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Swertia abyssinica species - complex differences in Afroalpine Mountain Systems as Inferred from AFLP

BY
Biructawit Bekele

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Abstract

Swertia abyssinica (Hochst.) *S. lugardae* (Bullock) and *S. pumila* (Hochst.) (Gentianaceae) are closely related taxa occurring in the high mountains of North East Africa. Amplified Fragment Length Polymorphism (AFLP) markers were used to assess the genetic variation between the higher and the lower altitude populations of the taxa within and between different mountain regions. In a principal coordinate analysis (PCoA), the resulted grouping of accessions of the first two taxa corresponds with their geographical locations rather than taxonomic classifications while *S. pumila* was far and distinct. Such groupings were inconsistent with previous study based on morphology. The PCoA result was supported by the AMOVA with higher variations among mountains (21.4 %) than variations within mountain among taxa (14.5 %). The considerable reduction in variation among taxa (7.03 %) in the AMOVA excluding *S. pumila* and its distinct position in the PCoA implies *S. pumila* as a different taxon. The evolutionary distance tree based on AFLP characters supported the results from AMOVA and PCoA. These results are in contradiction with the acceptance of three distinct species, but rather suggest as two distinct species. Thus the results suggest clinal morphological differences between the two, *S. abyssinica* and *S. lugardae*. Thus it is likely that the clinal variation is caused by primary cline than hybridization. Although there was non - significant variation between altitudes and weak correlation between them, it is difficult to conclude for the absence of genetic base for the clinal morphological variation.

Key words: *Swertia,* ecoclinal evolution, genetic diversity, glacial refugia, AFLP

1. INTRODUCTION

The afroalpine flora occurs in isolated enclaves on the upper parts of the high East African and Ethiopian mountains (Hedberg, 1957). The afroalpine flora inhabits a number of geographically and ecologically isolated temperate “islands”. These “islands” are comparable to oceanic islands because the montane forests and the savanna vegetation separate the high mountain enclaves from each other better than sea between islands in an ocean (Hedberg, 1970).

The isolation of these high mountains provides unique opportunities for studying evolutionary phenomena, that is, speciation in connection with geographical isolation and adaptation to extreme environmental conditions (Hedberg, 1975). The easily observable morphological adaptations occurring in the afroalpine flora will be related to its age in the environment concerned and to the extremeness of the latter (Hedberg, 1995).

Plant populations that occupy latitudinal and elevational climatic gradients usually exhibit clines in morphology, phenology and physiology (Clausen *et al*., 1940; Chapin and Chapin, 1981; Weber and Schmid, 1998; Gehring and Delph, 1999; Jonas and Geber, 1999; Clevering *et al*., 2001). Clinal variation is sufficiently prevalent to cause considerable taxonomic confusion as in the case of *Eucalyptus viminalis* and *E. dalrympleana* (Phillips and Reid, 1980).

Clinal variation in morphological traits can be explained by an ecological gradient such as humidity. Brochmann *et al*. (1995) studied the morphological variation in subspecies of *Frankenia ericifolia* (Frankeniaceae). There was genetically based clinal morphological variation in two of the subspecies paralleling a gradient in humidity ranging from arid to semiarid. The authors suggested that the clines were of primary origin (i.e. they evolved in response to the gradual changing humidity conditions). Other studies have also indicated that genetic and morphologic variability can be caused within a species by clinal variation, e.g. *Achilea lanulosa*, Claussen *et al*. (1948); *Potentilla glandulosa* (Clausen, 1951); *Chionochloa rigida* (Mark, 1965).

The genus *Swertia* L. (Genetianaceae) is a morphologically diverse but taxonomically distinct group of ca. 150 annual, biennial or perennial herbaceous species. Three species diversifications are recognized; Himalalya and the mountains of southern and western China where most of the species are concentrated (Meusel, 1978) in the latter, due to the presence of the taxa with ancestral character (i.e. tall perennial plants, pentamery, few flowered inflorescence, rugose seeds), the genus is likely to have its center of origin in south and western China (Ho *et al.,* 1994). Secondly, the North American species and the African species which occur in the afromontane and afroalpine archipelago- like regional center of endemism.

In this study we deal with the *Swertia abyssinica* species complex: *S. abyssinica* Hochst. *S. lugardae* Bullock and *S. pumila* Hochst. According to Nemomissa (1994), these three species are classified as afromontane and afroalpine taxa based on their altitudinal range.

The afromontane taxon, *S. abyssinica,* has an altitudinal range of 1500- 3350m and grows commonly in vegetation dominated by *Erica arborea.* The afroalpine taxa are *S. lugardae* and *S. pumila*, their altitudinal ranges extend from 2800- 4050 and 3070-3700m asl, respectively.

Although the species are accepted as being distinct, the differentiation of the *Swertia abyssinica* species complex has been problematic. In a previous study (Nemomissa, 1994) morphological characters have been used to separate the complex: growth habit (annual or perennial), leaf morphology (rosette or cauline), and anatomy, floral morphology and anatomy, fruit and seed (size, shape, numbers and surface) and their anatomy, karyology and palynology.

Morphological differences are observed based on altitudinal differences with respect to the height, branching habit, and flower color. *S. abyssinica* (Fig. 1 A), which is found at lower altitude, is taller, branches less and has light yellow flower color. The plants found at intermediate altitude (Fig. 1 B, C and F) were accordingly classified as *S. pumila* or *S. lugardae*, and are intermediate in height compared to the ones at lower and higher altitudes and branch more than the lower altitude species. The color of the stem and flower are violet. The high altitude plants growing above 3700m are regarded to be typical *S. lugardae* (Fig. 1 D & E) and have a cushion forming habit with dark violet stem and flower.

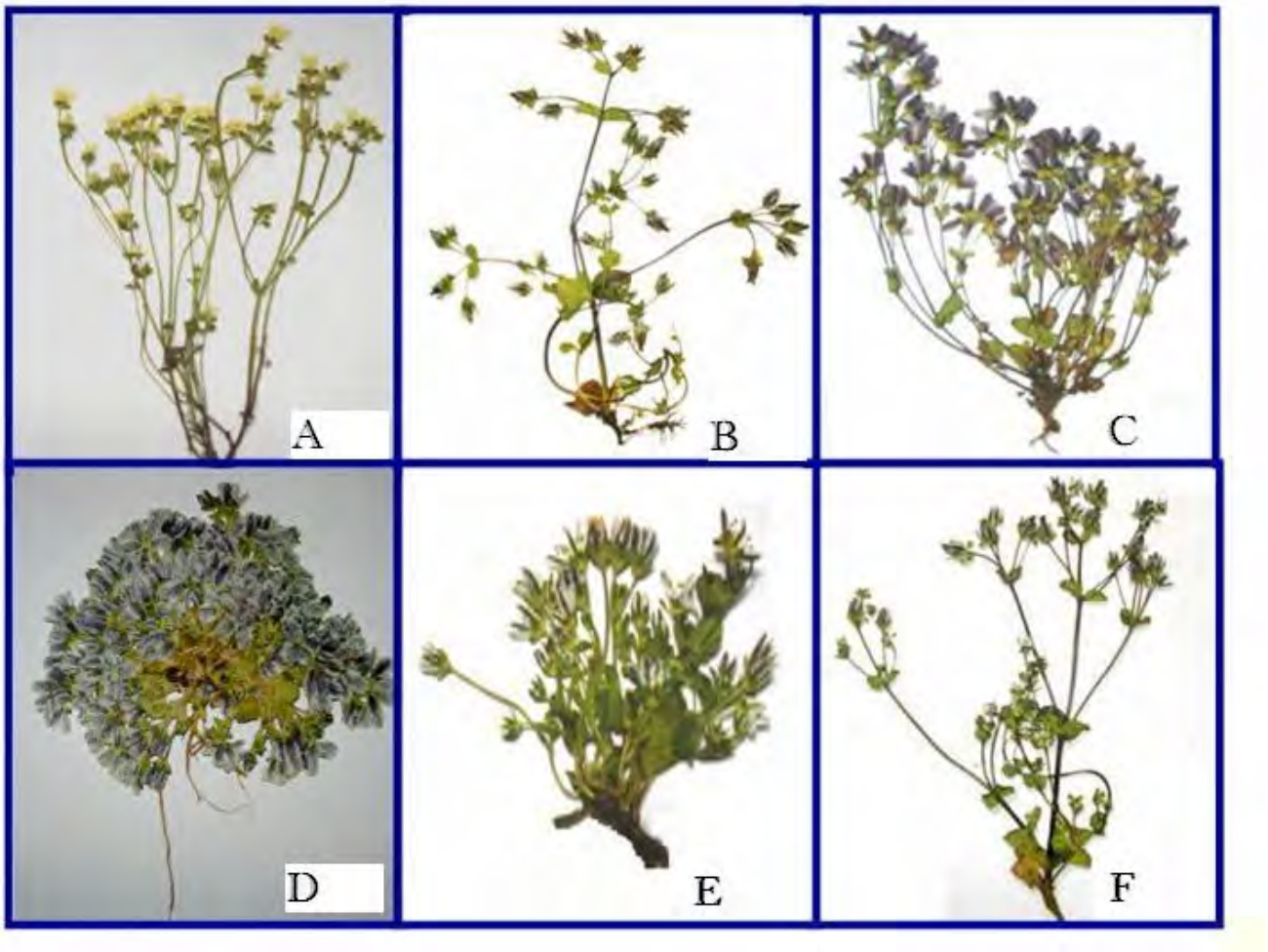


Figure 1. Variation in morphology in *Swertia abyssinica* species complex .A. *S. abyssinica* (lower altitude; Simen); B. *S. pumila* (intermediate; Bale); C. *S. lugardae* (intermediate; Bale); D. *S. lugardae* (uppermost; Bale); E. *S. lugardae* (uppermost; Siemen); F. *S. pumila* (intermediate; Bale)

In this paper, we analyze the population structure of the *Swertia abyssinica* species complex, and revise its taxonomy. We used Amplified Fragment Length Polymorphism (AFLP), to quantify genetic differentiation among the populations or species, and to address whether an ecocline or secondary hybridization contact accounts for the morphological differentiation. In the former case, we would expect relatively low diversity within mountain regions, and evidence of clinal evolution within each mountain region; in the latter case we would expect higher level of genetic diversity within

mountains, confirming secondary contact between previously isolated glacial gene pools. When islands are colonized, there will be a few individuals of the new population which result into immediate burst of genetic drift (Mayr, 1954). Colonization is associated with long periods during which population size are small, which may cause genetic drift followed by differentiation of the isolated populations (Barret, 1998). Such process may result in taxa more or less adapted to different habitat, which may also be spatially close. If there is an expansion in population size with no reproductive barriers, hybridization between previously isolated populations may occur (Nielsen, 2003).

The AFLP technique is a novel polymerase chain reaction (PCR)-based molecular assay (Vos ***et al.***, 1995) which has the capacity to detect a high number of polymorphic loci in a single assay. AFLP has been used as an efficient and reliable technique for evolutionary studies, e.g. Despres ***et al.,*** (2002) using AFLP markers addressed the geographic pattern of genetic variation in the European globeflower, ***Trollius europaeus*** L. (Ranunculaceae) and Tero ***et al.***, (2003), applied AFLPs to study genetic structure and gene flow in a metapopulation of an endangered plant species, ***Silene tatarica*** (L.).

2. LITERATURE REVIEW

2.1 The Afroalpine flora

The alpine flora, above the tree line, occur sporadically throughout the world, sometimes associated with mountain chains such as the Rocky Mountains and Andes, or other times as isolated flora on a single mountain peak, as for example Mt. Kinabulu in Borneo, or as a group of isolated mountain peaks, as in East Africa (Packer, 1974). In the tropical East Africa, there are a number of isolated high mountains in which several of them reach altitudes of 4000 m or more above sea level (Hedberg, 1970). These mountains lie in two groups, one eastern and one western. The vegetation of these mountains have a conspicuous zonation which starts with a montane forest belt followed by an ericaceous belt, and finally an afroalpine belt having its lower limit at 3500-4000 m above sea level (Hedberg, 1951).

The climate of the afroalpine belt is a very remarkable type, with nightly frosts all the year round, and with intense sunshine in daytime (Hedberg, 1964). The upper parts of the mountains harbor equatorial glaciers. Although there is resemblance, the alpine climates of the different high East African Mountains display substantial differences. Such kinds of differences are observed in the amount and seasonal distribution of cloudiness and precipitation, and in the size of the mean diurnal temperature amplitude (Scaetta, 1934). Hedberg (1995) has pointed out the gradual climatic change on each mountain from the lower limit of the alpine belt towards the summit. Furthermore, he indicated that there may be considerable differences in precipitation, insolation, diurnal temperature amplitude and other factors between different localities at the same altitude.

The afroalpine flora of East Africa, studied by Hedberg (1957), comprises 278 species in 39 families of which the four most important are the Compositae, Grammineae, Rosasea, and Scrophulariaceae. There are only some, 70-150 species of vascular plants in the alpine belts of the mountain that are capable of enduring such climate with " summer everyday and winter every night" (Hedberg, 1957). All these plants are adapted to withstand their environmental vicissitudes, and several of them have very conspicuous morphological adaptations (Hedberg, 1970). Most alpine vascular plants have a very slow growth rate due to decrease in the partial pressure of the carbon dioxide (Hedberg, 1995). Based on these morphological adaptations, Hedberg (1964) had recognized five distinct life forms adapted to tropical alpine conditions. These are giant rosette plants, tussock grasses, acaulescent rosette plants, cushion plants, and Sclerophyllous shrubs.

The endemism of 80% of the afroalpine taxa indicated that the high mountains of the tropical East Africa and Ethiopia have long been isolated from other high mountain and temperate flora (Hedberg, 1961). The distribution pattern of afroalpine vascular plants suggests the greater possibilities of exchange between the afroalpine biotas of the mountains within each of the two groups of mountains than between them (Hedberg, 1961). The closest relatives of most of the afroalpine plant are found in distant parts of the world, such as in the Mediterranean, than in the lower parts of the mountain (Hedberg, 1965). This suggests that the afroalpine floras are complex derivatives and most of the mountains harboring them are of volcanic origin and of unequal ages (Miocene to late Peiostocene) (Hedberg, 1970).

Temporal variation in the physical environment has played a major role in the evolution of tropical alpine plants (Smith and Young, 1987). Many aspects of morphology and physiology seem to provide escape from, or tolerance to extreme diurnal climatic fluctuations. Patterns of species distribution and species richness have been moduled in part by climatic fluctuation on a geologic time scale (Smith and Young, 1987).

2.2 The genus Swertia

The genus *Swertia* L. (Gentianaceae) is a morphologically diverse but taxonomically distinct group of 150 annual, biennial or perennial herbaceous species. At the base of rotate corolla lobes it has 1 or 2 nectaries. The taxa range from 2-4 cm to over 1.5 m in height. The flower (pentamerous or tetramerous) color may be blue, yellow, white, greenish or red, with various spots or stripes.

Swertia is present on all the continents, except, central and South America, Australia and New Zealand (Chassot *et al.*, 2001). The taxa are ecologically quite hetermorphic as the species occupy habitats ranging from hygrophytic, mesophytic or more or less xeric conditions, essentially in temperate zones but also in tropical Africa. They all occupy altitudinal ranges from 500 m to 6200 m. Most of the species are encountered in subalpine pastures, open forests, or along stream banks.

Most of the species occur in temperate regions of the northern hemisphere and the highest species diversity within the genus is in the Himalayas and in South-western China (Meusel *et al.*, 1978). The genus is, however, represented in tropical regions and in the southern hemisphere as well. Ho *et al.*, (1994) have proposed south western China as the

center of origin of the genus due to the high species diversity and the occurrence of taxa with presumed ancestral characters (i.e. tall perennial plants, pentamary, few-flowered inflorescences, rugose seeds). From there, *Swertia* has perhaps diversified and dispersed to south-east Asia as well as to Africa and North America, where they have formed two secondary centers of diversification. There are two taxa in the Arabian Pennisula and one species in Madagascar.

In Africa, the genus *Swertia* is distributed between 14^0 N and 30^0 latitude in sub-Saharan countries from West Africa to Madagascar (Nemomissa, 1998). The African taxa of the genus have three geographical distribution patterns: species which are widely distributed without any sign of infraspecific speciation due to continuous variations in the taxonomically useful character state, those species which are common to Ethiopia and tropical east Africa (TEA) and exhibit infraspecific segregation and the endemic species.

According to Whites (1983) division of the vegetation of Africa, all the African *Swertia* species occur in the Afromontane and Afroalpine archipelago-like regional center of endemism. The highest species concentrations are in the high mountains of North East and Tropical East Africa (Muesel *et al*., 1978). *S. abyssinica* occurs in the high mountains of North East and West Africa (Cameroon). It has relatively wider distribution in North East Africa than the remaining species in this region. *S. lugardae* mainly occurs in Siemen (Gondar) and Bale mountains. *S. pumila* is considered as endemic to North East Africa (Nemommisa, 1994). The three taxa were classified according to their altitudinal ranges by Nemomissa (1994) as afromontane and afroalpine taxa. The afromontane taxon includes *S. abyssinica* with an altitudinal ranges from 1500- 3350 m and grows

commonly in vegetation dominated by *Erica arborea.* The afroalpine taxa are *S. lugardae* and *S. pumila*, their altitudinal ranges extend from 2800- 4050 and 3070-3700 m, respectively.

The generic concept of *Swertia* has never been stable mainly because of macro-morphological characters, which do not exhibit clear enough patterns to unambiguously justify taxonomic units (Chassot *et al*., 2001). There are certain taxa which are recognized as synonyms of *Swertia,* for example *Frasera* Walter (Shah, 1990), but sometimes others describe it as a distinct genus (Card, 1931). Parallel evolution of the morphological traits such as the shape of glands, seed ornamentation and shape tends to mask the phylogenetic relationship of the species. Chassot *et al.,* (2001) have defined *Swertia* to be a paraphyletic taxon and Struwe *et al.,* (2002) have placed *Swertia* in the sub tribe Swertiinae.

2.3 Evolutionary diversification

Two contrasting views exist in evolutionary biology concerning the dominant forces driving diversification. On one hand, unique histories among organisms might play a major role in evolutionary outcomes, thus producing disparate results under similar selection i.e., historical contingency; (Price *et al*., 2000). Such differences in evolutionary outcomes presumably derive from clade-specific factors as well as chance historical happenings and localized environmental differences (Harvey and Pagel, 1991). In contrast, natural selection might play a dominant role in evolutionary diversification, thus producing similar outcomes under similar environmental circumstances (Schluter, 2000;

Losos *et al*., 1998). According to this view, natural selection could overwhelm the vagaries of history and produce evolutionary convergence. Probably more likely is that both unique histories as well as common natural selective forces play key roles in the evolutionary process (Johnson, 2002; Matos *et al.,* 2002; Ruzzante *et al*., 2003). Thus, when multiple groups of organisms face a common environmental gradient, their patterns of divergence might exhibit both shared and unique elements.

Geographical divergence is one means of evolutionary diversification in which the process occurs by isolating mechanisms, in part due to the restriction of gene flow between populations (Still *et al*., 2005). Among subpopulations of a widespread species, different ecological environments and independent evolution of populations through genetic drift may lead to divergence. In the absence of a barrier that restricts gene flow, adaptation to a new environment will be limited. Geographical barriers and/or restricted gene flow, coupled with differential selection pressures within the geographical range of the species, should provide the opportunity for divergence.

The evolutionary significance of geographic variation within species is one of the most controversial and perplexing questions in biology (Pimentel, 1959). According to Hammond (1990) five different phenomenons are included under the general subject of geographic variation. These include: genetic gradients within cohesive populations called primary clines, differentiation between populations resulting from geographic isolation, secondary intergradation between previously isolated populations along hybrid suture zones, vicariance and polytopic variation, and incipient speciation.

Evolutionary parallelism is generally treated together with convergence as the independent acquisition of similar attributes in distinct lineages (homoplasy). Simpson (1961) and Mayr (1969) defined parallelism in terms of possession of similar features in two or more lineages which share a common ancestry. Convergence would then be the development of similar features in unrelated taxa. Hull (1967) and Mayr (1969) have pointed out several problems with these concepts. The distinction between parallelism and convergence on the basis of proximity of common ancestry is problematic as the differences are relative. Parallelism has also been distinguished as homologous similarity. It is similarly difficult to differentiate parallelism on the basis of homology, as most parts are homologous in some respects, analogous in others (Gosliner *et al*., 1984).

Discovery of parallel evolution has recently accelerated, aided by molecular and ecological studies that have detected similar but independent evolutionary transitions in traits across multiple populations or closely related species in association with changes of environment. Examples include interspecific differentiation within the genus *Clarkia* along an elevational gradient (Jonas and Geber, 1999),

The recognized significance of parallel phenotypic evolution lies in its contribution to the study of natural selection. Natural selection has almost certainly played a crucial role in phenotypic evolution whenever the same traits of different populations and related species evolve repeatedly and consistently in association with similar transitions in environment (Rundle *et al*., 2001).

2.4 Molecular markers

Estimates of genetic variation are increasingly being based upon information at the DNA level. The development of molecular markers has made it possible to detect variation in plants. Protein markers were among the first group of biochemical markers exploited for genetic diversity assessment and genetic linkage map development (Ferguson and Grabe, 1986; Hash and Bramel-Cox, 1996). Such variation has, however, remained restricted to a few numbers of loci. However, the development of DNA-based molecular markers has clearly allowed the direct comparison of the genetic material of two individual plants and for detection of polymorphism. Among these molecular markers, hybridization based DNA markers such as restriction fragment length polymorphism (RFLP) and PCR based DNA markers such as random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLP), are included.

2.4.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphisms were the first DNA markers to come into wide spread use. RFLP refers to restriction fragments of different sizes produced by restriction enzymes from the same stretch of genomic DNA of different genotypes. Variations in fragment length between individuals or species can arise either when mutations alter restriction sites, or result in insertions / deletions between them (Burr ***et al.***, 1983). The experimental procedure of RFLP consists of (Karp ***et al.***, 1996) extraction and purification, genomic DNA is digested with restriction enzymes and separated according to the size of the DNA fragments on an agarose gel. After electrophoresis, the

DNA fragments are denatured and blotted on to a nitrocellulose or nylon membrane. Specific DNA fragments are identified by hybridization with a labeled probe. All DNA fragments on the membrane which have homology to the probe will be visualized. Because heterozygous are distinguishable, RFLPs are co-dominant markers. Their technical limitations are: (a) a good supply of probes are needed and, if heterologous probes are unavailable, cDNA or genomic DNA probes must be developed; (b) the blotting and hybridization steps are time consuming and difficult to automate and; (c) sufficient quantities (e.g.10 µg per digestion) of good quality DNA are required and RFLPs are, thus not applicable where very limited amounts of source material, DNA or preserved tissue are available.

2.4.2 Random Amplified Polymorphic DNA (RAPD)

The development of polymerase chain reaction (PCR) for amplifying DNA led to a revolution in the applicability of molecular methods and a range of new technologies were developed which can overcome many of the technical limitations of RFLPs. The most common version is RAPD (Random amplified polymorphic DNA) analysis, in which the amplification products are separated on an agrose gels in the presence of ethidium bromide and visualized under ultraviolet light (William ***et al***., 1990). Arbitrary primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anolles ***et al***., 1991) differ from RAPDs principally in primer length, primer to template ratio, the gel matrix used and in the visualization procedure. The enormous attractions of these arbitrary priming techniques are: (a) there is no requirement for DNA probes or sequence information for the design of specific

primers; (b) since the procedure involves no blotting or hybridizing steps, it is quick, simple and automatable and: (c) very small amounts of DNA (10 ng per reaction) are required. It is absolutely critical, however, to maintain strictly constant PCR reaction in order to achieve reproducible profiles (Karp *et al.*, 1996).

Although the difficulty of achieving robust profiles, particularly in RAPDs makes their reliability for 'typing' questionable, the data derived from RAPDs (or AP-PCR and DAF) have strength in distinguishing individuals, cultivars or accessions (Ayana *et al*., 2000; kjolner *et al*., 2004). RADPs have also been used in germplasm characterization (Ayana *et al*., 2000). In using arbitrary priming procedures, it should be realized that: (a) the markers are dominant and heterozygotes can not be detected; (b) in the absence of pedigree analysis, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci; (c) the presence of a band of apparently identical molecular weight in different individuals can not be taken as evidence that the two individuals share the same homologous fragment, although this assumption is commonly made and ; (d) single bands on the gel can sometimes be comprised of several co-migrating amplification products.

2.4.3 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) is a new molecular technique for fingerprinting DNAs of any origin and complexity. The AFLP method can visualize and detect hundreds of amplified DNA fragments simultaneously. The AFLP band pattern can be used for several purposes, such as monitoring inheritance of agronomic traits in plant and animal breeding, diagnostic of genetically inherited diseases, pedigree analysis, forensic typing, parentage analysis, screening of DNA markers linked to genetic traits and microbial typing.

2.4.3.1 Principle of the method

The AFLP is a technique that combines the power of RFLP with the flexibility of PCR-based technology by ligating primer-recognition sequences (adapters) to the restricted DNA (Vos ***et al***., 1995). Figure 1 outlines the four steps of the AFLP technique: DNA digestion, ligation, amplification and gel analysis. Genomic DNA is first digested by two restriction enzymes (usually ***EcoR***I and ***Mse***I), followed by ligation of double stranded oligonucleotide adapters to the restriction sites. PCR primers complementary to the adapters and restriction sites are used for the amplification of fragments that are flanked by the adapters. A subset of fragments is selectively amplified by PCR primers that have 2- or 3- base extensions into the restriction fragments. Only those fragments that perfectly match the primer sequences can be amplified by PCR. Therefore, the complexity of PCR amplicons is reduced. Polymorphisms are revealed by analysis of amplified fragments on a denaturing polyacrylamide gel, and comparisons of the patterns generated for each sample. Relative ease of implementation, large number of polymorphisms detected per

gel, small amount of genomic DNA required, and high reproducibility of DNA fingerprint patterns recommend AFLP as an attractive method to study DNA polymorphism in general.

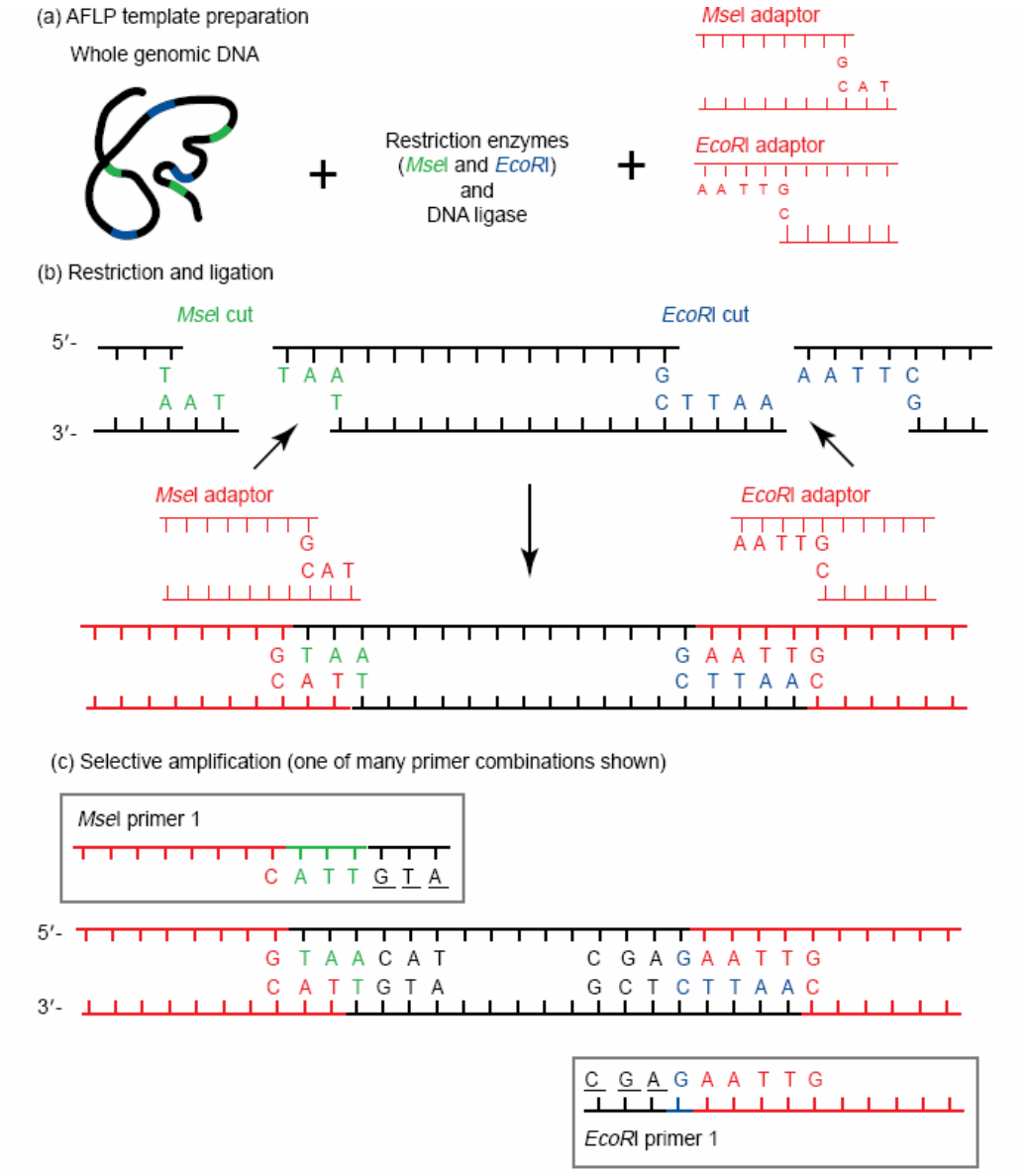


Figure 2. Generating amplified length polymorphism, a small amount of DNA (~50 ng) is digested with two restriction enzymes (a), and AFLP adapters are joined (ligated) to these ends (b). The end sequences of each adapted fragment now consist of the adapter sequence (in red) and the remaining part of the restriction sequence (in blue and green). These known end sequences serve as priming sites in the subsequent AFLP-PCR (Muller and Wolfenbarger, 1999).

2.4.3.1.1 Digestion of genomic DNA

Restriction fragments are generated using two restriction endonucleases, a 'rare'- cutting enzyme with 6 to 8 – base recognition, in combination with a 'frequent' – cutting enzyme of 4 –base recognition. The high degree of specificity of restriction enzymes results in production of a reproducible set of DNA fragments. The complexity of the genome and factors such as the methylation status of the DNA influence the choice of enzymes (Bachem *et al*., 1996). According to Vos *et al*. (1995), the reason for using two different enzymes are the following: (a) The frequent cutter will generate small DNA fragments, which will amplify well and are in the optimal size range for separation on denaturing gels (sequence gels), (b) the number of fragments to be amplified is reduced by using the rare cutter, since only the rare cutter fragments are amplified. This limits the number of selective nucleotides needed for selective amplification. (c) the use of two restriction enzymes makes it possible to label one strand of the double strand PCR products, which prevents the occurrence of 'doublets' on the gels due to unequal mobility of the two strands of the amplified fragments, (d) using two different restriction enzymes gives the greatest flexibility in 'tuning' the number of fragments to be amplified, and (e) large numbers of different fingerprints can be generated by the various combinations of a low number of primers. Examples of rare cutting enzymes employed in AFLP include *EcoR*I, *Ase*I, *Hind*III, *Apa*I and *Pst*I. The commonly used frequent cutters include *Mse*I and *Taq*I. The frequent –cutter generates small fragments within the desired size range of 100-1000 base pairs required for efficient PCR amplification and separation on denaturing polyacrylamide gels (Bachem *et al*., 1996).

2.4.3.2.2 Ligation of Adapters

Prior sequence knowledge is not required for AFLP. AFLP adapters consist of a core sequence and an enzyme specific sequence, and are double stranded nucleotide usually 10-30 base pairs long. T4 DNA ligase is employed to ligate the adapters complementary to the sticky ends of the corresponding restriction site (Figure 3). The sequence of the adapters and the adjacent restriction half-site and serve as primer binding sites for subsequent PCR amplification. Adapters are composed of two synthetic oligonucleotides that are in part complementary to each other and form a double-stranded structure in solution under appropriate conditions. Ligation does not restore the original restriction enzyme site because of a base change incorporated into the adapter sequence (Figure 3).

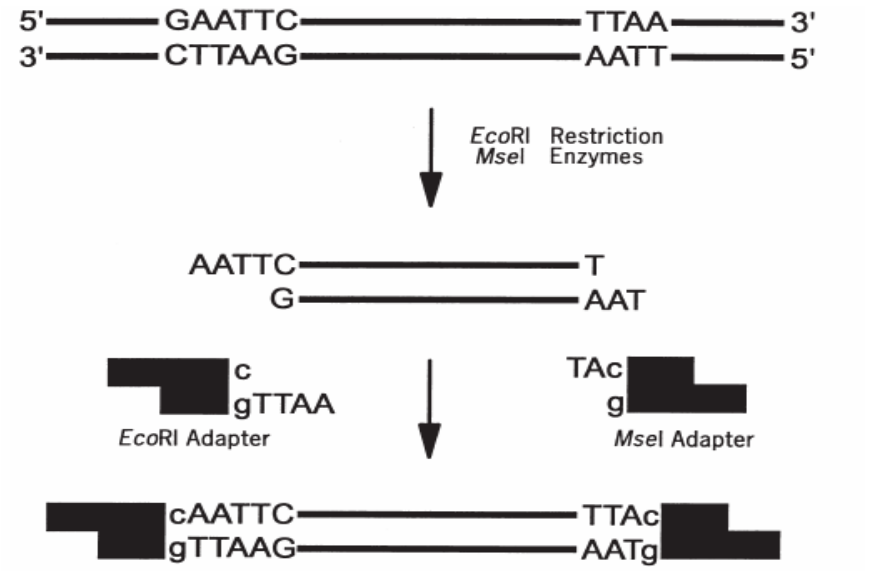


Figure 3. A schematic outlining of the ligation of adapters to the ends of a restriction fragment. Genomic DNA is first restricted by *Eco*RI and *Mse*I. Double stranded adapters, complementary to the short single- strand extension generated by the restriction enzymes, are ligated to the DNA fragment. The *Eco*RI and *Mse*I recognition sites are not restored by ligation because of a base change in the adapter sequence (shown in lower case) (Blears *et al*., 1998).

This change in the recognition site prevents restriction from taking place after ligation has occurred, enabling restriction and ligation reactions to be performed in the same tube. With these reactions occurring simultaneously, any fragment-to-fragment product is restricted (Mueller and Wolfenbarger, 1999). Furthermore, the adapters used are not phosphorylated, which causes only one strand to be ligated to the ends of the restriction fragments and prevent adapter to adapter ligation (Blears *et al.*, 1998)

The use of biotinlayted adapters, complementary to the rare cutting restriction half-site (e.g. biotin labeled *Eco*RI adapters) can be used to reduce the complexity of the DNA template, prior to amplification. According to Zabeau and Vos (1993) the selection of biotinylated restriction fragments cut by the rare-cutting enzymes are done by the use of streptavidin coated magnetic beads. The use of biotin-labeled adapters separates fragments having at least one rare-cutting restriction site from the large number of fragments having two frequent-cutting restriction sites (e.g. *Eco*RI - *Eco*RI and *Eco*RI - *Mse*I fragments are separated from *Mse*I - *Mse*I fragments). This step has proved to be important for generating high quality fingerprints of complex DNAs (Gaudeul *et al., 2000*) and for decreasing background smears on gels.

2.4.3.2.3 Selective amplification

AFLP primers for selective amplification contain three types of DNA sequence: the 5′ region corresponding to the adapter, the restriction site sequence and the 3′ selective nucleotides (Figure 4). Two AFLP primers are used; one primer is complementary to the adapter and adjacent rare-cutter restriction site sequence with one to three additional

selective nucleotides at the 3′ - end (e.g. *Eco*RI primer 3′ - XXX, where X denotes the selective nucleotides), and the second primer is complementary to the adapter and frequent-cutter recognition site sequence with an additional selective one- to three-base extension (e.g. *Mse*I primer 3′ - XXX) (Blears *et al*., 1998).

Following the restriction-ligation reaction, a limited number of ligated restriction fragments are selectively amplified by the AFLP primers (Figure 4). Only a subset of the template fragments, with complementary nucleotides extending beyond the restriction site, will be amplified under stringent annealing conditions (Figure 5) (Vos *et al*., 1995). The nucleotide extensions on the 3′ -end of the primers serve two purposes: (i) they allow a variety of restriction fragment subsets to be amplified; and (ii) they provide additional possibilities for polymorphisms to be detected beyond the restriction site itself (Blears *et al*., 1998).

Although fragments cut only by the frequent-cutter (e.g. *Mse*I - *Mse*I fragments) are predominant (> 90%), fragments cut by both enzymes (e.g. *Eco*RI - *Mse*I fragments) are preferentially amplified. There are two reasons for the efficiency: (i) the primer complementary to the rare-cutting restriction site and adapter (e.g. *Eco*RI primer) has a higher annealing temperature than the primer of the frequent-cutter (eg *Mse*I primer); and (ii) the fragments cut by both enzymes (eg *Eco*RI-*Mse*I fragments) are amplified using two primers (e.g. *Mse*I and *Eco*RI primers) preventing the formation of an inverted repeat

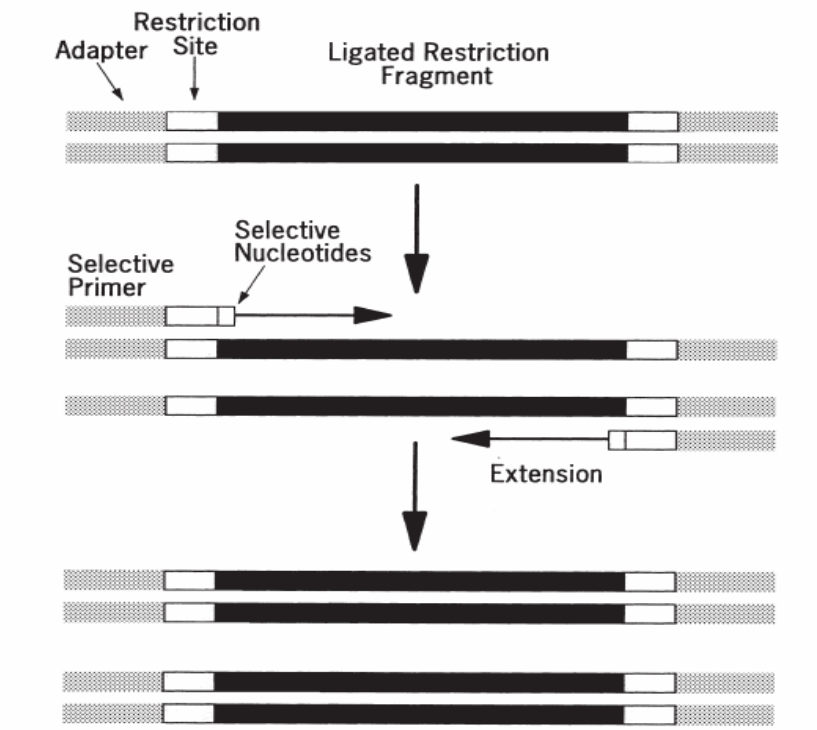


Figure 4. PCR amplification of a ligated restriction fragment. The AFLP primers have three regions, the 5'- end complementary to the oligonucleotide adapter sequence, the restriction site sequence and the 3' selective nucleotides. The horizontal arrows indicate the direction of DNA synthesis. With each PCR cycle, the amount of DNA is doubled (Blears *et al.*, 1998)

at the ends (Vos *et al*., 1995). Base-pairing of the ends of the fragment forms a stem loop structure which competes with primer annealing. The PCR conditions are important features of the AFLP technique. Most restriction digestion techniques generate such complex patterns that differences cannot be discerned. However, by increasing the efficiency of the primer complementary to the rare-cutter, only a subset of the fragments (< 10%) are efficiently amplified (e.g. *Eco*RI – *Mse*I fragments) to allow polymorphism to be revealed.

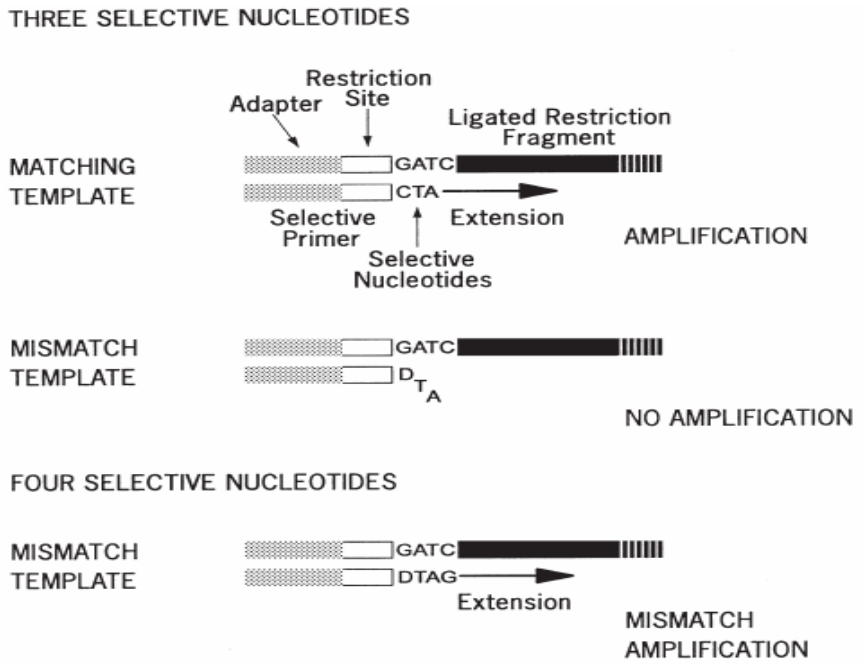


Figure 5. Diagram illustrating the selectivity of AFLP primers. The first example shows successful extension of a primer with three selective nucleotides matching the template sequence. The arrow indicates the direction of DNA synthesis. In the second example, there is a mismatch between the three selective bases and the restriction fragment preventing extension of the primer. The final example illustrates the mismatch amplification that can occur with primers having four selective bases. D= G, A or T (Blears *et al.*, 1998)

The number of amplified fragments is determined by the complexity of the genomic DNA, the choice of enzymes, as well as the number and type of selective nucleotides in the PCR primers (Gaudeul *et al., 2000*). As the number of selective nucleotides is increased, the complexity of the DNA fingerprint decreases (Vos *et al.*, 1995). The length and nature of the base extension on the 3′ - end of the primers can be manipulated to generate fingerprints of desired complexity.

For small genomes of $10^6 – 10^7$ base pairs (bp), one to two selective nucleotides on the 3′- end of each primer maybe sufficient to reveal polymorphism. More complex genomes ranging from $10^8 – 10^9$ bp will require additional selective nucleotides to yield the desired number of amplified fragments. Typically, the ideal number of amplified restriction fragments ranges from 50 – 100 (Vos *et al*., 1995).

Although the complexity is reduced with each additional selective nucleotide, the selectivity is maintained with nucleotide additions to a maximum of three selective bases. The fingerprints generated are merely a subset of the previous pattern. However, selectivity is lost with a 4-base extension. This loss of selectivity was demonstrated by Vos *et al.* (1995). When additional bands were amplified using four selective bases, which had not been detected in the corresponding fingerprint with primers having three selective bases. This indicates a tolerance of mismatches during amplification by primers with 4-base extensions (Figure 5).

2.4.3.2.3 Gel analysis

The DNA samples are denatured by heating at 90 - 95^0C for 3 - 5 min, prior to loading the amplified products into polyacrylamide gels. Only one strand is labeled to prevent the occurrence of double bands or ‘doublets’ on the gel due to unequal mobility of the two strands of an amplified fragment. Two labeling techniques, using either a radioactive label or a fluorescent dye are employed. The primer corresponding to the rare cutting restriction enzyme is labeled by phosphorylating the 5’ – end with [γ-^{32} P or -^{33} P] ATP using T4 polynucleotide kinase (Blears *et al*., 1998).

By electrophoresis the amplified fragments are separated on a 4.5 % or 5% polyacrylamide gel and the AFLP patterns are visualized by autoradiography. The resulting markers can be scored using an automatic genetic analyzer (Gaudeul ***et al., 2000***).

2.4.4 Advantages of AFLP

The AFLP technique has several advantages over other DNA fingerprinting systems. The most important of these are the capacity to inspect an entire genome for polymorphism and its reproducibility. AFLP can be applied to any DNA samples including human, animal, plant and microbial DNAs, giving it the potential to become a universal DNA fingerprinting (Mueller and Wolfenbarger, 1999). It is capable of detecting small sequence variations using only small quantities of genomic DNA (0.05 - 0.5 µg). The capacity to reveal many polymorphic bands in one lane is a major advantage of AFLP markers. The numerous bands on a gel are analyzed simultaneously making AFLP an extremely efficient technique. For example AFLP has allowed detecting highest number of polymorphisms in a single assay than other marker types in a study of relationship among early European maize inbreds (Lubbersted ***et al***., 2000). AFLP is superior interms of the number of sequences amplified per reaction and their reproducibility. The markers produced are reliable and more reproducible within and between laboratories (Jones ***et al***., 1997). This technique also allows generating unlimited number of markers by simply varying the restriction enzymes, the nature and number of selective nucleotides (Blears ***et al***., 1998).

2.4.5 Limitations of AFLP

The technique demands genomic DNA of high purity to ensure complete digestion by the restriction endonucleases. Incomplete restriction of DNA generates partial fragments, predominantly of high molecular weight. Amplification of fragments that are not fully digested generates an altered banding pattern, and may be misinterpreted as false polymorphisms (Vos *et al.*, 1995). Other studies have indicated poor reproducibility of AFLP fragments as a result of incomplete digestion of genomic DNA (Goulao *et al.*, 2001; Arnau *et al.*, 2002)

The AFLP procedure is insensitive to the template DNA concentration, although it is affected by DNA quality. The protocol is optimized such that the amplification reaction ceases when the labeled primer is exhausted (Vos *et al.*, 1995). This ensures that fingerprints of equal intensity are produced despite variation in template concentration. However, at very high template dilutions (Picogram quantities), the nucleotide sequences flanking the restriction site will no longer be random for a small pool of restriction fragments and variations in the banding patterns may be observed (Blears *et al.*, 1998).

AFLP markers usually detect variation in anonymous nuclear sequences and show predominantly dominant Mendelian inheritance, which limits their usefulness for population analysis (Paglia and Morgante, 1998; Lerceteau and Szmidt, 1999; Nikaido *et al.*, 1999). They share many limitations with RAPDs with respect to band homologies and identities (Karp *et al.*, 1996).

2.4.6 Comparison to other DNA markers

AFLP has advantages over other molecular based techniques for DNA fingerprinting including restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD).

The major differences between AFLP and RFLP technique is that PCR amplification is used for the detection of restriction fragments in AFLP analysis. However, in PCR-RFLP technique, a modified RFLP technique, PCR amplification is followed by restriction digestion. In AFLP, to detect the presence or absence of restriction fragments, fragments are run on a denaturing polyacrylamide gel while RFLP displays length differences of restriction fragments on agarose or polyacrylamide gels following hybridization. Due to the nature of RFLP technique, only the restriction site is scanned for differences in DNA sequences. The selective nucleotides included in AFLP provide additional possibilities for polymorphism to be detected beyond the restriction site itself. AFLP has the capacity to detect more point mutation than RFLP but insertions and deletions are detected at approximately the same frequency (Beker *et al*., 1995). In a single hybridization experiment, RFLP can detect, at most, a few genetic loci compared to 100 - 200 loci detected using AFLP (Meksem *et al.,* 1995). In addition to greater number of polymorphisms per reaction, AFLP is also superior interms of efficiency as it does not require template DNA sequencing. No prior sequence knowledge is required to produce fingerprints.

When comparing the RAPD technique to AFLP which uses primers specific to the adapters, the RAPD utilizes arbitrary primers having no known homology to the target sequences. These arbitrary primers are used to randomly amplify segments of the target DNA. RAPD analysis is easier to perform than AFLP; however it is very sensitive to reaction conditions, template DNA concentration and purity, and PCR temperatures profiles resulting in low reproducibility (e.g. Mcunier and Grimont, 1993; Scheweder *et al*., 1995). AFLP analysis uses stringent annealing conditions which guarantee a better reproducibility (Folkertsma *et al*., 1996). This technique also exhibits other problem which is, competitive priming (Hallde *et al*., 1996). In the study of AFLP vs. RAPD markers in clonal diversity of *Saxifraga ceruna* (Kjolner *et al*., 2004), they concluded that although RAPD can still be used, AFLP analysis has a superior efficiency.

2.4.7 Applications

In the study by Vos *et al.*, 1995 the method was evaluated by using organisms with genomes widely differing in complexity (bacteria, yeast, plant and humans) demonstrating its broad applicability. It has been used for marker assisted plant breeding, for the construction of high density linkage map, genetic diversity and phylogenetic analysis and population genetics.

Vos *et al*. (1995) were primarily interested in genome mapping, i.e. construction of high density genetic maps of either genomes or genome fragment; 'it can bridge the gap between genetic and physical maps'. Since then, many studies have applied this technique to mapping studies, e.g. *Oryza* (Zhu *et al.*, 1998), *Zea* (Xu *et al*., 1999) and

Solanum (Bradshaw *et al*., 1998). Xu *et al*., (1999) suggested that using AFLP is the most efficient way to generate a large number of markers that are linked to target genes.

The AFLP technique has widely been used for assessing plant genetic diversity. AFLP marker was employed by Kiambi *et al.* (2005) to study contrasting genetic diversity among *Oryza longistaminata* (A. Chev et Roehr) populations from different geographic origins. The study has clearly demonstrated the usefulness of AFLP in studying diversity in rice populations and its power of resolution in discriminating between populations and individuals within populations. Other studies have also pointed out the use of AFLP in diversity assessments, e.g. Ethiopian barleys (Bjornstand *et al*., 1997), Eragrostis (Ayele *et al*., 1999), weedy rice (Federici *et al*., 2001), Bentgrass (Vergara and Bughrara, 2003). Gaudeul *et al*. (2000) have demonstrated the efficiency of AFLP markers in allowing a quick and a reliable assessment of intraspecific genetic variability in an endangered alpine plant, *Ergynium alpinum* L. Negash *et al*., (2002) have also indicated the usefulness of AFLP in characterization of Enset clones.

2.5 Objectives of the Study

The present study, employing the most efficient technique, amplified fragment length polymorphism (AFLP), aims in testing the following hypothesis:

- Whether high altitude populations of *Swertia abyssinica* species complex in different mountains are more related to each other than to low altitude populations in the same mountain? or

- Whether high altitude populations of *Swertia abyssinica* species complex are most closely related to low altitude populations in the same mountains?

3. MATERIAL AND METHODS

3.1 Plant Materials

The current study was conducted in five Ethiopian afroalpine mountain systems (Fig. 6).

These are Simen and Bale Mountains, Mt. Choke, Mt. Chilalo and Mt. Kaka.

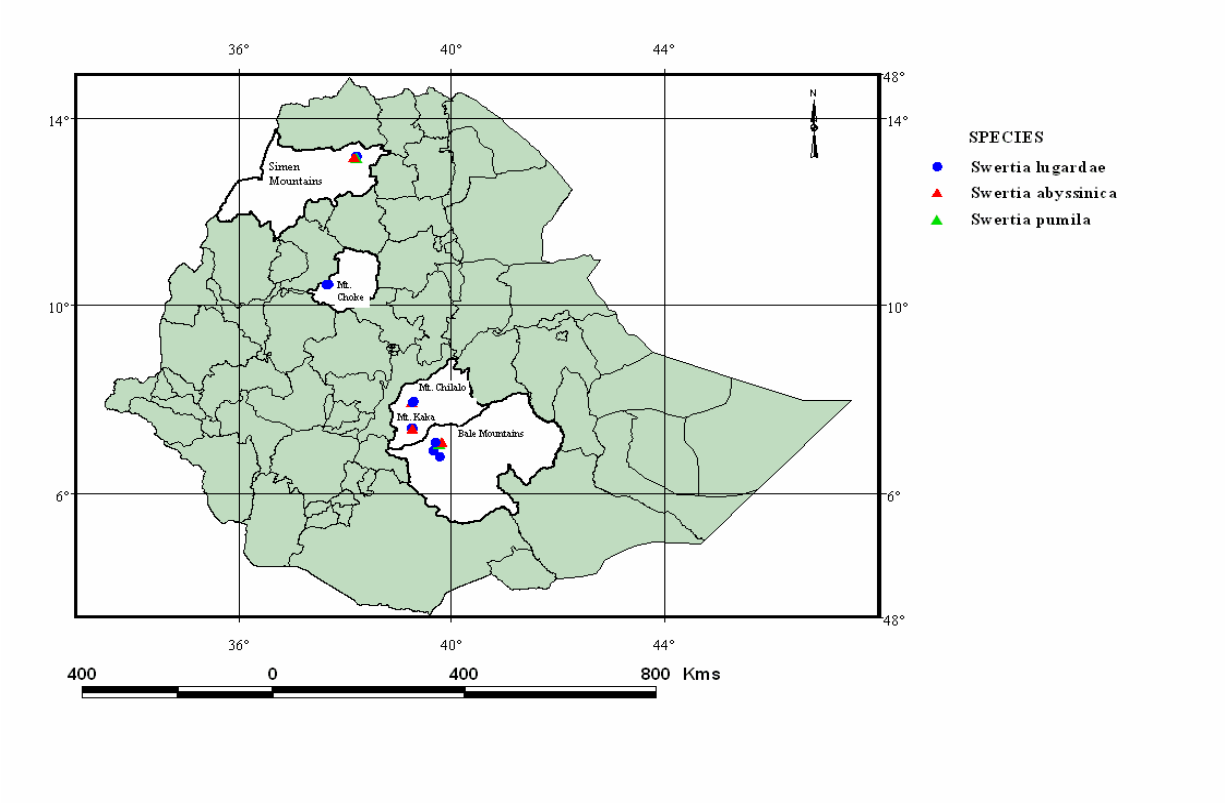


Figure 6. Geographical locations of the Study Mountains where the investigated populations of *S. abyssinica*, *S. lugardae*, and *S. pumila* were collected. The white color shows administrative regions (Simen Mountains in Gonder; Mt. Choke in Gojam; Mt. Chilalo and Kaka in Arsi and Bale Mountains in Bale administrative regions) of sampling locations

Young leaf materials of *Swertia abyssinica*, *S. lugardae* and *S. pumila* were collected and dried on silica gel (Table 1). From each mountain, specimens were collected at three distinct localities. Collections were made at different altitudinal levels: lowermost (3000 - 3250 m); intermediate (3250 – 3500 m); and uppermost (3500 – 4000 m). At each locality, 11 individuals per species per population were collected. A total of 28 such local population samples were collected. In each population, a random individual was collected twice. These replicates were labeled X throughout the analysis procedure and used to assess replicability.

Table 1. Plant materials examined in the current study with accession numbers, locality, altitude and coordinates

Species	accession No.	locality	altitude, m	longitude	latitude
Swertia abyssinica	AFR-003	Bale Mountains	3270	$07^0$09595	$039^0$79225
Swertia lugardae	AFR-005	Bale Mountains	3510	$06^0$77409	$039^0$75500
Swertia lugardae	AFR-008	Bale Mountains	3983	$06^0$89388	$039^0$60788
Swertia pumila	AFR-012	Bale Mountains	3120	$07^0$08731	$039^0$78528
Swertia pumila	AFR-016	Bale Mountains	3332	$07^0$04575	$039^0$75152
Swertia pumila	AFR-019	Bale Mountains	3359	$07^0$04135	$039^0$74963
Swertia lugardae	AFR-029	Mt. Kaka	3817	$07^0$36887	$039^0$18170
Swertia lugardae	AFR-031	Mt. Kaka	3764	$07^0$36773	$039^0$18508
Swertia lugardae	AFR-033	Mt. Kaka	3690	$07^0$36624	$039^0$18676
Swertia lugardae	AFR-035	Mt. Kaka	3475	$07^0$36320	$039^0$19721
Swertia abyssinica	AFR-040	Mt. Kaka	3488	$07^0$37099	$039^0$19388
Swertia abyssinica	AFR-043	Mt. Kaka	3428	$07^0$36743	$039^0$19680

Swertia lugardae	AFR-047	Mt chilalo	3670	$07^0$93860	$039^0$22117
Swertia abyssinica	AFR-052	Mt chilalo	3123	$07^0$91828	$039^0$18772
Swertia abyssinica	AFR-053	Mt chilalo	3160	$07^0$91997	$039^0$19028
Swertia abyssinica	AFR-054	Mt chilalo	3097	$07^0$9221	$039^0$18350
Swertia lugardae	AFR-309	Mt chilalo	3402	$07^0$92331	$039^0$20.557
Swertia lugardae	AFR-226	Simen mountains	3683	$13^0$16.361	$038^0$06.421
Swertia lugardae	AFR-227	Simen mountains	3591	$13^0$15.999	$038^0$06.463
Swertia lugardae	AFR-231	Simen mountains	3942	$13^0$17.197	$038^0$08.760
Swertia lugardae	AFR-232	Simen mountains	3358	$13^0$15.347	$038^0$06.717
Swertia lugardae	AFR-234	Simen mountains	3339	$13^0$15.380	$038^0$06.640
Swertia abyssinica	AFR-235	Simen mountains	3242	$13^0$13.919	$038^0$02.419
Swertia abyssinica	AFR-239	Simen mountains	3281	$13^0$14.089	$038^0$02.959
Swertia abyssinica	AFR-244	Simen mountains	3261	$13^0$13.912	$038^0$02.217
Swertia lugardae	AFR-255	Choke mountain	3304	$10^0$43.363	$037^0$47.387
Swertia lugardae	AFR-256	Choke mountain	3524	$10^0$43.718	$037^0$47.847

3.2 DNA extraction and Analysis

Total genomic DNA was extracted from the dried leaf material using a modified cetyltrimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987). A small amount of leaf was ground using a grinding machine at a frequency of 22 for 5 minutes and centrifuged at 13.000 rpm for 2 minutes. 700 µl of CTAB solution (12.1 g Tris, 5.8 g Na_2 EDTA, 81.9 g NaCl, 20 g CTAB and 4 g PVP disolved in a liter of water) containing 200 µl of mercaptoethanol per 100 ml was added to the finely ground leaves and incubated at 60 ^{0}C in a water bath with shaking for 30 min. The solution was mixed with 500µL chloroform/Isoamylalcohol (24: 1), shaked, allowed to stand for 5 min. and centrifuged at 13.000 rpm for 2 min. The supernatant was then mixed with 350 µL of isopropanol, shaked and put in a fridge for 5 min. It was then centrifuged at 13.000 rpm for 2 min and the supernatant was decanted. The pellet was washed with 70 % ethanol, followed by 2 min centrifugation at 13.000 rpm. The ethanol was then removed and pellet dried in a vacuum centrifuged for 15 min. The pellet was resuspended in 100 µL TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0) with 2 µL RNAase (100 mg/ml). Finally, the solution was vortexed and allowed to stand at room temperature for at least 30 min in order to resuspend the DNA and digest the RNA.

AFLP profiles were obtained according to Gaudeul *et al.* (2000) with some modifications. The procedures were as follows: Restriction and ligation to adapters were carried out in one reaction. 5.5 µL of DNA from each sample were digested with 40 U of *Eco*RI (cutting at 5' – GAATTC - 3') and 50 U of *Mse*I (cutting at 5' – TTAA - 3') in a final volume of 11 µL. The reaction mix contained 10 x T4 ligase buffer with 0.5 M

NaCl, 1 mg/ml BSA, 5 U T4 DNA ligase and water. The adaptor pairs *Mse*I and *Eco*RI were denatured at 94 ^{0}C for 5 minutes and annealed by slowly cooling down to room temperature before they were added to the reaction mix. The restriction - ligation reaction was carried out at 37 ^{0}C for 3 hours.

Digested and ligated DNA samples were diluted 10 times with distilled water. The pre-selective PCR was carried out in a total volume of 12.5 µL containing 1.5 µL diluted restriction ligation product, 1.25 µL Taq buffer, 0.75 µL $MgCl_2$, 1 µL dNTPs, 0.25 µL *Eco*RI + A and 0.25 µL *Mse*I + C primers, 0.075 µL AmpliTaq and 7.45 µL water and amplification was performed on a Biosystem 9700 thermocycler (Applied Biosystems) with the following program: 72 ^{0}C for 2 min, followed by 30 cycles of 94 ^{0}C for 30 sec, 56 ^{0}C for 30 sec, 72 ^{0}C for 2 min, and 72 ^{0}C for 10 min. Successful amplification of target sequence was verified on a 1 % agarose gel loading 5 µL of pre - selective product with 2 µL of loading buffer. The pre - amplified product was then diluted 20 times and used as a template for selective amplification.

The selective amplification reaction was performed in a final volume of 12.5 µL containing 2.5 µL of 20 x diluted pre - selective product, 1.25 µL of 10 x Taq buffer, 1.25 µL of 25 mM $MgCl_2$, 0.1 µL of 1 µM *Eco*RI + XXX (marked with a fluorescent dye), 0.25 µL of 5 µM *Mse*I + XXX, 0.10 µL BSA 0.10 µL of 5 U AmpliTaq and 5.95 µL water. Amplifications were carried out on the same thermocycler as the pre - selective PCR and the PCR conditions were 95 ^{0}C for 10 min, 13 cycles with a denaturation at 94 ^{0}C for 30 sec, an annealing temperature decreasing from 65 - 56 ^{0}C for 1 min and

extension at 72 ^{0}C for 1min, followed by 23 cycles with 94 ^{0}C for 30 sec, 56 ^{0}C for 1 min, 72 ^{0}C for 1 min; and final extension at 72 ^{0}C for 10 min.

The selective PCR product was mixed with 11.7 µL formamide and 0.3 µL GS Rox 500 (Applied Biosystems) size standard, and then denatured for 5 min. at 95 ^{0}C and put on ice immediately for 5 min. Electrophoresis was carried out on an ABI 3100 capillary sequencer (Applied Biosystems).

Twelve primer pairs were tested on six individuals in the selective PCR. Three of them were chosen for the clarity of the produced bands, most variability and good reproducibility. The three different E and M primers each with three selective nucleotides at the 3'- end used were: E - ACC/ M - CAG, E - ACA/M - CAC and E - AGA/M - CTC (Table 2). Reproducibility of each primer pair was checked by carrying out the whole AFLP protocol twice.

Table 2. Sequences of adapters and primers used for the AFLP analysis

Adapters	
*Eco*RI adaptor	5'-CTC GTA GAC TGC GTA CC-3' 3'-AAT TGG TAC GCA GTC TAC-5'
*Mse*I adaptor	5'-GAC CAT GAG TCC TGA G-3' 3'-TAC TCA GGA CTC AT-5'
Pre selective primers	
*Eco*RI	5'-GAT GAG TCC TGA GTA AC-3'
*Mse*I	5'-GAC TGC GTA CCA ATT CA-3'
Selective primers	
E-ACA (Green)	5'-GAC TGC GTA CCA ATT C**AC A**-3'
E-ACC (Yellow)	5'-GAC TGC GTA CCA AAT C**AC C**-3'
E-AGA (Blue)	5'-GAC TGC GTA CCA ATT C**AG A**-3'
M-CAC	5'-GAT GAG TCC TGA GTA A**CA C**-3'
M-CAG	5'-GAT GAG TCC TGA GTA A**CA G**-3'
M-CTC	5'-GAT GAG TCC TGA GTA A**CT C**-3'

3.3 Band Scoring

Raw data were collected using the ABI prism GeneScan Ver. 3.7 analysis software (Applied Biosystem). Amplification products were sized in reference to the size standard using the local Southern method, which allowed samples from different gels to be aligned accurately. Aligned data were imported into Genographer (Ver 1.6.0, Benham, 2001; http://hordeum.oscs.montana.edu/genographer) for visualization and scoring. Fragments between 100 and 500 bp having a single peak were scored as presence or absence and recorded as 1 and 0, respectively. They were used to construct a presence/absence data matrix with individual plants featuring in rows and fragments in columns.

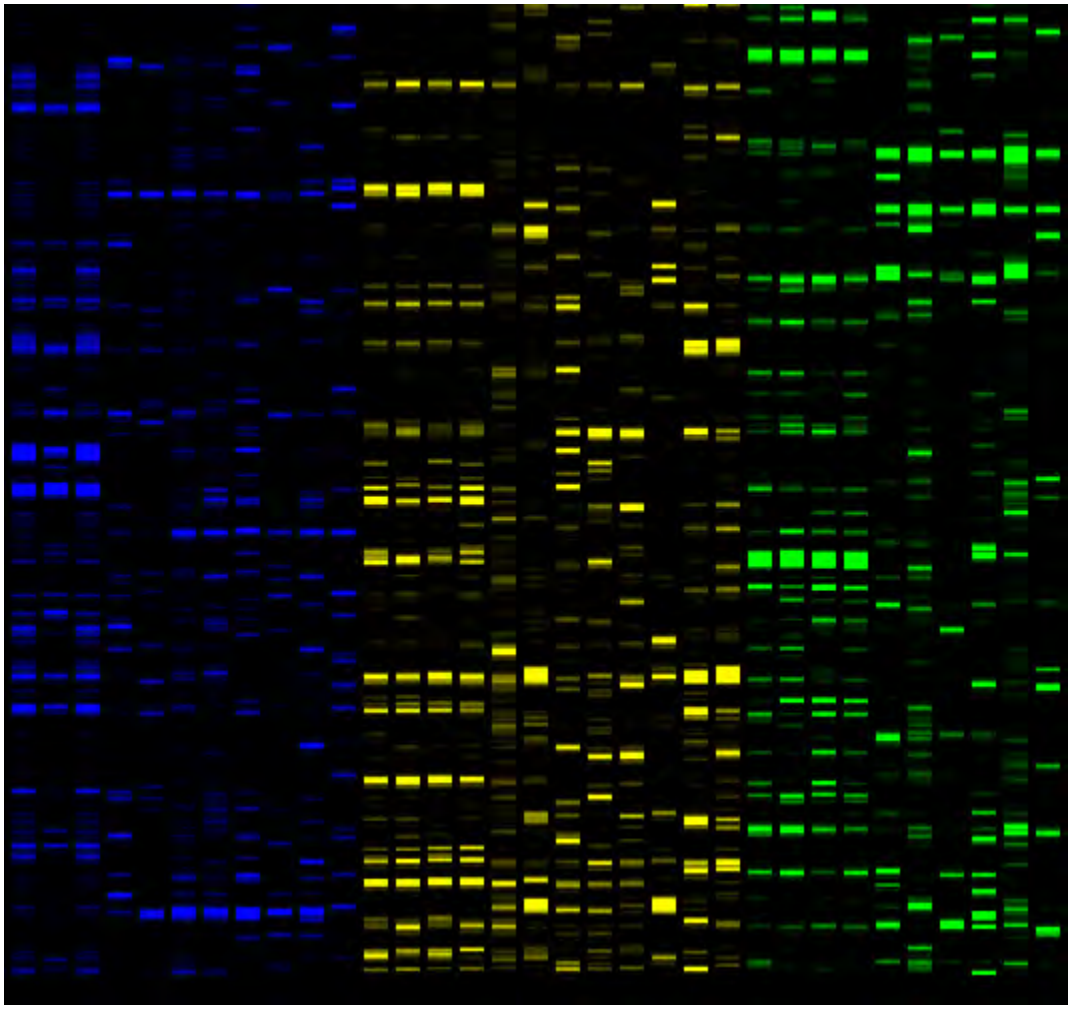


Figure 7. A sample gel picture of the three primer combinations: Blue (E - AGA/M - CTC), yellow (E - ACC/M - CAG) and green (E - ACC/M - CAG).

In addition to the individuals replicated throughout the analyses (X individuals), some individuals were replicated in the PCR to check if certain bands were formed due to the artifacts of the reactions. All replicates were then used to estimate an error rate for each primer combinations as the average number of errors per replicate divided by the total number of markers. A total of 240 individuals were subjected in the final analysis.

3.4 AFLP data analysis

Genetic diversity within populations was estimated as the average proportion of pair wise differences between individuals using the software Arlequin 2.0 (Schneider *et al.,* 2000). The genetic structure of the population at two hierarchic levels was analyzed by an Analysis of Molecular Variance (AMOVA). AMOVA is a statistical tool originally developed for population genetics (Excoffier *et al*., 1992) and based on the scheme of analysis of variance. The proportions of variance among groups, among populations within groups and within populations were calculated from the complete AFLP matrix. For the AMOVA, the populations were grouped according to mountain regions and according to taxa. AMOVA was performed using the software Arlequin 2.0 (Schneider *et al.,* 2000). A principal coordinate analysis (PCoA) was conducted to graphically represent a matrix of genetic similarities estimated as DICE coefficient (DICE, 1945) using NTSYS ver 2.02 (Rholf, 1992). A neighbor-joining tree was constructed using the program TREECON for windows 1.3 b, a software package for the construction and drawing of evolutionary distance trees (Van der Peer and De Wachter, 1997). For the neighbor-joining tree, we used Nei and Li's (1979) genetic distance, which corresponds to the DICE distance used for the PCoA, and a bootstrap analysis was run to estimate the support for the branches. Nei and Li's genetic distance is computed as follows:

$$GDxy = 1 - \frac{2Nxy}{Nx + Ny}$$

Where Nxy is the number of fragments (bands) shared among individuals x and y, and Nx is the number of fragments in individual x, and Ny is the number of fragments in individual y.

4. RESULTS

4.1 AFLP polymorphism

Using the three primer combinations, a total of 372 polymorphic bands were scored, 121 from the green primer combination, 130 from the blue and 121 from the yellow primer combination. After removing markers which were obviously not reproducible, replicability was 96%, 95% and 97% for the green, blue and yellow primer combinations, respectively.

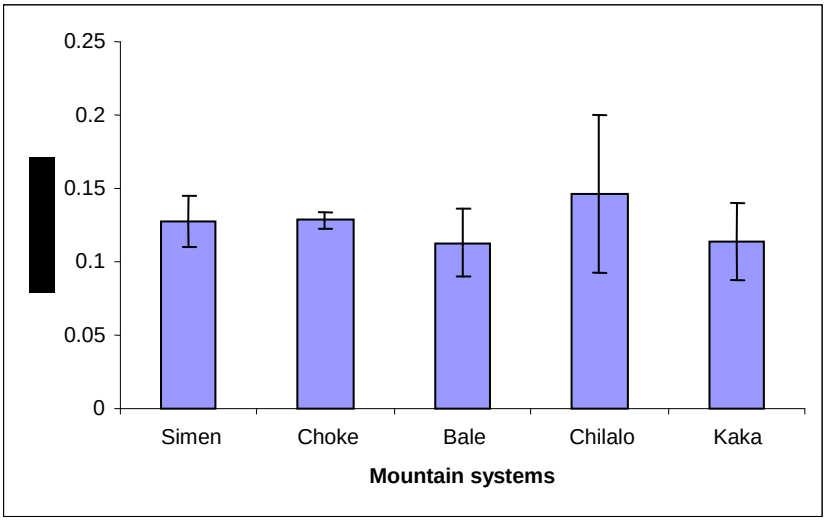
4.2 Genetic diversity

The genetic diversity was about equally distributed between the mountains and the taxa (Table 3; Fig. 8a & b). On average, populations which were furthest apart geographically differed somewhat in diversity. From all the mountains, diversity was relatively higher in Chilalo Mountain (Fig. 8a) and there seems the diversity was not distributed according to any particular pattern.

Table 3. Genetic diversity within the populations of *S. abyssinica, S. lugardae* and *S. pumila* based on AFLP markers

Species	accessions	No. of Individuals	Proportion of variable markers	Gene diversity
S. abyssinica	Bale_003	5	0.259541985	0.128244275
S. lugardae	Bale_005	4	0.236641221	0.127226463
S. lugardae	Bale_008	6	0.290076336	0.124681934
S. pumila	Bale_012	6	0.179389313	0.077862595
S. pumila	Bale_016	3	0.133587786	0.089058524
S. pumila	Bale_019	2	0.129770992	0.129770992
S. abyssinica	Chilalo_052	3	0.129770992	0.086513995
S. abyssinica	Chilalo_053	2	0.198473282	**0.198473282**
S. abyssinica	Chilalo_054	3	0.27480916	**0.183206107**
S. lugardae	Chilalo_309	3	0.175572519	0.117048346
S. lugardae	Choke_255	2	0.122137405	0.122137405
S. lugardae	Choke_256	4	0.244274809	0.132951654
S. lugardae	Choke_258	8	0.377862595	0.130179935
S. lugardae	Kaka_031	2	0.122137405	0.122137405
S. lugardae	Kaka_033	3	0.118320611	0.078880407
S. lugardae	Kaka_035	3	0.167938931	0.111959288
S. abyssinica	Kaka_043	4	0.259541985	0.141221374
S. lugardae	Siemen_226	2	0.103053435	0.103053435
S. lugardae	Siemen_227	2	0.15648855	0.15648855
S. lugardae	Siemen_231	8	0.389312977	0.127998909
S. lugardae	Siemen_232	4	0.270992366	0.148854962
S. lugardae	Siemen_234	4	0.217557252	0.115776081
S. abyssinica	Siemen_235	5	0.251908397	0.122900763
S. abyssinica	Siemen_239	4	0.236641221	0.125954198
S. abyssinica	Siemen_244	3	0.175572519	0.117048346

On average intra-population diversity was the same in the two taxa, *S. abyssinica* and *S. lugardae*. It was somewhat lower in the three populations of *S. pumila* (Fig. 8 B). Taking all the three taxa together, diversity was also about the same in all mountain systems (Fig. 8A). The highest values were obtained for two populations, AFR_053 and AFR_054 from Chilalo (Table 3).



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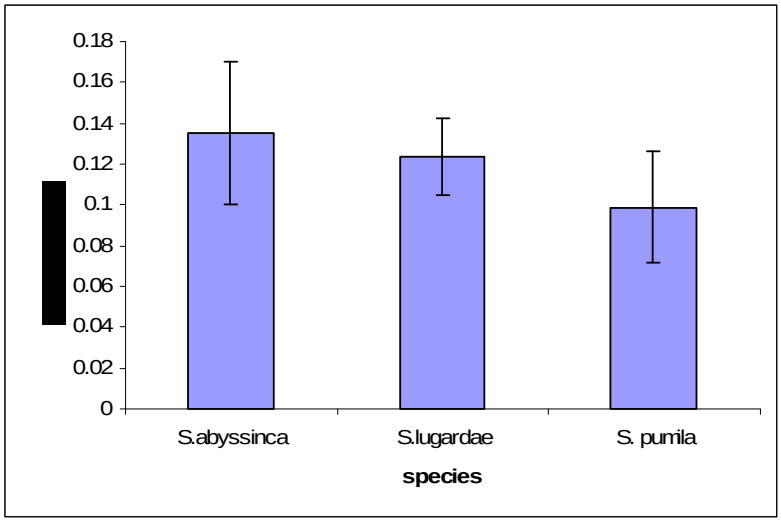

B

Figure 8. A- Average diversities per mountain system, B- Average diversities per species

4.2 Between and within mountain relationships

The PCoA showed that populations were primarily grouped according to their geographic origin. Figure 9 shows a two dimensional plot of the AFLP data set, the axis represents 14.75 % of the variation and illustrates the separation of Simen and Choke from the rest. The second principal coordinate accounts for 7.99 % of the variation. It mostly separates one part of the populations of Bale from the rest of the Bale populations. The populations of Simen Mountains were clearly separated from the other mountains while the populations from Choke Mountains were found between Simen and the remaining mountains. Populations from Kaka, Chilalo, and Bale mountains were not clearly separated, they showed overlap. The PCoA also shows that part of the populations of Bale mountains were clearly separated from the rest. These individuals corresponded to *Swertia pumila*.

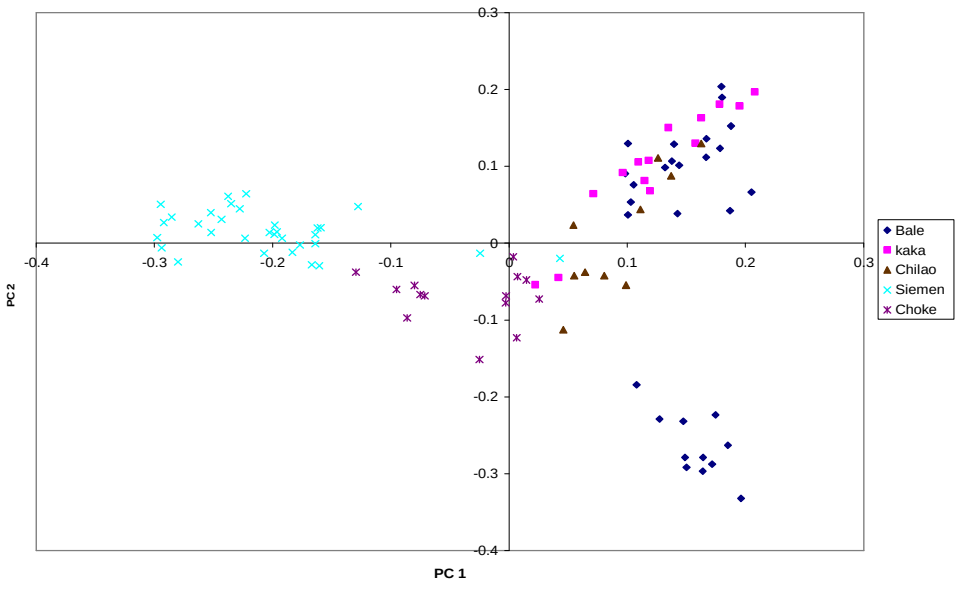

Figure 9. Principal Coordinate Analysis (PCoA) based on similarity of 25 populations of *Swertia abyssinica* species complex

To see the genetic differentiation between the species, the PCoA was re-colored according to the hypothetical species (Fig. 10). Two of the species, *S. abyssinica* and *S. lugardae* showed overlapping while *S. pumila* was distant and clearly differentiated. Within the two first species, individuals were grouped according to the mountain system they were sampled in.

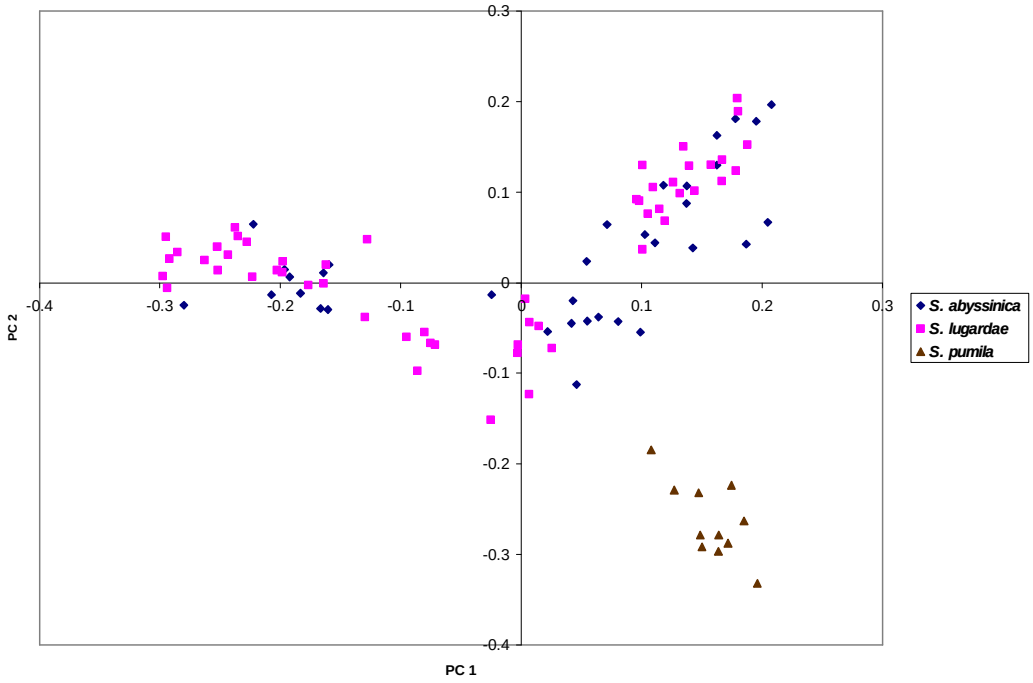

Figure 10. Principal Coordinate Analysis, recolored based on the species

4.3 Genetic structure at different hierarchical levels

There was clear genetic differentiation both among mountains and among populations within mountains using a significance test based on 10100 permutations. The AMOVA based on grouping by geographical locations (mountains) showed that there was more variation among mountains (21.40 %) than among populations within mountains (14.50

%), while the within population variation was 64.10 % (Table 4). At all levels, the variations were highly significant (P< 0.0000). The higher variation observed among mountains revealed a clear phylogeographic structure.

Table 4. Analysis of Molecular Variance (AMOVA) based on mountain and species grouping. d.f., degree of freedom; SSD, sum of squared deviations; VC, variance components; %var, percent of variation; *P*, probability and Φ, Phi-statistics

Source of Variation	df	SSD	VC	%Var	*P*	Φ
Grouping by Mountains						
Among mountains	4	514.808	5.372	21.40	0.0000	Φct=0.213
Among population Within mountains	20	589.055	3.642	14.50	0.0000	Φsc=0.184
Within population	70	1126.600	16.094	64.10	0.0000	Φst=0.359
Grouping by Species						
Among species	2	214.253	2.484	9.89	0.0021	Φct=0.098
Among population Within species	22	889.610	6.546	26.06	0.0000	Φsc=0.289
Within population	70	1126.600	16.094	64.05	0.0000	Φst=0.359

The AMOVA result based on grouping by species showed 9.89% of variation among species (Table 4). This grouping showed considerably less genetic differentiation than the geographical grouping and was significant at P< 0.0021. It also showed high variation among populations within species and within populations with 26.06% and 64.05% of variation, respectively. The differentiations among populations within species and within population were highly significant (P<0.0000).

Because of the distinct position of *S. pumila* on the PCoA, AMOVA was also carried out excluding *S. pumila.* This analysis revealed 24.60 % variation among groups and 7.03 % among populations within groups showing high reduction in variation within groups (Table 5). This indicated that the majority of the variation was contributed by this species, *S. pumila*. The within population variation accounts for 68.38 % of the total variations. There were high significance (P< 0.0000) variations at all levels.

Table 5. Hierarchical analysis of molecular variance based on AFLP data with two different groupings i.e. mountain and taxa with exclusion of *Swertia pumila*

Source of Variation	df	SSD	VC	%var	P	Φ
Grouping by Mountains						
Among groups	4	478.865	6.006	24.60	0.00000	Φct=0.245
Among population	17	390.404	1.716	7.03	0.00000	Φsc=0.093
Within groups						
Within population	62	1035.267	16.697	68.38	0.00000	Φst=0.316
Grouping by taxa						
Among taxa	1	46.018	0.075	0.33	0.319	Φct=0.003
Among population	19	752.728	6.274	27.19	0.00000	Φsc=0.272
Within taxa						
Within populations	57	953.600	16.729	72.49	0.00000	Φst=0.275

AMOVA by species, excluding *S. pumila* (Table 5), showed very small differences among taxa (0.33 %; P< 0.319) while the variations among populations within taxa were (27.19 %) with significant differentiations (p< 0.0000). The within population variations accounted for the 72.49 % of the total variations and was significant at p< 0.0000. The non significant differentiation among taxa and the very small variations indicated the existence of low genetic differentiations between the two species, *S .abyssinica* and *S. lugardae*, may suggest their being one. The other possible reason might be if the markers and the primers used are unable to detect their differences.

4.4 Genetic differentiation along altitudinal gradient

Due to the morphological differentiation along altitudinal gradients, AMOVA was performed based on three different altitude ranges (Lower 3000 - 3250 m; intermediate 3250 - 3500 m; and upper 3500 – 4100 m) basing on Hedberg (19 51) classification of the vegetation of tropical East Africa. Bale and Kaka were overlapping on the PCoA plot (Fig. 7), and differentiation among these two mountains was not significant. Also differentiation with Chilalo was not significant either thus they were considered as one system in the AMOVA. The Simen was considered alone while Choke was not included in the analysis since there are too few populations.

Table 6. AMOVA based on altitudinal differences (Lower 3000 - 3250 m; intermediate 3250 – 3500 m; and upper 3500 – 4100 m) between the combined populations from Bale, Kaka and Chilalo (upper AMOVA) and between populations of Simen (lower AMOVA)

Source of Variation	df	SSD	VC	%var	P	Φ
Bale, Kaka and Chilalo						
Among altitudes	2	121.016	1.333	5.91	0.072	Φct=0.059
Among populations within altitude	11	384.686	5.830	25.84	0.0000	Φsc=0.274
Within populations	35	539.033	15.400	68.25	0.0000	Φst=0.317
Simen Mountains						
Among altitudes	2	44.635	0.078	0.44	0.355	Φct=0.004
Among populations within altitudes	5	100.142	0.977	5.52	0.036	Φsc=0.055
Within populations	24	399.942	16.664	94.04	0.003	Φst=0.059

The analysis revealed small variation among altitudes (5.91 %; P< 0.072). The variation among populations within altitudes explained 25.84 % (P< 0.0000) of the total variation, and the largest amount of variation (68.25 %; P< 0.0000) was within populations (Table 6). Altitudinal AMOVA for Simen (Table 6), showed 0.44 % (P< 0.355) of variation among altitudes, 5.52 % and 94.04 % of the total variations among populations within altitudes and within populations, and significant at p< 0.036 and 0.003, respectively. The variations among altitudes were not significant for both of the analyses and among populations within altitude was not significant for Simen.

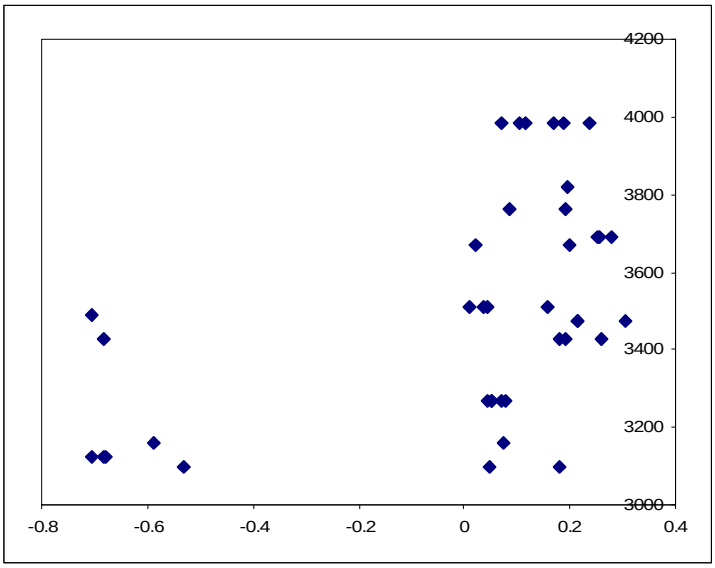

Figure 11. A principal coordinate analysis based on altitude differences for the Bale mountains, Mount Kaka and Chilalo

The PCoA has demonstrated very weak correlation of the observed morphological variations with the altitude (Fig. 11 and 12). As mentioned earlier Bale Mountains, Mount Kaka and Chilalo (Fig. 11) were considered as one mountain system. Simen was considered as the other mountain system in the analysis (Fig. 12). The results

demonstrated that there is no genetic base for the morphological differentiations along altitude but we can not entirely exclude the possibility of a genetic base for the cline since the technique used, AFLP, is a random finger printing method.

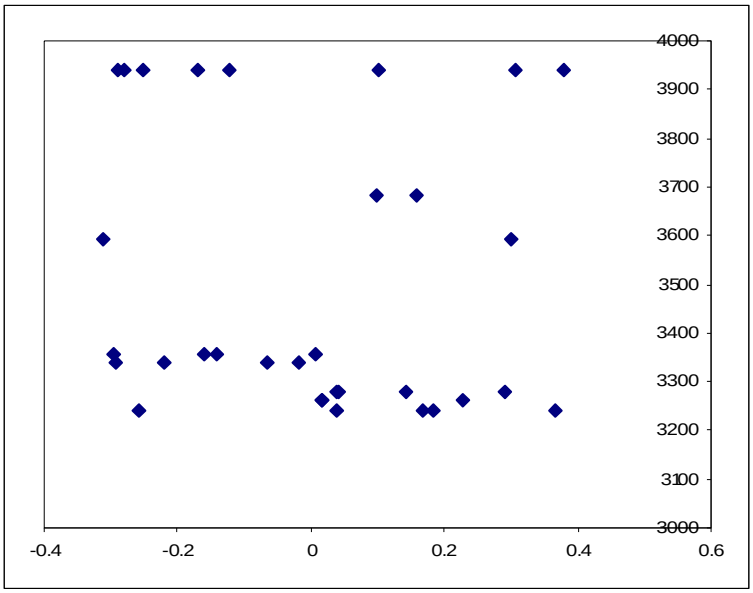

Figure 12. Principal coordinate analysis of individuals from Simen mountains in relation to altitude

A Neighbor Joining tree among all individuals of the three species has been estimated using the Nei and Li distance measure implemented in TREECON (Fig. 13). In the tree also geographical genetic differentiation was observed, the species were forming clusters according to their geographical locations and also showed a dispersed pattern of distribution. The cluster formed by part of Bale, choke and Kaka populations have a higher bootstrap support. While the geographic cluster formed by Simen was supported less by a bootstrap value.

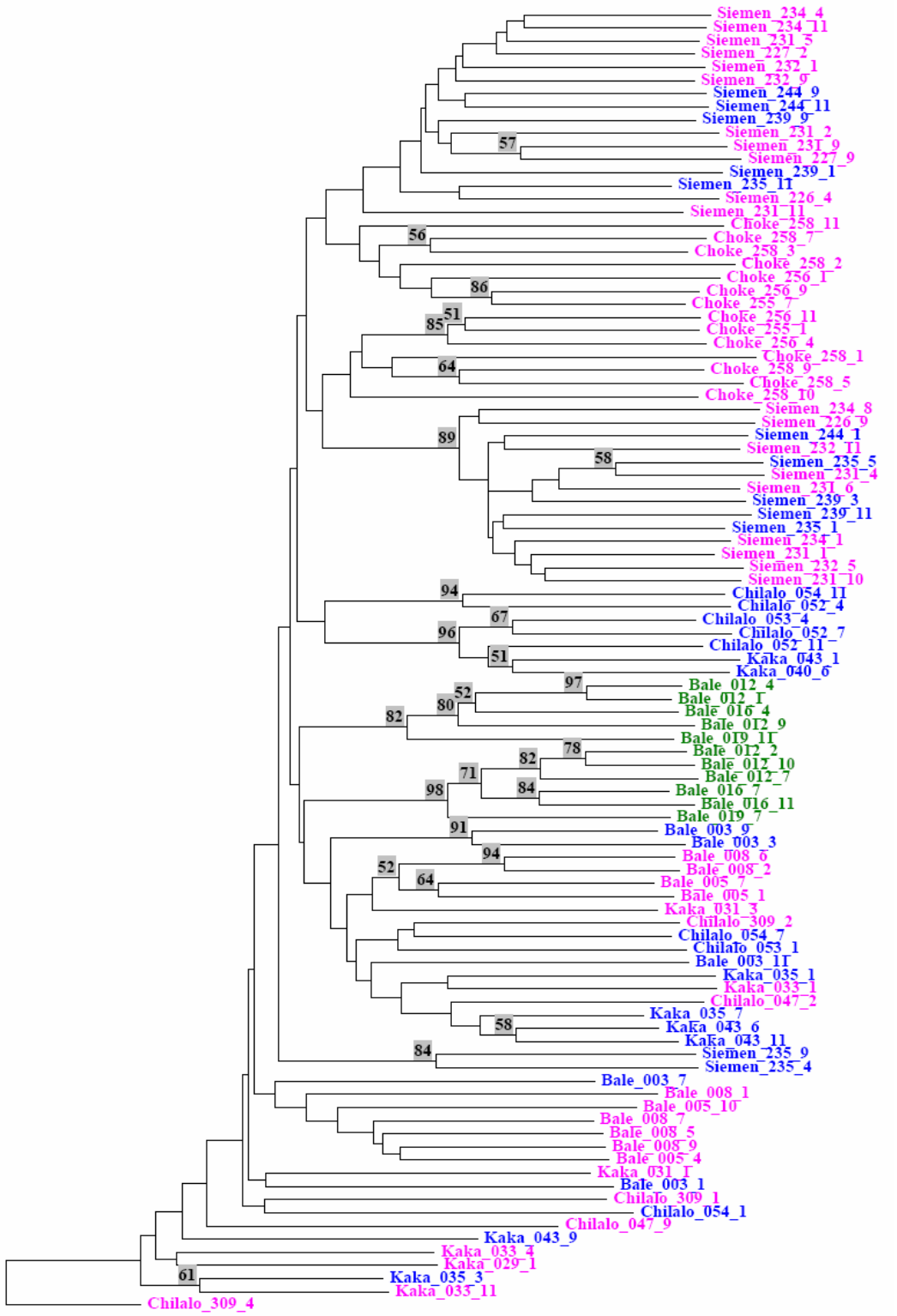

Figure 13. Neighbor joining tree based on Nei and Li distance. Only bootstrap values >50% are shown (1000 replicates). Numbers after location refers to populations (see fig. 5; table 1).The green color indicates *S. pumila* while blue and fuchsia is *S. abyssinica* and *S. lugardae*, respectively

In the neighbor joining tree of the population of *S. abyssinica,* only part of the populations have showed grouping according to their geographical location while others were dispersed (Fig. 14). All the clusters formed have a high bootstrap support. The same grouping patterns have also been observed between the populations of *S. lugardae* (Fig. 15). Those populations which are geographically closer have a closer grouping. Part of the clusters formed by Bale, Kaka, Chilalo, Siemen and Choke has high bootstrap value. In both trees it seems like that the populations of *S. abyssinica* and *S. lugardae* are more similar within their mountain systems.

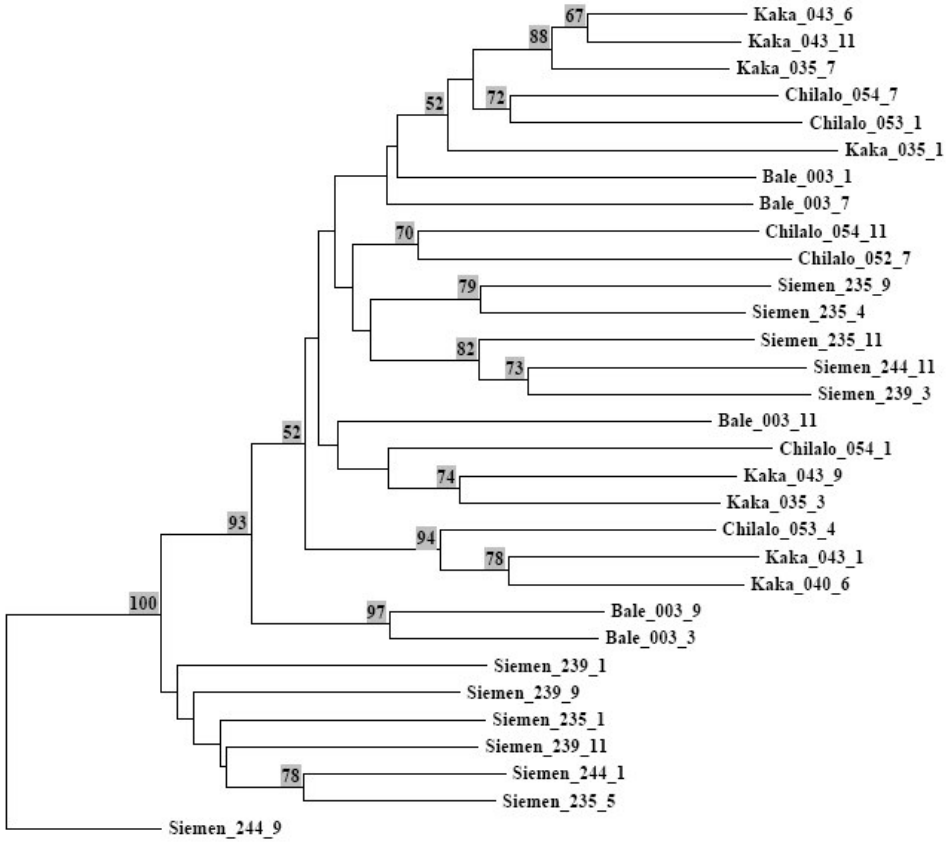

Figure 14. Neighbor joining tree based on Nei and Li distance estimation for the populations of *S. abyssinica* from the four different mountain systems. Only bootstrap value > 50 % are shown (1000 replicates)

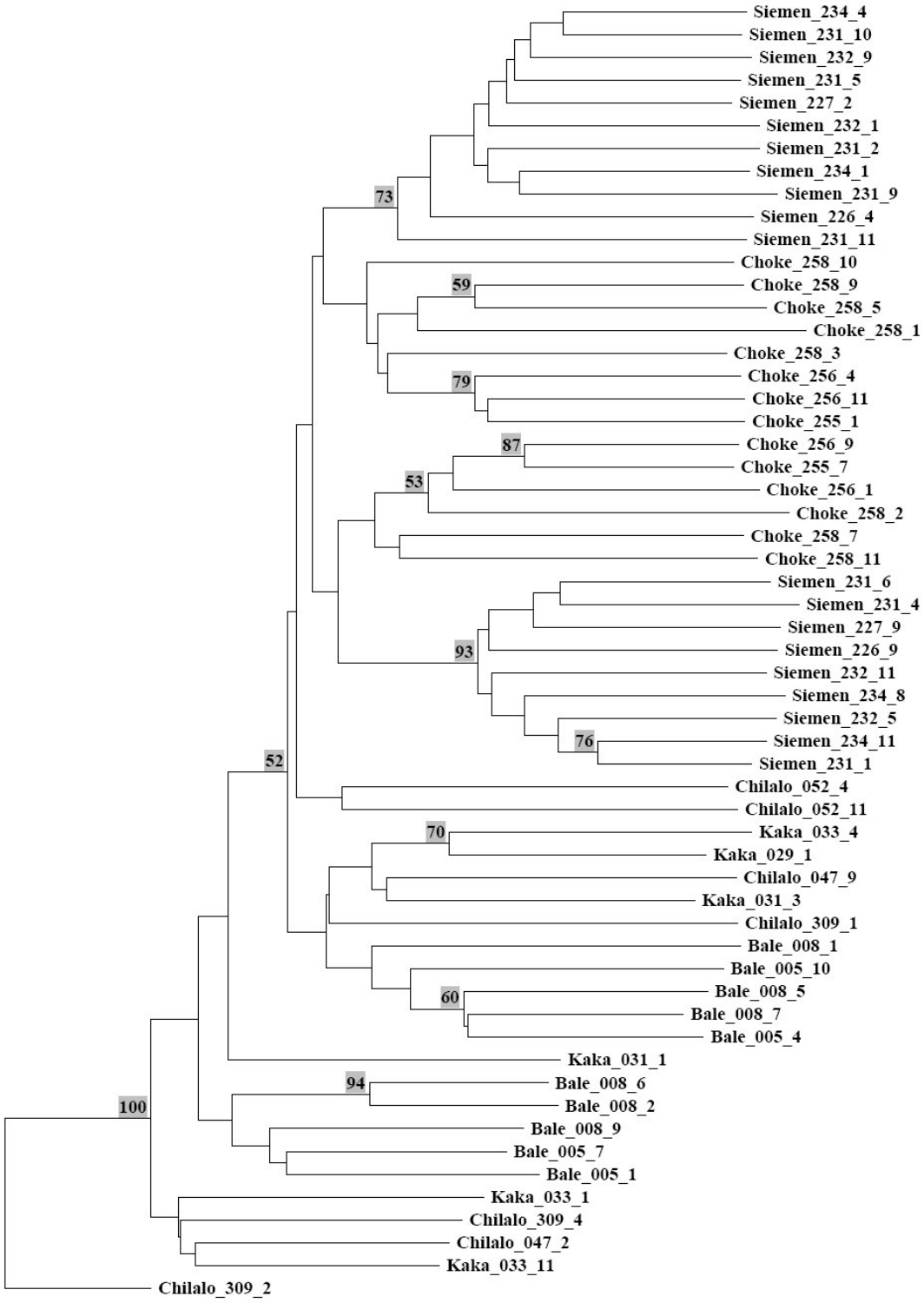

Figure 15. Neighbor joining tree for the populations of *S. lugardae* from the five different afroalpine mountain systems based on Nei and Li (1979) distance estimation. Bootstrap value >50 % are shown (1000 replicates)

5. DISCUSSIONS

The presence of low genetic differentiation between the populations of *S. abyssinica* and *S. lugardae* does not support the general acceptance of *S. abyssinica,* and *S. lugardae* as two distinct taxa. The PCoA analysis has shown that samples of *S. abyssinica* and *S. lugardae* have grouped according to their mountains of origin while *S. pumila* was clearly distinct from them. Furthermore, in the PCoA, the species *S. abyssinica* and *S. lugardae* did group together and clearly overlapped. The overlapping had showed a pattern in which axis 2 (Fig. 10) separates two forms of *S. lugardae*, mainly *S. lugardae* in its left side and mixed with *S. abyssinica* on the other side. Although the genetic differentiation found between *S. abyssinica* and *S. lugardae* is low, it can not entirely be concluded as their being one rather the differentiation might put *S. abyssinica* at a subspecies level. The position of the samples of *S. pumila* on the PCoA plot, being far and distinct from the two other species, does not support it to be included as same species with *S. lugardae* and *S. abyssinica*. Furthermore, the distance based tree indicated the grouping of the taxa according to their geographical origin. The cluster formed by *S. pumila* was clear and supported highly by the bootstrap value than the clusters formed by the others.

The grouping of the populations in respect to their mountain of origin can be explained by the high percentage of variation among the mountains, which accounted for 21.40 % of the total variation. This further showed that there may be less genetic differentiation among species within their mountains of origin than between the mountain regions. A lower amount of genetic variation was explained by the within mountain/ among

populations of the same mountain origin, and there was high within population variation, which represented 64.10 % of the total variation.

Although a relatively low proportion of the total variation was observed among populations within groups (14.50 %), the populations were significantly different from each other. Particularly, *S. pumila*, which had a distinct position on the PCoA plot, was highly differentiated from the two other taxa. The AMOVA analysis, excluding *S. pumila* resulted in a considerable decrease of variations among populations within groups, 7.03% (Table 5). This suggests that the higher variation observed between the taxa was largely contributed by this species and it indicated low level of differentiation of the two other taxa, *S. abyssinica* and *S. lugardae*. This result as well as the PCoA, do not support the inclusion of this species into a larger species together with the other two. The variations found indicated that *S. pumila* might be a different species.

The pattern of variation observed, grouping of the species according to their mountain origin rather than their taxonomical classifications as in the *S. abyssinica* and *S. lugardae*, may indicate local adaptation of the taxa to their respective environment. The observable morphological adaptations of the afroalpine flora can easily indicate the occurrence of the species in an extreme habitat, alpine (Hedberg, 1995) since they have very conspicuous morphological adaptations to withstand the climate with “summer everyday and winter every night” (Hedberg, 1957). Furthermore, temporal variation in the physical environment has played a major role in the evolution of tropical alpine plants (Smith and Young, 1987).

The morphological variation in the two taxa, ***S. abyssinica and S. lugardae***, clearly parallels an altitudinal gradient. Brochmann ***et al.*** (1995) in ***Frankenia ericifolia*** have indicated clinal morphological variations parallel with environmental gradients, in their case humidity. Jonas and Geber (1999) have reported clinal variations of morphological traits in ***Clarikia unguiculata*** along environmental gradients. Afroalpine flora exhibits a great deal of broad adaptation to local conditions through very conspicuous morphological adaptations to withstand the physical severity of the environment (Hedberg, 1970).

A similar pattern was also reported in connection to avian life history variation (Badyaev, 1997). The same author has indicated that elevation induced variation in the life history traits has been attributed to changes on climate, duration of breeding seasons , predation and food limitations . Afroalpine environment exhibits an increasing order of climate change with increasing altitude (Hedberg, 1964). This gradual change in climate along altitudinal gradient certainly induces clinal differentiation which might be apparent in ***S. abyssinica*** and ***S. lugardae.***

The morphological differentiations of the two taxa, ***S. abyssinica*** and ***S. lugardae*** might be in response to the altitudinal gradients. Altitudinal clines in morphology are often found in species that occur over steep environmental gradients (Neuffer and Hurka, 1986; Jonas and Gebere, 1999). The observable morphological differences in the two taxa, that is the gradual reduction in plant height and increase of branching with increasing altitude

is likely to be ecoclinal, (Mark, 1965) in *Chionocholoa rigida* and (Clausen *et al*., 1948) in *Achiliea lanulosa* have reported such cases.

In the current study, pair wise differentiation (Table 6) between samples from different altitudes within each mountain system was not significant and also the morphology had weak correlations with the altitude (Fig. 11 & 12). This does not mean that there is no genetic base for the cline because the genes coding for the adaptive traits may not be included in to the AFLPs (which is a random fingerprinting method). It needs to be studied further by including common garden experiment.

5.1 Parallel ecoclinal evolution

The congruence between the AMOVA and PCoA may be demonstrating that the morphological characters used to define *S. abyssinica* and *S. lugardae* are not revealed to uphold taxonomic distinctiveness. The cluster analysis may corroborate this finding that *S. abyssinica* and *S. lugardae* may not be distinct biological species but rather exhibit clinal differentiation. The clinal morphological variation among the populations of the two taxa from the different altitudes of each studied mountains may suggest primary cline (i.e. they evolved due to the gradually changing environmental (temperature, humidity and other factors) conditions) rather than secondary, which are produced when two taxa are isolated for some period of time and form cline when contact between the two taxa is reestablished.

Although the morphologically intermediate populations occur in an intermediate altitudinal zone, the possibility of hybrids is unlikely. The low AFLP differentiation between the taxa reflects clinal variations rather than crossing events between previously differentiated species. The existence of similar ecoclines of the two taxa in the different mountain systems supports the parallel ecoclinal evolution hypothesis. Nielsen *et al.* (2003), studied morphological and molecular variation in populations of *Scalesia divisia* and *S. incise* (Asteracea). The two populations represent introgression between two recently diverged species and also the putative hybrid population reflects crossing events between divergent progenitors. They indicated that the patterns of variations in both morphological and molecular markers reflect introgression between two previously geographically isolated species. Their hypothesis was based on the fact that there is high within population variation and that the morphologically deviating populations had an intermediate position between the two species in all the analyses. However, in our case the patterns of genetic and morphologic variation in *S. abyssinica* and *S. lugardae* may likely reflect adaptation to a local environment and indicate a primary cline than a secondary contact. While the genetic differentiation observed for *S. pumila* indicated it being a different taxon. Nevertheless we can not entirely conclude since we used only one marker with a limited number of primers.

5.2 How many species comprise Swertia abyssinica species complex?

The genetic differentiation between the populations of this species - complex, which comprises of *S. abyssinica*, *S. lugardae* and *S. pumila*, does not give a support to their alpha taxonomy, i.e., the acceptance of *S .abyssinica*, *S. lugardae* and *S. pumila* as three

distinct species. It is to be noted that these three species were recognized as distinct species based purely on morphological traits (Nemomissa, 1994 & 1997). The molecular data have revealed that the populations of *S. abyssinica* and *S. lugardae* have grouped according to their geographical origin regardless of their alpha taxonomy, suggesting a mere ecotypic differentiation rather than taxonomic distinctiveness. However, this is only a suggestion because we have not done detailed morphological analysis in addition to the molecular data on hand. Further more, the grouping pattern also indicated that populations of the same mountains are more closely related to each other than to any one of the populations from the different mountains. In the reciprocal experiment of Chapin and Chapin (1981) in *Carex aquatilis* growth was most successful in the geographic region in which each population had evolved. AMOVA (cf. Table 5) has revealed a low genetic differentiation (0.33 %, $p< 0.000$) between *S. abyssinica* and *S. lugardae*, indicating that these two taxa may have comprised a single biological species with clines locally adapted along an altitudinal gradients of the five mountains systems of Ethiopia. According to our result, the relative high differentiations between and within populations of the aforementioned taxa may be suggesting the presence of gene flow along the altitudinal gradient. Biological species exhibit internal genetic cohesion and homeostasis maintained by gene flow throughout all of the reproductively linked populations of the species (Mayr, 1963). The absence of genetic differentiation between *S. abyssinica* and *S. lugardae* which can regard them as distinct species is the manifestation of internal genetic cohesion of their population. However we make no conclusions since this also varies with the section of the genome studied.

On the high mountains of tropical Africa, which often referred to as “islands in the sky”, morphological clinal variations was reported for a number of afroalpine species e.g. *Dispsacus pinnatifidus* and *Cineraria grandifolia* (Hedberg, 1957). However apart from the decrease in a statue in the latter species, there is no apparent morphological marker which facilitates species distinction unlike in the *S. abyssinica* species complex. It is to be noted that environmental conditions for growth of plants are generally considered to become severe with increasing altitude i.e. wind speed, intensity of solar radiation and UV increase with rising altitude (Taguchi and Wada, 2001). Ecoclinal differentiation along altitudinal gradient was found to be due to environmental factors e.g. humidity (Brochmann *et al*., 1995) and edaphic factors (Gregor, 1946).

The Pleistocene glaciations have considerably resulted in a downward shift of the vegetations of the afroalpine environment (Coeteze, 1964) and those species which are at the lower most part of the montane forest belt must have had a more chance for intermountain dispersal than those e.g. above 3000 m (Hedberg,1970). The retreat of the glaciations has created new open potential ecological niches for low altitude taxa and the latter gradually ascended the mountains through adaptation. Generally, the afroalpine flora is of a recent origin and exhibits insular evolution (Hedberg, 1970). Various biochemical data have revealed a general tendency of oceanic island plant genera to have a low between species differentiation in comparison to their continental relatives due to recent speciation (Crawford *et al*., 1987).

The morphological deviating populations in the PCoA (Fig. 9) have a distinct position and belong to the *S. pumila.* A clear separation of populations of *S. pumila* from those of *S. abyssinica* and *S. lugardae* coupled with high contribution of variance component between taxa perhaps reflects its being distinct taxon. Although our data suggests the distinctiveness of *S. pumila*, we can not completely exclude the possibility of secondary cline which could result from hybridization. In fact, establishing of the origin of *S. pumila* is beyond the scope of this study which needs further detailed investigations such as detailed Microsatellite DNA markers and experimental approaches.

6. CONCLUSIONS

The pattern of morphological variation found among populations of *S. abyssinica* and *S. lugardae* may be due to ecological gradients. *S. pumila*, because of its higher genetic differentiations, could be classified as distinct taxon. In view of the altitudinal differences between the selected populations of *S. abyssinica* and *S. lugardae* on each mountain system and the rather gradual reduction in plant height and increase of branching with increasing altitude, it may appear that the differentiation in height and branching habit is ecoclinal rather than secondary contact. According to our data, it might be reasonable to classify *S. abyssinica* and *S. lugardae* as belonging to one species because of the low genetic differentiation between the putative species and the grouping of the populations in respect to their mountains of origin.

The AFLP technique has been applied for the differentiation of the *Swertia abyssinica* species complex, which enabled analysis of the components of this complex and provided more information than the previous study. In the current study, although the results demonstrated that there is no genetic base for the cline in the morphology of the two taxa, *S. abyssinica* and *S. lugardae*, we can not entirely conclude so, since we used a random finger printing method. Therefore, it needs further investigations employing other parameters, including detailed morphological analysis which may need cultivation under the same environmental conditions, which will allow distinguishing phenotypic plasticity from genetically determined ecoclinal variation.

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