alpine environment, a unique and little-studied ecosystem occupying the high mountains of eastern Africa. We analysed amplified fragment length polymorphisms (AFLPs) from 11 mountain systems in Ethiopia and Tropical East Africa to infer the phylogeographical history of the species. A total of 191 individuals were investigated from 25 populations. Principal coordinate analysis and population structure analyses revealed three major phylogeographical groups: the Ethiopian mountains and one group on each side of the Rift Valley in Tropical East Africa, respectively: Elgon–Cherangani and Kenya–Aberdare–Kilimanjaro–Meru. Analysis of Molecular Variance showed 55.7% variance among the three groups, suggesting an old divergence. Together with a clear geographical substructure within the main groups, this pattern indicates gradual expansion and supports the montane forest bridge hypothesis, stating that the area occupied by forest was larger and more continuous in previous interglacials and earlier in the present interglacial. Genetic diversity was lower in Ethiopia than in the other two main groups, possibly due to an ancient founder effect when Ethiopia was colonized from the south.**

Keywords: AFLP, afro-alpine, afro-montane, conservation, dispersal, *Lobelia giberroa*, phylogeography

Received 9 June 2006; revision received 17 October 2006; accepted 13 November 2006

Introduction

The afro-alpine region consists of the scattered high mountains of Ethiopia and Tropical East Africa (Kenya, Tanzania and Uganda), which were called 'islands in the sky' by Hedberg (1969, 1970). Most mountains are of volcanic origin and of unequal ages ranging from the Miocene to Late Pleistocene (Hedberg 1970). Many of them are high, reaching altitudes between 3500 and 6000 m, and have three vegetation belts: the montane forest zone, the ericaceous zone and the afro-alpine zone. The flora is famous for its large numbers of geographically vicariant and locally endemic taxa — its giant senecios and giant lobelias are as renowned as the finches of the Galapagos Islands (Hedberg 1969). It offers beautiful examples of distinct adaptations to different altitudes as well as evolutionary differentiation in the highly structured environment of mountain islands. Whereas many species, notably among giant lobelias and giant senecios, are restricted to a narrow geographical range and some occur only in a particular altitudinal belt of a single mountain, other species are widespread and have managed to colonize most of the afro-alpine region (Knox 1993).

The climatic fluctuations of the Pleistocene contributed to shaping the distribution of the afro-alpine species (Livingston 1962; Hamilton 1982; Mohammed & Bonnefille 1998; Gottelli *et al*. 2004), as observed in the northern part of the world (Hewitt 2000). Whereas the impact of the Pleistocene climate fluctuations on the phylogeographical history of northern hemisphere species has been amply studied (see e.g. Taberlet *et al*. 1998; Hewitt 2000; Brochmann *et al*. 2003 for reviews), only a few studies have addressed this topic in Africa (e.g. Bowie *et al*. 2006 in addition to references above); especially few studies have been

Correspondence: Mulugeta Kebede. Fax: +251 11 155 2350; E-mail: kmulugetak@yahoo.com

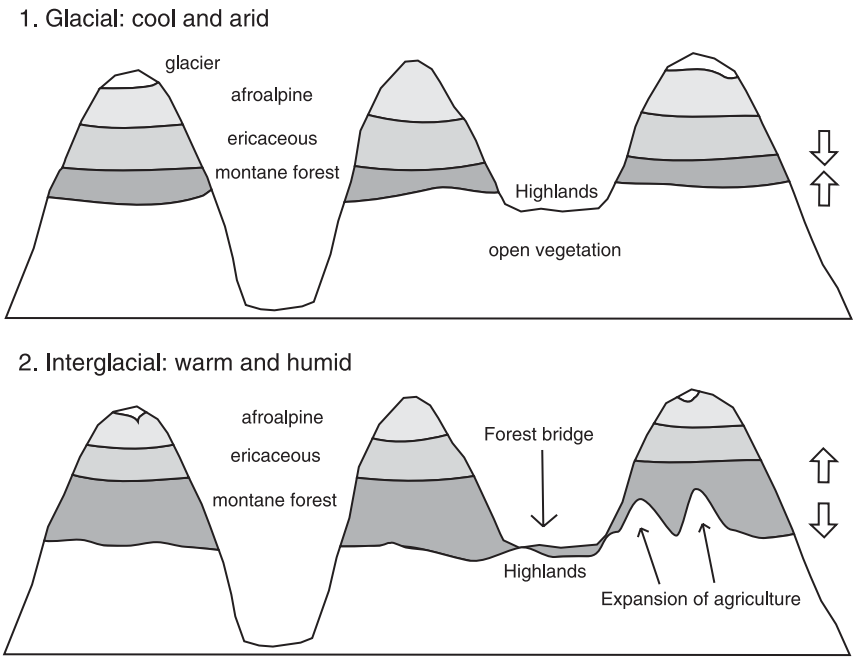

Fig. 1 Schematic representation of range shifts of the three vegetation belts of the afro-alpine region in response to climate fluctuations during the Pleistocene. 1, During glaciations, glaciers formed on some mountain tops and the afro-alpine and ericaceous zones expanded. The lower limit of the montane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

undertaken in plants. The climate changes and vegetation history of eastern Africa have been documented from studies of sediment cores (e.g. Bakker 1962; Mohammed & Bonnefille 1998; Trauth *et al*. 2005), demonstrating cycles with varying temperature and humidity. Traces of extensive earlier glaciations on some of the mountains, such as the Bale mountains, Kilimanjaro and Mount Kenya, also provide information about past climates in the region. Despite changing climate, the mountains provided relatively stable habitats where older species survived by altitudinal range shifts and new lineages were generated (Fjeldså & Lovett 1997; Hewitt 2000; Tzedakis *et al*. 2002).

During humid periods (interglacials at high latitudes) the montane forest expanded to cover low-lying ridges and highlands. Aridification in response to glaciation at higher latitudes and altitudes, on the other hand, favoured the expansion of open vegetation and reduced the montane forest, which became more fragmented (Fig. 1). Major periods of aridity, estimated from study of deep sea cores, peaked near 2.8, 1.7 and 1.0 million years ago (deMenocal 1995, 2004). More recently, periods of aridity roughly corresponded to high latitude ice ages. Thus, during the last glaciation, the African tropics were colder and drier (Bonnefille *et al*. 1990) and the present vegetation belts of the afro-alpine region were pushed down by more than 1000 m (Moreau 1963; Flenley 1979). However, whereas the afro-alpine and ericaceous vegetation zones were larger than at present (e.g. Gottelli *et al*. 2004), the montane forest zone was contracted and fragmented (Fig. 1). The upper limit of the forest was pushed down due to expansion of glaciers and alpine vegetation at the top of the mountains (Jolly *et al*. 1997; Ryner *et al*. 2006). The lower limit was in many places pushed up by expansion of open vegetation in the lowlands due to aridification. Despite this general trend, local climatic variability resulted in cool and relatively humid periods and local microclimates in some areas, creating favourable patches for montane forest at lower altitudes also during glacial periods (Osmaston & Harrison 2005). Thus some pollen records indicated a local lowering of forest vegetation during the last glacial maximum (Vincens *et al*. 2005).

In the recent past, the climate was not the only factor to govern the extent of montane forest in Africa. Its lower limits on most mountains have been pushed up in historical time by the expansion of agriculture. Thus, up to 35% of the Ethiopian highlands were covered by montane forest until a few hundred years ago (EFAP 1994). For Tropical East Africa, Hedberg (1969) suggested that without human impact, the montane forests of Elgon, Cherangani, Aberdare and Mount Kenya could have been in direct contact, a suggestion that we will discuss in this study. At present, many mountain massifs are used for extensive agriculture on almost all sides. The montane forest belt has therefore become highly fragmented and reduced to small patches.

The giant lobelias of eastern Africa comprise 21 species (Knox & Palmer 1998). They are distributed throughout the mountains of the afro-alpine region. They grow in the alpine and ericaceous vegetation belts, with exception of *Lobelia giberroa*, which is restricted to the montane forest. *L. giberroa* is the most widespread species of the giant lobelias. Whereas most other giant lobelias have restricted geographical ranges (Knox 1993; Knox & Palmer 1998), *L. giberroa* is found from Eritrea to northern Malawi and inland to eastern Congo (Knox 1993). The distribution of

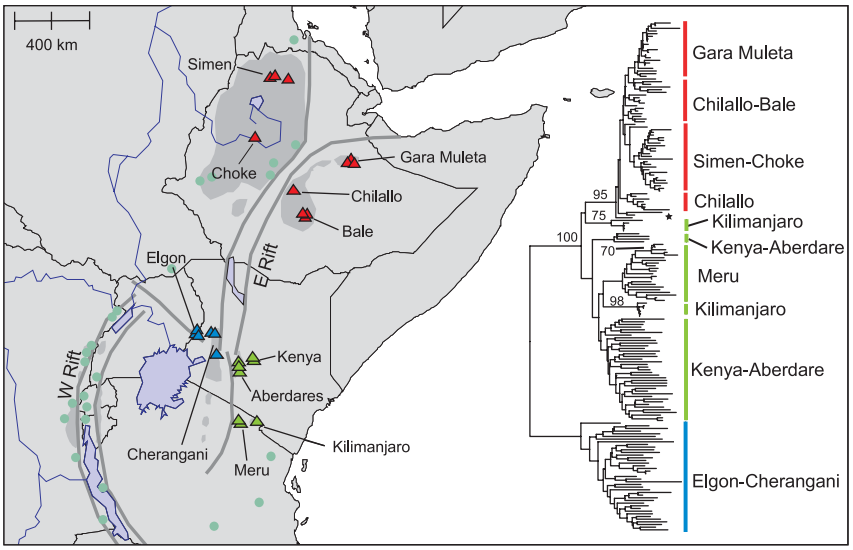

Fig. 2 Geographical distribution (green dots; redrawn from Knox 1993) and sampling localities (triangles) of *Lobelia giberroa*, and neighbour-joining tree of the AFLP phenotypes of the individual plants (based on the simple matching coefficient). Shaded areas represent mountains and highlands and thick grey lines the Rift Valley. The three major genetic groups identified in *L. giberroa* (also cf. Fig. 2) are indicated by different colours both on the map and the tree. Bootstrap support above 50% is indicated for geographical groups (not shown for small groups). The star shows three individuals from Ethiopia which were not placed in one of the clusters within the Ethiopian group. They were from Choke, Bale and Chilallo.

this species corresponds to the distribution of highlands in eastern Africa (Fig. 2), and it is the only lobelia found on most large and small mountains throughout the region. It grows in small patchy groups along streams or in moist depressions from 1550 to 3000 m, occasionally as low as 1260 m and as high as 3350 m (Knox 1993). Larger populations occur in disturbed areas, both natural (land slides and lava flows) and man-made (road cuts, grazed areas and clearings for cultivation). The plants have a giant–rosette growth form and can reach 9 m when in flower. Giant lobelias are pollinated by sunbirds, such as *Nectarinia johnstonii*. They have very small, numerous and narrowly winged seeds, probably dispersed by wind. In a phylogenetic study of the genus *Lobelia* based on chloroplast DNA (cpDNA) restriction analysis, Knox & Palmer (1998) found high genetic polymorphism and two distinct clades within the monophyletic *L. giberroa*. The first clade was distributed in the Western Rift mountains (eastern Congo, western Uganda, Rwanda, Burundi and northern Malawi), but also on Mount Elgon and in the Cherangani Hills along the Eastern Rift. The second clade occurred on the Eastern Rift mountains (Kenya and northeastern Tanzania), including Ethiopia (only two samples analyzed). The place of origin of the species was inferred to be situated either in the Western Rift or in the southern highlands of Malawi and southern Tanzania.

The high degree of endemism in the afro-alpine region is not surprising, considering the island-like structure of this habitat, and highlights how rare and difficult dispersal among mountains is for most species. Widespread species such as *L. giberroa* may have dispersed more recently or have a sufficient level of gene flow to persist as one species. They are thus a good model to address dispersal mechanisms in this highly fragmented region. Hedberg (1969) suggested cyclones as massive long-distance dispersal agents in the region. However, cyclones are rare in eastern Africa (only two cyclones were recorded with an interval of 80 years, Sansom 1953). Alternatively, species may have dispersed more gradually. The montane forest bridge hypothesis states that dispersal among adjacent mountains was more likely when montane forest was more widespread, both during previous interglacials and in earlier times of the present interglacial, before the impact of agriculture (Hedberg 1969). In this case, populations on mountains connected by highlands, where montane forest could have grown, would be expected to be more closely related than populations on more isolated mountains. Such a historical montane forest bridge seems likely in the extensive Ethiopian highlands, on each side of the Rift Valley, and may have connected Mount Kenya, the Aberdares, the Cherangani Hills and Mount Elgon in Tropical East Africa (Hedberg 1969); for *L. giberroa*, however, a recent connection over the Rift in this region is very unlikely in the light of the phylogeographical pattern described by Knox & Palmer (1998). However, gradual dispersal may also proceed by stepping-stone dispersal from one habitat patch to the next. This model requires the assumption of dispersal over the barriers of dry habitat between montane forest patches of adjacent mountains and predicts that genetic differentiation depends solely on geographical distance. If, on the other hand, most dispersal was due to random events like cyclones or independent rare long-distance dispersal, random genetic patterns with little genetic structure would be expected.

Here we analyze the spatial genetic structure of the giant *L. giberroa*, using amplified fragment length polymorphism (AFLP), and investigate the hypothesis that montane forest bridges forming during interglacials were important for dispersal in this species. With a much more extensive sampling in the Ethiopian mountains than in the Knox & Palmer (1998) study, we are also able to address the origin and the genetic structure of *L. giberroa* in the northern part

Table 1 Locality data, number of individuals analysed for AFLPs (n) and gene diversity (D) of the investigated populations of *Lobelia giberroa*

Pop.	Country	Mountain	Lat./Long.	Altitude (m)	n	D
L056	Ethiopia	Simen	N13.22166/E038.99902	2370	5	0.0277
L057	Ethiopia	Simen	N13.29127/E038.09282	2470	11	0.0345
L058	Ethiopia	Simen	N13.29853/E038.12611	2590	5	0.0428
L500	Ethiopia	Choke	N10.42490/E037.47320	2850	6	0.0351
L120	Ethiopia	Gara Muleta	N09.22051/E041.78170	2520	5	0.0358
L122	Ethiopia	Gara Muleta	N09.23278/E041.74273	2590	11	0.0267
L124	Ethiopia	Gara Muleta	N09.23316/E041.74327	2600	5	0.0220
L312	Ethiopia	Chilallo	N07.92914/E039.16980	2850	11	0.0397
L125	Ethiopia	Bale	N06.70370/E039.72004	2310	6	0.0516
L126	Ethiopia	Bale	N06.61483/E039.73801	1840	4	0.0318
L127	Ethiopia	Bale	N06.65274/E039.73223	1920	5	0.0439
L104	Tanzania	Meru	S03.22517/E036.79781	2630	11	0.0650
L105	Tanzania	Meru	S03.22582/E036.79834	2470	11	0.0637
L088	Tanzania	Kilimanjaro	S03.20025/E037.51874	2400	11	0.0523
L134	Kenya	Aberdare	S00.39531/E036.73089	2960	11	0.0746
L154	Kenya	Aberdare	S00.50920/E036.64869	2950	5	0.0867
L155	Kenya	Aberdare	S00.72276/E036.67835	2820	5	0.0902
L158	Kenya	Kenya	S00.17617/E037.19933	2820	11	0.0948
L182	Kenya	Kenya	N00.00469/E037.24118	2610	11	0.0849
L186	Kenya	Elgon	N01.07194/E034.72719	2540	5	0.0798
L200	Kenya	Elgon	N01.06383/E034.70394	2920	5	0.0855
L201	Kenya	Elgon	N01.06134/E034.68774	3000	11	0.0910
L213	Kenya	Cherangani	N01.20582/E035.28007	2580	11	0.0792
L214	Kenya	Cherangani	N01.16556/E035.33190	2710	4	0.0713
L224	Kenya	Cherangani	N00.05275/E035.53882	2730	5	0.1017

of its range. In addition, we analyze the general phylogeographical pattern, compare it to the results of Knox & Palmer (1998) and try to identify important areas for conservation of the species based on hotspots of intraspecific diversity.

Materials and methods

Sampling

Plants were collected from 11 mountain systems in Ethiopia and Tropical East Africa (Fig. 2) in 2003 and 2004. Whenever possible, three distant populations and 11 individuals per population were sampled per mountain system. A total of 191 individuals were collected from 25 populations (Table 1). In each population, leaf material was sampled in silica gel, if possible from individuals at 25 m intervals along 250 m straight line transects. A duplicate (from a randomly chosen plant sampled twice) marked 'X' was collected for each population. Voucher specimens for each population are deposited at the National Herbarium of Addis Ababa University, Addis Ababa, Ethiopia.

DNA isolation and AFLP

DNA was extracted from silica gel dried leaf material using the CTAB method following Doyle & Doyle (1987) with minor modifications (Schönswetter *et al*. 2002). From each mountain system where three populations had been sampled, five individuals from each of two populations and 11 individuals from the third population were subjected to AFLP analysis. If less than three populations had been sampled, all available individuals were analysed. The duplicates, as well as negative controls, were included to test for reproducibility and contamination.

AFLP analysis was performed according to Gaudeul *et al*. (2000), but reaction volumes in the polymerase chain reaction (PCR) were reduced by 50%. Twelve primer pair combinations were tested on four plants from two different mountain systems. AFLP profiles with many polymorphic markers and well separated fragments were selected. A second primer test was carried out using six primer pair combinations chosen from the first primer test on 16 individuals sampled from three geographical regions (Ethiopia, Kenya and Tanzania). Finally, three of the primer pair combinations were chosen, which produced a manageable number of fragments that were well separated, and reproducibility was confirmed. The final AFLP analysis was carried out with the three primer pair combinations *Eco*RI AGA (6FAM)-*Mse*I CAC, *Eco*RI ACA (VIC)-*Mse*I CAT, and *Eco*RI AGC (NED)-*Mse*I CTG. For each individual, 2.0 µL 6-FAM, 2.0 µL VIC and 4.0 µL NED labelled selective PCR products were mixed with 11.7 µL formamide and 0.3 µL GENESCAN ROX 500 size standard and run on an ABI 3100 sequencer (Applied Biosystems).

Raw data were analysed using the ABI prism GENESCAN version 3.7 analysis software (Applied Biosystems) and imported for scoring into GENOGRAPHER (version 1.6 available at http://hordeum.oscs.montana.edu/genographer/). Fragments in the size range of 50–500 bp were scored as present (1) or absent (0). The duplicates were used to test the reproducibility of the markers. The average reproducibility, calculated as the average proportion of correctly reproduced bands over all replicates (Bonin *et al*. 2004), was 98% for the three primer pair combinations. A negative correlation between fragment length and fragment frequency reflects a high probability of size homoplasy (Vekemans *et al*. 2002). In our dataset, the correlation was not significant (Pearson's $r = -0.072$, $P > 0.05$), not indicating any serious problems with size homoplasy.

Data analyses

Principal coordinate analysis (PCO) was used to visualize pairwise similarities between the AFLP multilocus phenotypes. Analyses were executed in NTSYS-pc (Rohlf 1990), using the simple matching and Dice similarity coefficients. The dataset was also subjected to a neighbour-joining analysis based on simple matching and Nei & Li (1979) genetic distances using the software TREECON 1.3b (Van de Peer & De Wachter 1994). The Nei & Li (1979) and Dice coefficients are equivalent and take into account only similarity in presence of fragments. They are thus more conservative than the simple matching coefficient, which takes into account both presence and absence of fragments. The trees were midpoint rooted and branch support was estimated with 1000 bootstrap replicates. In addition, a maximum parsimony analysis was carried out using the software TNT (Goloboff 1999). We performed a traditional search, starting with 10 random addition sequences and using TBR branch swapping. Support was estimated from 1000 bootstrap replicates.

Genetic diversity was estimated for each population using Nei's unbiased diversity estimator for each marker, $H_e = [1 - (P_0^2 + P_1^2)]\, n/(n - 1)$, where n is the sample size, P the frequency of each band's presence (1) and absence (0), respectively,; and computing the average over all markers (Nei 1978). The average gene diversity within the mountain systems and major groups was calculated from the intrapopulation estimates. Total genetic diversity was estimated for groups by pooling all samples from the populations concerned. Analyses of molecular variance (AMOVA) were computed with the software ARLEQUIN 2.0 (Schneider *et al*. 1997) to quantify genetic differentiation at different hierarchical levels. Furthermore, we plotted pairwise estimates of genetic differentiation between mountain massifs (estimated as $F_{ST}/(1 - F_{ST})$, Rousset 1997) against the natural logarithm of geographical distance to assess a pattern of isolation by distance. For this analysis, all samples from one mountain massif were pooled. The significance of the relation was tested by a Mantel test in ARLEQUIN 2.0 (1000 permutations).

As an alternative approach, the population structure was examined by genetic mixture analysis using the programs BAPS version 3.2 (Corander *et al*. 2006) and STRUCTURE version 2.1 (Pritchard *et al*. 2000). BAPS is a program for Bayesian inference of population structure, which infers the optimal number of clusters as well as the cluster each individual belongs to. The analysis was carried out using a maximum possible number of groups of 25 (k). The program STRUCTURE implements a model-based clustering method using Markov Chain Monte Carlo estimation. By comparing the likelihood of the data estimated in different runs for different numbers of groups (K) it is possible to identify the optimal K. Individuals are assigned (probabilistically) to one of the clusters defined by allele frequencies at each locus. Our data were analysed with STRUCTURE at the Bioportal, University of Oslo (http://www.bioportal.uio.no), with K ranging from one to 10, 10 replicate runs for each K, and a burn-in period of 2×10^5 and 10^6 iterations. The no admixture model and uncorrelated allele frequencies were assumed for the analysis. The AFLP data were coded as recommended in the user manual. Similarity coefficients among pairs of STRUCTURE runs were calculated according to Rosenberg *et al*. (2002), using the R-script AFLPdat (Ehrich 2006).

Results

The AFLP analysis of 191 individuals from 25 populations of *Lobelia giberroa* provided a total of 173 markers, of which 132 (76.3%) were polymorphic. The PCO plot based on the simple matching similarity coefficient (Fig. 3a), where the first and second axis explained 32.8% and 17.1% of the variation, respectively, grouped the populations into three main geographically distinct gene pools: the Ethiopian group, the Elgon–Cherangani group and the Mount Kenya–Aberdare–Kilimanjaro–Meru group. The Elgon–Cherangani group had the most divergent position and the main division in the dataset was thus observed across the eastern Rift Valley in Tropical East Africa. The third axis explained 4% of the variation (Fig. 3b) and split two of the major groups into subgroups. The Ethiopian group was divided into three subgroups: Simen–Choke north of the Rift Valley and Chilallo–Bale and Gara Muleta south of the Rift Valley. South of the Rift Valley, the subdivision was in accordance with geography, as Chilallo and Bale are quite close to each other, whereas Gara Muleta is situated at the other end of a long, narrow mountain ridge (Fig. 2). Two divergent subgroups were observed in the third main group: the Kilimanjaro–Meru subgroup and the Kenya–Aberdare subgroup, each of which were found on two geographically close mountains. The PCO based on the Dice similarity

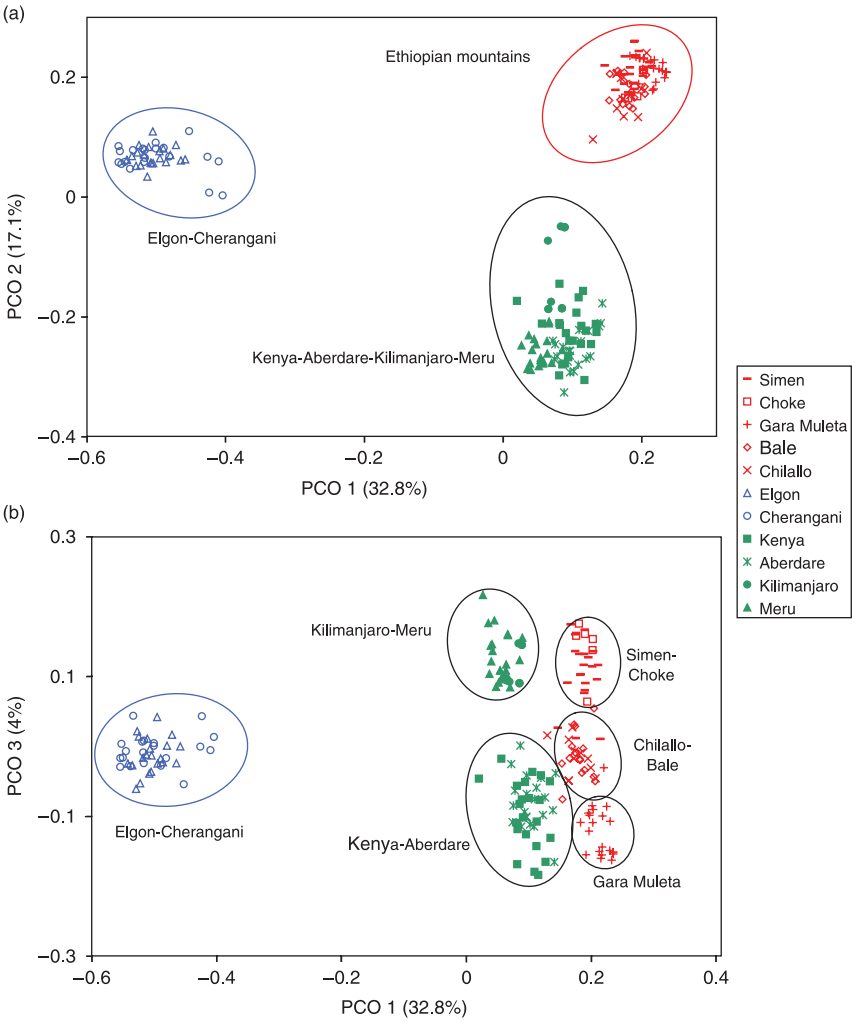

Fig. 3 Principal coordinate analysis (PCO) of individual AFLP phenotypes of *Lobelia giberroa* from 25 populations based on the simple matching coefficient. (a) Axes 1 and 2 (b) Axes 1 and 3.

Table 2 Average intrapopulation gene diversity, total gene diversity estimated from the pooled samples and number of private AFLP markers for the three main groups of *Lobelia giberroa*

Group	Number of mountains	Number of populations	Average gene diversity ± SD	Total gene diversity	Private markers
Ethiopian mountains	5	11	0.036 ± 0.009	0.058	4
Kenya–Aberdare–Kilimanjaro–Meru	4	8	0.077 ± 0.015	0.103	6
Elgon–Cherangani	2	6	0.085 ± 0.011	0.099	8

coefficient showed exactly the same structure (not shown). In the neighbour-joining analysis (Fig. 2), the Ethiopian group and the Elgon–Cherangani group had high bootstrap supports. The Mount Kenya–Aberdare–Kilimanjaro–Meru group was, however, not supported and did not form a distinct cluster. The topology and the support values were nearly identical when based on the simple matching and Nei and Li's coefficients. The maximum parsimony analysis confirmed the support for the Ethiopian group (67%) and Elgon–Cherangani (100%). The Mount Kenya–Aberdare–Kilimanjaro–Meru group formed an unresolved polytomy situated between the two other groups. The subgroups revealed by the third axis of the PCO plot were not supported by the tree-based analyses.

We detected four private AFLP fragments in the Ethiopian mountains, eight in Elgon–Cherangani and six in Kenya–Aberdare–Kilimanjaro–Meru (Table 2). The average intrapopulation gene diversity was 0.0605. The highest value was observed in population L224 from Cherangani Hills (D = 0.1017; Table 1). The average gene diversity in the major groups (Table 2) was relatively high in the Elgon–Cherangani (0.085) and Kenya–Aberdare–Kilimanjaro–Meru (0.077)

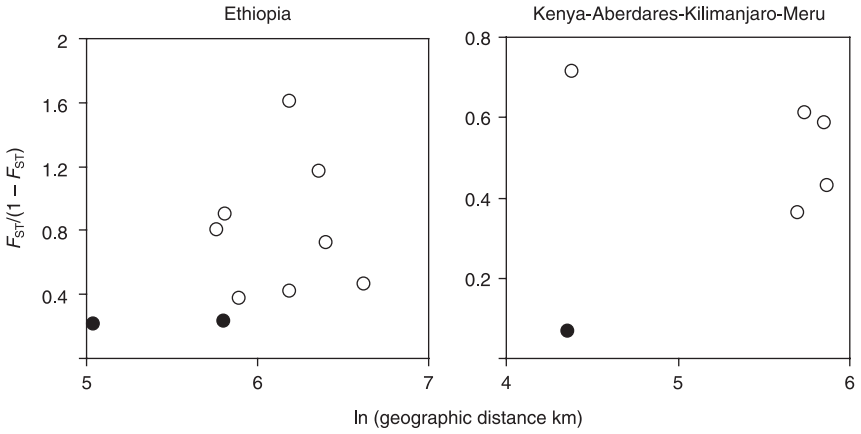

Fig. 4 Relationship between pairwise genetic distances ($F_{ST}/(1 - F_{ST})$) and geographical distances between mountain massifs within two of the main groups. For this analysis, all samples from each mountain massif were pooled. The black dots represent comparisons between mountains where a historical montane forest bridge is likely: Chillalo–Bale and Simen–Choke in Ethiopia, and Kenya–Aberdares in Tropical East Africa. All other comparisons are represented by open circles.

Table 3 Analyses of molecular variance (AMOVA) of the AFLP data for *Lobelia giberroa*. *P*-values were estimated in a permutation test (10 000 permutations)

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

groups and lowest in the Ethiopian mountains (0.036). The same was true for the total diversity estimated per region (Table 2). The difference in average diversity between Ethiopia and the southern mountain massifs was significant ($P < 0.001$, *t*-test).

In a hierarchical AMOVA, the largest proportion of the genetic variation was found among the three main groups identified by PCO with an estimate of 55.71% (Table 3). There was also considerable differentiation among subgroups. In separate AMOVA analyses for each main group, 36.7% of the variation was found among the subgroups in Ethiopia and 16.8% between Kenya–Aberdare and Kilimanjaro–Meru. Differentiation between Elgon and Cherangani was not significant (Table 3). In the Ethiopian mountains, the genetic differentiation across the Rift Valley corresponded to 31.5% of the variation ($P = 0.003$), whereas 27.9% of the variation was found between Gara Muleta and Bale–Chilallo ($P = 0.029$) and 30.0% ($P = 0.029$) between Simen–Choke and Bale–Chilallo. Between the two divergent subgroups Simen–Choke and Gara Muleta, across the Rift Valley, differentiation was even higher; at 50.6% ($P = 0.027$). Among mountain massifs within subgroups, on the other hand, differentiation was lower and not significant (Simen–Choke: 14%, $P = 0.25$; Bale–Chilallo: 18.4%, $P = 0.25$). Because the dataset was clearly divided into three phylogeographical groups, we only addressed isolation by distance within each group (excluding Elgon–Cherangani, where samples were available from only two mountains). There was an indication for an increase of genetic differentiation with geographical distance in Ethiopia but not in Kenya–Aberdares–Kilimanjaro–Meru (Fig. 4). In the latter group, there was much higher differentiation between Kilimajaro and Meru ($F_{ST} = 0.417$) than between Kenya and Aberdares ($F_{ST} = 0.062$). In both cases the Mantel test was not significant ($P = 0.124$ in Ethiopia and $P = 0.127$ in Kenya–Aberdares–Kilimanjaro–Meru).

The analysis with the program BAPS also showed clustering of the populations into three main groups. In addition, the populations from Kenya–Aberdares were separated from Kilimanjaro–Meru. However, the genetic distance between these two groups (estimated in a Külbach–Leibler distance matrix by BAPS; not shown), was much smaller than the values among the other clusters. The result of the STRUCTURE analysis was congruent with the results from other analyses. The populations were grouped into the same three main gene pools (Fig. 2). The graph of the likelihood of the AFLP data estimated from the different runs showed a clear point of inflection for K = 3 (Fig. 5a). Up to K = 3, outputs from the program were also absolutely identical, as shown on the plot of similarity coefficients against K (Fig. 5b). For larger values of K, the likelihood values were slightly higher but there was no convergence for a particular configuration of clusters.

Discussion

The PCO and genetic mixture analyses clearly revealed three main groups, which correspond to three geographically

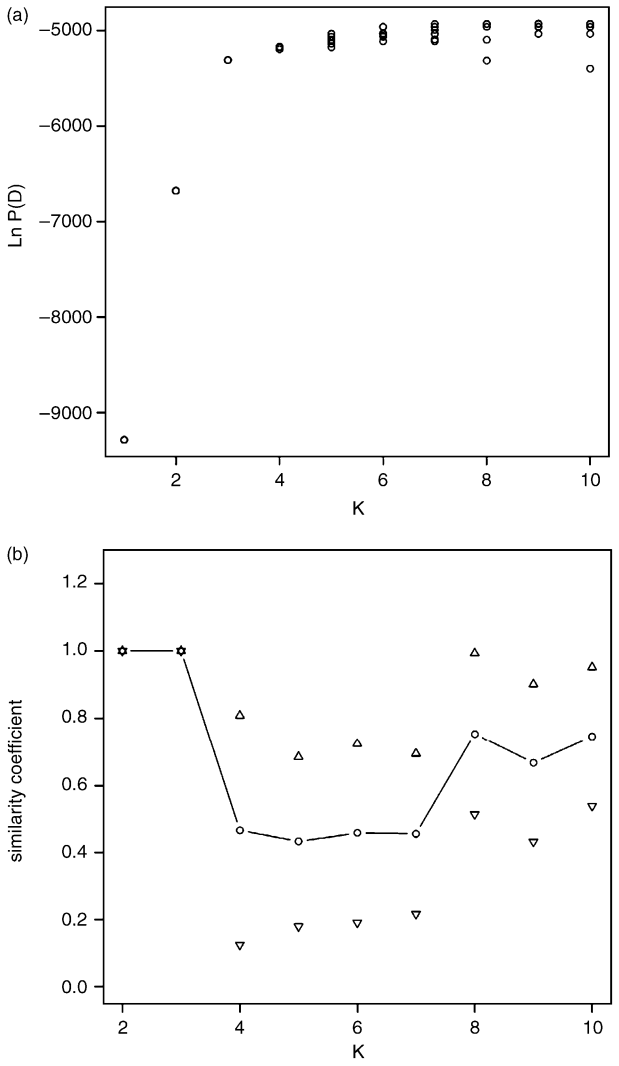

Fig. 5 STRUCTURE analysis of the AFLP data for *Lobelia giberroa*. (a) Estimated likelihood for values of K ranging from one to 10. (b) Similarity coefficients of the results from different runs of STRUCTURE calculated according to Rosenberg *et al*. (2002). Dots represent the average similarity coefficient for the pairwise comparisons among 10 runs and triangles show the standard deviation.

distinct regions: the Ethiopian mountains, Kenya–Aberdares–Kilimanjaro–Meru on the eastern side of the Rift Valley, and Elgon–Cherangani on the western side of the Rift in the southern part of the study area. In the neighbour-joining and parsimony trees, the Ethiopian group and Elgon–Cherangani were identified with substantial bootstrap support, whereas the samples from Kenya–Aberdares–Kilimanjaro–Meru did not form a supported group. Based on the PCO and the AMOVA results, two of the groups were divided into geographically separated subgroups. Altogether, this phylogeographical pattern corresponds to expectations from a gradual dispersal model and indicates that *Lobelia giberroa* did not colonize its large range by random long-distance dispersal mediated, for example, by cyclones.

In Ethiopia, one genetic subgroup was identified north of the Rift Valley and two spatially separated groups south of it. Within two of these groups, the mountains are connected by extensive highlands, which may have supported larger montane forests in the past, whereas the third was restricted to one more distant mountain, Gara Muleta, connected to Chilallo and Bale only by a narrow mountain ridge. In the northern Ethiopian subgroup, the two mountain systems (Simen and Choke) are geographically quite distant, but they are connected via highlands and harbour genetically very similar populations. This pattern is most consistent with the montane forest hypothesis, but pure isolation by distance cannot be excluded statistically based on our sampling. However, whereas isolation by distance predicts that the differentiation between Gara Muleta and Chilallo, between Chilallo and Choke and between Choke and Simen should be about equal because of nearly equal geographical distances (Figs 2 and 4), the observed differentiation was more than twice as large for the mountain pairs not connected by highlands than for Simen and Choke, which may have been connected by a forest bridge (F_{ST} = 0.444 between Gara Muleta and Chilallo, 0.473 between Chilallo and Choke, and only 0.191 between Choke and Simen; estimates based on pooled samples for each mountain massif).

In the mountains of Tropical East Africa, our data did clearly not support a montane forest bridge across the Rift Valley between Cherangani and Aberdares, as postulated by Hedberg (1969). On the contrary, we observed the largest genetic discontinuity in our dataset between these two mountain regions, in agreement with Knox & Palmer (1998). Although the montane forest occurs as an altitudinal vegetation belt in the afro-alpine environment, its distribution is strongly influenced not only by altitude but also by precipitation (Hedberg 1951). Notably, even if *L. giberroa* has quite large altitudinal range, it requires high humidity (Knox 1993; Lüttge *et al*. 2001). It is thus not possible to infer its past distribution or that of the montane forest only on the basis of altitude. The bottom of the Rift Valley between Cherangani and Aberdares is quite high in some places (up to 1600 m, Chorowicz 2005), but this region is much drier than the bordering mountain ranges, which attract most of the rain clouds. A phylogeographical barrier has been reported in this region also for a montane forest bird, the olive sunbird (*Nectarinia olivacea/obscura*; Bowie *et al*. 2004), in agreement with the absence of a historical forest bridge.

On each side of the Rift Valley, on the other hand, the populations from adjacent mountain massifs were genetically closely related (Elgon and Cherangani; Aberdares and Kenya), indicating that montane forest bridges were important for the dispersal of *L. giberroa* in Tropical East

Africa as well. The high differentiation observed across the Rift Valley compared to the absence of differentiation between the pairs of mountains on each side indicates that the pattern is not due to pure isolation by distance. The likely importance of a past montane forest bridge is further supported by the large differentiation observed between the two isolated volcanoes Meru and Kilimanjaro, which are separated by about 50 km of arid lowland. Differentiation between these two mountains was much higher than between Aberdares and Kenya (Fig. 4) or between Elgon and Cherangani, although the geographical distances were similar. Altogether we conclude that our data, both in Ethiopia and Tropical East Africa, are best explained by the montane forest bridge hypothesis.

Our genetic data seem consistent with the hypothesis that this contact among forest patches was as recent as the present interglacial. There is considerable evidence that the climate was more humid in large parts of eastern Africa during the first part of the Holocene (e.g. Kuper & Kropelin 2006), suggesting that montane forest bridges could have been extensive during this period. In some regions it is also likely that forest patches were in contact as long as the habitat was not fragmented by agriculture (Hedberg 1969). However, because it is difficult to estimate a time frame for divergence from AFLP data, our data do not provide firm evidence against an older age for the last montane forest bridge in some regions, such as during the previous interglacial.

The divergence among the major groups is likely to be old for at least two reasons. First, all three groups harbour private AFLP markers. There is thus no indication of recent colonization of one region from another. Second, AMOVA revealed high differentiation among them (55.71%; Table 3). It is thus likely that the three lineages identified here survived several glacial periods, when the montane forest was contracted by aridification in separate refugia. Furthermore, we suggest that the subgroups found both in Ethiopia and in Kenya–Aberdares–Kilimanjaro–Meru may descend from distinct refugial populations during the last ice age, which expanded to occupy their respective ranges over montane forest bridges at the beginning of the present interglacial.

The relative genetic distances among the three main groups, of which the Elgon–Cherangani group is the most divergent (Fig. 2a), is consistent with the cpDNA restriction analysis data of Knox & Palmer (1998). Their study showed that *L. giberroa* from Mount Elgon and the Cherangani Hills had cpDNA haplotypes belonging to the clade of the Western Rift, whereas other mountains of the Eastern Rift, where our Kenya–Aberdare–Kilimanjaro–Meru group is located, and the two Ethiopian individuals included belonged to the other cpDNA clade. As we lack samples from the Western Rift region, however, we cannot confirm that the populations from Elgon–Cherangani are more closely related to the Western Rift populations than to the rest of our material.

From the conservation point of view, the identification of three highly divergent gene pools in this part of the range of *L. giberroa* indicates that these should be treated as distinct evolutionarily significant units (ESUs). Thus, conservation of this unique plant, and indeed of the montane forest ecosystem in general, should be a priority in all three regions. Furthermore, despite the higher genetic diversity observed in Tropical East Africa, the distinctness of the Ethiopian populations (including four private markers) clearly support their conservational value and their status as an independent ESU.

The Ethiopian mountains

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

The average gene diversity within populations was lower in the Ethiopian mountains than in the southern part of our study area, and among the three main groups, Ethiopia had fewest private markers. A lower level of genetic diversity may reflect a founder effect or result from leading edge colonization, where diversity is lost gradually because of repeated colonizations of new land by only a few individuals (Hewitt 1996; Petit *et al*. 2002). In this case, fewer private markers would also be expected. Genetic diversity may, however, also be reduced by habitat fragmentation. Today, *L. giberroa* typically occurs only in isolated patches due to the strong impact of agriculture on the slopes of most mountains. In a highly fragmented habitat, diversity will be lost from each patch; but at the same time, the isolated populations will diverge from each other due to genetic drift. Thus in total, over the whole area, gene diversity can still be quite large due to high differentiation. As this is not the case in the Ethiopian populations of *L. giberroa*, where both intrapopulation and total genetic diversity are lower than in the southern samples (Table 2), we conclude that the relatively low diversity in these populations is most likely caused by their colonization history. This interpretation is supported by the distribution of genetic diversity within Ethiopia. Genetic variability was indeed somewhat higher in southern Ethiopia (Bale and Chilallo) than elsewhere in the country. This might indicate that this

part of Ethiopia was colonized first by *L. giberroa* coming from the south and subsequently served as the source for further colonization northwards.

Gillett (1955) suggested that the high mountain flora of southern Ethiopia shows a stronger resemblance to that of the East African mountains than to the mountains of northern Ethiopia. This is clearly not the case for intraspecific differentiation in *L. giberroa*. The closest relatives of the southern Ethiopian populations of *L. giberroa* were found in northern Ethiopia, and all Ethiopian populations together formed a distinct genetic cluster. Nevertheless, the strongest subdivision among Ethiopian populations was found across the Rift Valley, highlighting the importance of this valley as a dispersal barrier for montane plants.

Conclusions

Our results have demonstrated that there is a strong phylogeographical structure among the populations of the only widespread species of the giant lobelias, *Lobelia giberroa* in the afro-alpine region. The observed structure corresponds to expectations from the hypothesis of montane forest bridge formation between mountains during interglacial periods. Early divergence among the three main lineages was inferred. Since our samples were all from the Eastern Rift, we could not resolve the history of this ecologically important species in its entire range. However, this first phylogeographical study of an afro-montane plant has contributed considerably to our understanding of the history of the afro-montane flora.

Acknowledgements

We thank the Bale Mountains National Park, the Simen Mountains National Park, the Tanzanian Commission for Science and Technology and the National Museums of Kenya for permission to conduct field work. Particularly, we are grateful to A. Assefa for company during most of the field work and to Dr A. M. Muasya and Dr E. Knox for facilitating field work in Kenya. We also thank the Institute of Biodiversity Conservation and Research for permission to take samples from Ethiopia to Norway for laboratory analyses and two anonymous referees for useful comments on a previous version of the manuscript. The study was funded by the NUFU (The Norwegian Programme for Development, Research and Higher Education) project 54/2003 to S. Nemomissa and C. Brochmann ('AFROALP — Afroalpine "islands" as natural laboratories: dynamics and units of plant biodiversity') and partly by grant 150322/720 to C. Brochmann from the Research Council of Norway.

References

Bakker FMZ (1962) A Late-glacial and Post-glacial climatic correlation between East Africa and Europe. *Nature*, **194**, 201–203.

Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P (2004) How to track and assess genotyping errors in population genetics studies. *Molecular Ecology*, **13**, 3261–3273.

Bonnefille R, Roeland JC, Gulot J (1990) Temperature and rainfall estimates for the past 40 000 years in equatorial Africa. *Nature*, **346**, 347–349.

Bowie RCK, Fjeldså J, Hackett SJ, Crowe TM (2004) Molecular evolution in space and through time: mtDNA phylogeography of the Olive Sunbird (*Nectarinia olivacea/obscura*) throughout continental Africa. *Molecular Phylogenetics and Evolution*, **33**, 56–74.

Bowie RCK, Fjeldså J, Hackett SJ, Bates JM, Crowe TM (2006) Coalescent models reveal the relative roles of ancestral polymorphism, vicariance, and dispersal in shaping phylogeographical structure of an African montane forest robin. *Molecular Phylogenetics and Evolution*, **38**, 171–188.

Brochmann C, Gabrielsen TM, Nordal I, Landvik JY, Elven R (2003) Glacial survival or *tabula rasa*? The history of North Atlantic biota revisited. *Taxon*, **52**, 417–450.

Chorowicz J (2005) The East African Rift System. *Journal of African Earth Sciences*, **43**, 379–410.

Corander J, Marttinen P, Mäntyniemi S (2006) Bayesian identification of stock mixtures from molecular marker data. *Fishery Bulletin*, in press.

Doyle JJ, Doyle JL (1987) A rapid procedure for DNA purification from small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

EFAP (1994) Ethiopia Forestry Action Program. Final Report, Ministry of Natural Resources Development and Environmental Protection, Addis Ababa.

Ehrich D (2006) AFLPdat: a collection of R functions for convenient handling of AFLP data. *Molecular Ecology Notes*, **6**, 603–604.

Fjeldså J, Lovett JC (1997) Geographical patterns of old and young species in African forest biota: the significance of specific montane areas as evolutionary centres. *Biodiversity Conservation*, **6**, 325–346.

Flenley J (1979) *The Equatorial Rain Forest: a Geological History*. Butterworth, London.

Gaudeul M, Taberlet P, Till-Bottraud I (2000) Genetic diversity in an endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), inferred from amplified length polymorphism markers. *Molecular Ecology*, **9**, 1625–1637.

Gillett JB (1955) The relation between the highland floras of Ethiopia and British east Africa. *Webbia*, **11**, 459–469.

Goloboff P (1999) Analyzing large data sets in reasonable times: solutions for composite optima. *Cladistics*, **15**, 415–428.

Gottelli D, Marino J, Sillero-Zubiri C, Funk SM (2004) The effect of the last glacial age on speciation and population genetic structure of endangered Ethiopian wolf (*Canis simensis*). *Molecular Ecology*, **13**, 2275–2286.

Hamilton AC (1982) *Environmental History of East Africa. A Study of the Quaternary*. Academic Press, London.

Hedberg O (1951) Vegetation belts of the east African mountains. *Svensk Botanisk Tidskrift*, **45**, 140–202.

Hedberg O (1969) Evolution and speciation in a tropical high mountain flora. *Biological Journal of the Linnaean Society*, **1**, 135–148.

Hedberg O (1970) Evolution of the afroalpine flora. *Biotropica*, **2**, 16–23.

Hewitt GM (1996) Some genetic consequences of ice ages and their role in divergence and speciation. *Biological Journal of the Linnaean Society*, **58**, 247–276.

Hewitt GM (2000) The genetic legacy of Quaternary ice ages. *Nature*, **405**, 907–913.

Jolly D, Taylor D, Marchant R *et al.* (1997) Vegetation dynamics in central Africa since 18 000 yr BP: pollen records from the

interlacustrine highlands of Burundi, Rwanda and western Uganda. *Journal of Biogeography*, **24**, 495–512.

Knox EB (1993) The conservation status of the giant senecios and giant lobelias in Eastern Africa. *Opera Botanica*, **121**, 195–216.

Knox EB, Palmer JD (1998) Chloroplast DNA evidence on the origin and radiation of the giant Lobelias in Eastern Africa. *Systematic Botany*, **23**, 109–149.

Kuper R, Kropelin S (2006) Climate-controlled Holocene occupation in the Sahara: Motor of Africa's evolution. *Science*, **313**, 803–807.

Livingston DA (1962) Age of deglaciation in the Ruwenzori range, Uganda. *Nature*, **194**, 859–860.

Lüttge U, Fetene M, Liebig M, Rascher U, Beck E (2001) Ecophysiology of niche occupation by two giant rosette plants, *Lobelia gibberoa* Hemsl and *Solanecio gigas* (Vatke) C. Jeffrey, in an afromontane forest valley. *Annals of Botany*, **88**, 267–278.

deMenocal PB (1995) Plio-Pleistocene African climate. *Science*, **270**, 53–59.

deMenocal PB (2004) African climate change and faunal evolution during the Pliocene-Pleistocene. *Earth and Planetary Science Letters*, **220**, 3–24.

Mohammed MU, Bonnefille R (1998) A late glacial/late Holocene pollen record from a highland peat at Tamsaa, Bale Mountains, South Ethiopia. *Global and Planetary Change*, **16–17**, 121–129.

Moreau RE (1963) Vicissitides of the African biomes in the late Pleistocene. *Proceedings of the Zoological Society of London*, **141**, 395–421.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Nei M, Li W (1979) Mathematical model for studying genetic variance in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA*, **76**, 5269–5273.

Osmaston HA, Harrison SP (2005) The Late Quaternary Glaciation of Africa: a regional synthesis. *Quaternary International*, **138–139**, 32–54.

Petit RJ, Csaikl UM, Bordacs S (2002) Chloroplast DNA variation in European white oaks: phylogeography and patterns of diversity based on data from over 2600 populations. *Forest Ecology and Management*, **156**, 5–26.

Pritchard JK, Stephens M, Donnely PJ (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Rohlf F (1990) *NTSYS-PC. Numerical Taxonomy and Multivariate Analysis System, Version 2.02*. Exeter Software, Setauket, NY.

Rosenberg NA, Pritchard JK, Weber JL *et al.* (2002) Genetic structure of human populations. *Science*, **298**, 2381–2385.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**, 1219–1228.

Ryner MA, Bonnefille R, Holmgren K, Muzuka A (2006) Vegetation changes in Empakaai Crater, Northern Tanzania, at 14 800–9300 cal yr BP. *Review of Palaeobotany and Palynology*, **140**, 163–174.

Sansom HW (1953) The Lindi Cyclone 15 April 1952. *Memoirs of the East African Meteorological Department*, **3**, 1–16.

Schneider S, Kueffer J, Roessli D, Excoffier L (1997) *Arlequin 2.000: a Software for Population Genetic Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva, Switzerland.

Schönswetter P, Tribsch A, Barfuss M, Niklfeld H (2002) Several Pleistocene refugia detected in the high alpine plant *Phyteuma globulariifolium* Sternb. and Hoppe (Campanulaceae) in the Europian Alps. *Molecular Ecology*, **11**, 2637–2647.

Taberlet P, Fumagalli L, Wust-Saucy A-G, Cosson J-F (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453–464.

Trauth MH, Maslin MA, Deino A, Strecker MR (2005) Late Cenozoic moisture history of east Africa. *Science*, **309**, 2051–2053.

Tzedakis PC, Lawson T, Frogley MR, Hewitt GM, Preece RC (2002) Buffered Tree Population Changes in a Quaternary Refugium: Evolutionary Implications. *Science*, **297**, 2044–2047.

Van de Peer Y, De Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Application in Bioscience*, **10**, 569–570.

Vekemans X, Beauwens T, Lemaire M, Roldan-Ruiz I (2002) Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasy and of a relationship between degree of homoplasy and fragment size. *Molecular Ecology*, **11**, 139–151.

Vincens A, Buchet G, Williamson D, Taieb M (2005) A 23 000 yr pollen record from Lake Rukwa (8 degrees S, SW Tanzania): New data on vegetation dynamics and climate in Central Eastern Africa. *Review of Palaeobotany and Palynology*, **137**, 147–162.

Mulugeta Kebede is a PhD student working on the biogeography of afro-alpine plants with emphasis on afro-montane elements. Dorothee Ehrich is a postdoctoral researcher in the C. Brochmann laboratory and works on molecular ecology and phylogeography of arctic plants and animals. Sileshi Nemomissa is Associate Professor of systematics at Addis Ababa University and works on the biogeography and systematics of afro-alpine plants. Pierre Taberlet is interested in intraspecific phylogeographies of plants and animals and is involved in development of new molecular techniques for population studies. Christian Brochmann works on the biogeography and evolution of the arctic-alpine flora.
