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# Reconstructing South African Afromontane Forest History with Bryophyte Phylogeography



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## Abstract

Forests occur as fragmented patches throughout Africa, however the basis for the disjunction of afro-montane forests remains uncertain. The genetic structure of organisms should reflect their history, and in turn the history of their environment. Thus a phylogeographical study of forest-faithful mosses could provide insight into the fragmentation of forests. In this study, patterns of genetic variation in four forest-faithful mosses (*Leptodon smithii*, *Pyrrhobryum spiniforme*, *Aerobryopsis capensis* and *Neckera valentiniana*) were investigated. Data was analysed using a combination of population genetics (AMOVA, population F statistic, mismatch distribution) and descriptive phylogeographical approaches (haplotype tree, NCA, haplotype frequencies). *L. smithii* was sampled extensively (29 sites, n = 1 - 17), while the other three species were sampled less rigorously and only provided further insight into the patterns already acquired. Sequence data was obtained from ITS1 (nuclear) and *trnL-F* (chloroplast) for all species except *N. valentiniana* (ITS1 only). The genetic variation in *L. smithii* collected from the South African sites showed that there was population structuring (which indicated forest patch separation), but also evidence for previously widespread forests and dispersal between forest patches. Forests at Fernkloof and the combined Jonkershoek and Nursery Ravine Forests have been separated from the other sampled forests in South Africa for a long time. A range expansion inferred from the mismatch distribution corresponds to between 18 000 and 45 000 years ago. Recombination in ITS1 showed that dispersal through spores has occurred. Support for dispersal for this species also came from the Kenyan, Madeiran and Australian samples. Although the sample size was minimal, the genetic variation observed in *P. spiniforme* and *A. capensis* corroborated the geographical patterns obtained from *L. smithii*. *A. capensis* had high levels of variation within a small area in the southern Cape whilst most of the variation in *P. spiniforme* came from length variation within ITS1. *N. valentiniana* did not have any variation at the sampled locus, which suggested recent dispersal following a genetic bottleneck. The differences in genetic variation among the species studied highlighted the importance of comparative studies. As different organisms have different histories, future studies would need to include a range of diverse organisms, in order to reconstruct forest history accurately. These results show that forests had a complex history, with many processes involved. Although this study described hypotheses of dispersal, population structuring and previously widespread forests, techniques have recently been developed to also test them. Future studies would need to first define *a priori* hypotheses, which could then be explicitly tested using statistical phylogeographic approaches.

Cover: Near the top of Skeleton Gorge, Table Mountain, South Africa (photo credit: Clare Vander Willigen)

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# Chapter 1

## Introduction

### 1.1 Afromontane forests – a fragmented phytochorion

White (1983) defined a number of phytochoria for the African continent, based on patterns of richness in endemic floras at the species level. He further defined a centre of endemism as a phytochorion that has more than 50% of its species confined to it, and greater than 1000 endemic species.

One of the phytochoria defined by White (1983) is the afromontane archipelago-like centre of endemism; unlike the other phytochoria, which are restricted to well-defined geographical areas, this has a disjunct distribution. Islands of afromontane vegetation occur from Sierra Leone in the West, to Somalia in the East, Sudan in the North, and the Cape Peninsula in the South. A 'sea' of lowland vegetation surrounds the islands (Figure 1). Afromontane forests are thus found as small, fragmented patches throughout Africa. Afromontane forest is only one part of the Afromontane phytochorion, as it includes other vegetation types such as grassland and bushland. Forests were defined as "a continuous stand of trees, with a canopy between 10-50 m high. The vegetation usually consists of several layers, the tree crowns overlap and trees dominate" (White 1983). Forests are not restricted to the Afromontane phytochorion, but are also part of other regions such as the Zanzibar-Inhambane and Tongoland- Pondoland regional mosaic, which were not studied.

### 1.2 History of forests in Africa

In southern Africa forest patches cover less than 5000 km<sup>2</sup> (0.25% of land area) (Midgley *et al.* 1997). Some patches are less than 1 km<sup>2</sup>, while bigger areas are found in the Southern Cape and the Lowveld escarpment (Lubke and McKenzie 1996). There are three reasons given as to why forests are fragmented in the landscape today (these reasons may apply only to specific areas): 1) existing patches are relicts of widespread forests (Acocks 1953, Levyns 1964); 2) the patches represent recent invasion of forests (Meadows and Linder 1989, 1993, Cowling 1983); or 3) forests have always been patchy (Midgley *et al.* 1997). The origin of disjunctions in the afromontane flora thus remains controversial (White 1981).



Figure 1: Distribution of the afro-montane phytochorion (highlighted in black), taken from Meadows and Linder (1993).

Forests are generally found in areas where soil moisture is not limited for an extended period of time (Low and Rebelo 1996, Cowling 1983). Therefore in South Africa forests are generally found in protected ravines. The Cape Fold Mountains provide many disjunct habitats that are fire-free and sufficiently moist for the development of forest vegetation (Geldenhuys 1989, Lubke and McKenzie 1996).

Climatic modelling of forest in Natal shows that afro-montane forests are found in areas that have large differences in summer and winter rainfall and lower temperatures (than Tongolond-Pondoland forests) (Eeley 1999). Afro-montane forests are usually found at high altitudes, but in the Cape they are found a few hundred meters above sea level, due to the influence of latitude (White 1983).

As forests are limited by climate, the past climate affected their distribution. During the Pleistocene the climate fluctuated between glacial/interglacial periods. While widespread glaciation never occurred in Africa, the effects of the glacial cycles were felt. The climate fluctuated between wetter and drier and warmer and colder, however the precise pattern of change is unknown. Both a biogeographic study of Samango monkeys and palynological studies suggest that before the last glacial maximum (LGM) forests were widespread (Lawes 1990, Scott 1997).

The LGM (18 000 years before present) had different effects in different areas (White 1983, Meadows 2001). It was characterized by aridity and cool temperatures, which were not always coincidental (Geldenhuys 1989; Meadows and Linder 1989, 1993, Scott 1997). Forests were confined to refugia, such as those of the Eastern Cape, coastal scarp of Natal and eastern Transvaal (Lawes 1990). In the Cape and Kalahari it was wetter than it is now. Forests were widespread in the Lake Malawi catchment area, and were present at Vankervelsvlei in the Southern Cape during the LGM (Irving 1998, DeBusk 1998).

Prior to 12000 years BP tropical Africa is thought to have experienced an arid phase when forests were restricted to small refugia (Hamilton and Taylor 1991), followed by a major change to a wet climate, with subsequent increase in forests (Hamilton 1981). In southern

Africa the warming after the LGM was accompanied by an increase in precipitation (Partridge *et al.* 1990).

Throughout southern Africa there is evidence for forests fluctuating in a mosaic with other vegetation types, with fynbos around Knysna and the Cape Town area, and with grasslands at higher altitudes (Coetzee *et al.* 1983, Irving 1998, Deacon 1983, Meadows and Linder 1993). Forest patches found in mountain refugia in arid areas such as Graaff-Reinet and the Swartberg might indicate that forests must have been more widespread, and with the onset of drier weather the forests were restricted to moister mountain areas (Levyns 1964). Alternatively, these forest patches could also be an indicator of long distance dispersal.

Fluctuations in climate still occurred during the Holocene. Pollen analysis of afro-montane plateaus in Malawi, Zimbabwe and South Africa suggest that forests only appear in these areas after the Holocene. Pollen cores dating back to 12000 BP, and clay deposits from the same interval, indicate that forests covered a small area even then, and grasslands were much more dominant. Therefore grasslands are not relictual (Meadows and Linder 1989, 1993). However pollen samples from Lake Malawi have low levels of montane forest pollen throughout the Holocene, and higher levels throughout the LGM, which suggests that forests were continuous during the last glacial maximum, but were fragmented during the Holocene (DeBusk 1998).

Evidence that forests were once connected comes from similar species compositions and low levels of endemism. The Southern Cape forests are relatively similar to the Amatole, Transkei and northeastern forests, but are currently separated by broken mountain ranges, dry open valleys and extensive lowlands. The Sundays and Fish River valleys, in the Eastern Cape, are seen to be gaps, as they are large valleys with xerophilous vegetation (Geldenhuys 1989, Lawes 1990). Forests in Malawi are now disjunct, but their species composition is similar (DeBusk 1998).

### 1.3 Reconstructing forest history from bryophyte phylogeography

Reconstruction of palaeoenvironments and of species distributional history is often attempted through analysis of fossil or sub-fossil data like sediment cores containing fossil pollen.

However in Africa reconstruction of vegetation history is rendered difficult by a shortage of

quality sites, due to the lack of favourable preservation conditions. What information is available is geographically limited. We therefore know very little about the past and there is little evidence regarding where forests survived during glacial times (Hamilton and Taylor 1991, Eeley *et al.* 1999, Meadows 2001).

Phylogeography, pioneered by Avise *et al.* (1987), is defined as "a field of study concerned with the principles and processes governing the geographic distribution of genealogical lineages, especially those within and among closely related species" (Avise 2000). With phylogeographic data it is possible to test hypotheses that cannot be addressed by traditional paleoecological approaches, e.g. a disjunct population could have originated from either long distance dispersal or via migration from a cryptic refugium. It is therefore useful for studying patterns of distribution change when the fossil record is inadequate (Cruzan and Templeton 2000).

Ongoing population-level processes (e.g. gene flow, selection) and historical events (e.g. climate-induced fragmentation) produce the present day genetic structure of organisms. For a given level of genetic exchange, populations having the most recent common ancestry will be genetically more similar than those having a more distant common ancestry. If there is no genetic exchange, then shared common ancestry will be the only determinant of genetic similarity (Schaal *et al.* 1998, Posada *et al.* 2000). If it is possible to discriminate between ongoing and historical processes, then it should be possible to test hypotheses concerning species history (Templeton 1995, Avise *et al.* 1987).

Previous methods that determined genetic structure among and within populations, (e.g. F-statistics) did not include the historical information that is available from genetic data, whereas modern methods that incorporate gene genealogies, such as nested clade analysis and maximum likelihood analyses of gene flow, do. Coalescent theory embodies the formal mathematical and statistical treatment of gene genealogies within and among closely related species. It is used to model relationships among random samples of genes (Posada and Crandall 2001). Looking back in time, all haplotypes eventually coalesce to a common ancestor because the extant alleles of a gene are all derived from a single common ancestral allele that existed at some point in the past (Avise 2000, Schaal and Olsen 2000). From coalescent theory, a direct relationship is expected between age, frequency and distribution of haplotypes; older alleles should be more frequent and widespread (Posada and Crandall 2001).

Interspecific trees show hierarchical relationships between species, and are usually strictly dichotomous, since species are products of reproductive isolation. Intraspecific trees, however, are not necessarily hierarchical, as they are the result of reproduction. Furthermore, ancestral haplotypes are generally present in high numbers (Crandall and Templeton 1996). Many processes that are generally ignored in interspecific trees affect intraspecific trees, such as effective population size, allele frequency arrays, and patterns of gene flow. Population genetics was previously based on the assumption that populations had reached equilibrium between gene flow and random genetic drift, which is unlikely in organisms where recent history is a major determinant of genetic structure. Intraspecific trees can be used to study population processes within a temporal, non-equilibrium framework (Schaal *et al.* 1998).

The phylogeographic pattern of an organism actually shows the history of the particular DNA sequence. Phylogeographic inferences can be strengthened if co-distributed organisms have congruent phylogeographic patterns. This is because the most likely explanation for congruent patterns is shared environmental history. Comparative phylogeographic studies in Australia, Europe and North America have elucidated how climate change has effected vegetation change and caused historical barriers, and have identified glacial refugia (Arbogast and Kenagy 2001, Hewitt 1999, Soltis *et al.* 1997, Moritz 1996).

In the case of forests, it should thus be possible to reconstruct the historical background by studying the genetic structure of organisms faithful to these habitats. One such group is the Bryophyta, a large number of species of which are confined to forests, at least in their African range. This study uses a forest-faithful moss, *Leptodon smithii* to test the hypotheses discussed above. Although time constraints precluded the possibility of analyzing more than one species in detail, three other mosses were evaluated for their future potential in testing forest history hypotheses. Future studies could aim to include these in a comparative context.

#### 1.4 Potential genetic markers

Genetic markers are simply heritable characters with multiple states at each character (Sunnucks 2000). Which marker to use is decided by what information is required.

This study used direct DNA sequences as the results are comparable across a wide range of taxa and it is possible to determine ancestor-descendant relationships (Sunnucks 2000). Another benefit of DNA sequences is that they are PCR assayable, so only small amounts of DNA are needed.

In animals mitochondrial DNA (mtDNA) is used extensively in phylogeography, as it mutates rapidly. However in plants mtDNA mutates very slowly – as much as six times slower than nuclear DNA (Ennos *et al.* 1999). mtDNA in plants also undergoes many structural alterations, thus making homology assessment very difficult (Parker *et al.* 1998). The main difficulty with plant phylogeography has been to find DNA sequences with sufficient levels of ordered, neutral variation (Schaal and Olsen 2000). Whilst other markers such as microsatellites and DNA fingerprinting have higher levels of variation, their results are not comparable across a range of taxa and usually are not directly interpretable in terms of genealogy. Chloroplast DNA (cpDNA) mutates approximately seven times slower (Parker *et al.* 1998) than animal mtDNA, but non-coding and single copy regions of cpDNA have increased mutation frequency (Ennos *et al.* 1999, Schaal *et al.* 1998).

Although nuclear DNA generally has higher levels of variation, its usefulness is limited because the dominant life history stage in most organisms is diploid so many individuals will carry more than one haplotype (Schaal *et al.* 1998). In bryophytes this is not problematic as the dominant life history stage is haploid.

Two commonly used nuclear regions are the ITS regions of the nuclear ribosomal RNA cistron (Shaw *et al.* 2002). ITS1 is generally 300-600 bp in length and is frequently more variable than ITS2, which is typically 150 –300 bp in length. The level of variation observed in ITS sequences varies tremendously among bryophyte genera, and is not predictable. For example, in *Weymouthia* no sequence divergence of ITS2 was found at the population level (Quandt *et al.* 2001), while Chiang and Schaal (1999) found extremely high divergence in the ITS2 region of different *Hylocomium splendens* populations from North America.

Nuclear DNA is biparentally inherited, whilst cpDNA is generally uniparentally inherited. Therefore the nuclear genome has a bigger effective population size, which results in increased

coalescent times. As different genomes have different histories, a study is more robust if it includes regions from different genomes (Parker *et al.* 1998). Therefore this study used sequences from the nuclear and chloroplast genomes.

### 1.5 Objectives of the study

This study seeks to test hypotheses of forest history in southern Africa by examining genetic variation in forest-faithful mosses.

In particular the study aims to:

1) Investigate the phylogeographic pattern of a forest-restricted moss, *Leptodon smithii*, that has been shown previously to display genetic variation at the intra-specific level for the chosen markers (Mwafongo 2002), 2) use the phylogeographic pattern to infer the history of the forest patches, 3) use genetic parameters to determine genetic variation among and within populations of *L.smithii* and 4) determine the extent of genetic variation in *Neckera valentiniana*, *Aerobryopsis capensis* and *Pyrrhobryum spiniforme*, to determine their potential for comparative forest phylogeography.

## Chapter 2

### Methods

#### 2.1 Sample species

The study species are all restricted to forest habitats. They are by no means an exhaustive list of forest-faithful mosses, but they are all found throughout South African forests. The bryophytes selected cover a range of breeding systems, life forms and habitat preferences. Thus trends relating to ecological criteria could be investigated with respect to genetic variation.

All accessions used in this study are listed in Appendix one. For *Leptodon smithii* multiple individuals were assayed in five populations in the Western Cape and Limpopo Province; whilst for other areas of South Africa, only a few samples were available. To provide a more global context, samples from Madeira, Kenya and Australia were also included. The other three mosses were sampled on a small scale to test their viability for elucidating forest history. There are thus only a few samples and populations per moss.

#### *Leptodon smithii* (Hedw.) Weber & Mohr

*Leptodon smithii* is found in forests and woodlands, growing on bark and on rocks (Magill and van Rooy 1998). It prefers drier forest habitats, e.g. on tree trunks on the edge of forests, and is mostly absent from the wetter forest types. The moss forms tufts, with a creeping primary stem. The secondary stems are upright, and display regular branching. Both the stems and the branches curl over when dry. The plants are desiccation tolerant. *Leptodon smithii* is dioicous. It is found in South Africa from Limpopo and Gauteng in the north, in a broad band along the coast to the south Western Cape (Figure 2). Globally it has a very widespread distribution. It is found in Africa, Asia, North and South America, Australasia and Europe (Figure 3).

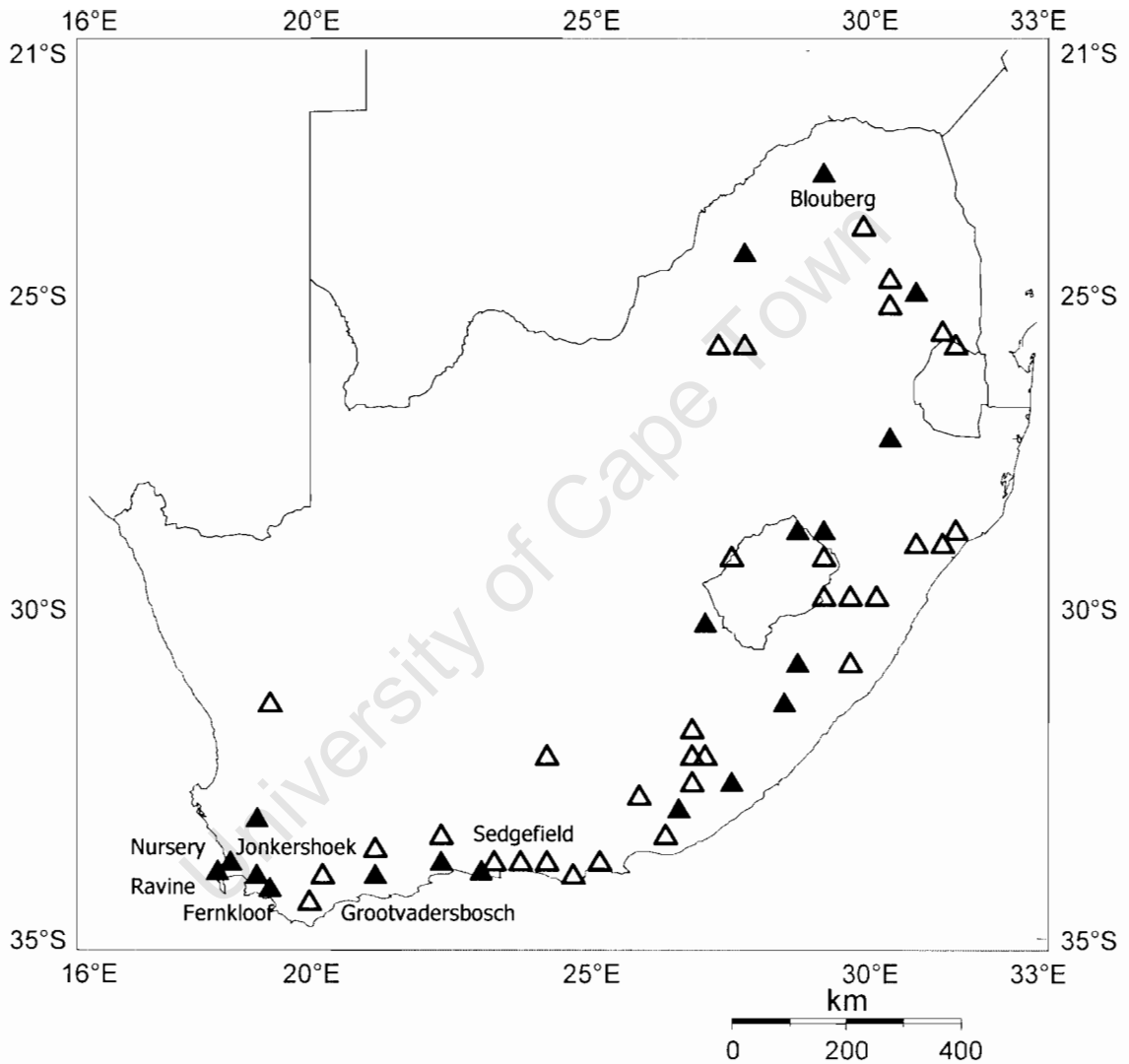


Figure 2: Distribution of *Leptodon smithii* in South Africa (Magill and van Rooy 1998). Solid triangles are sampling localities for this study, and open triangles are other sites where it has been found in South Africa. The labeled localities had multiple individuals sampled from the population.

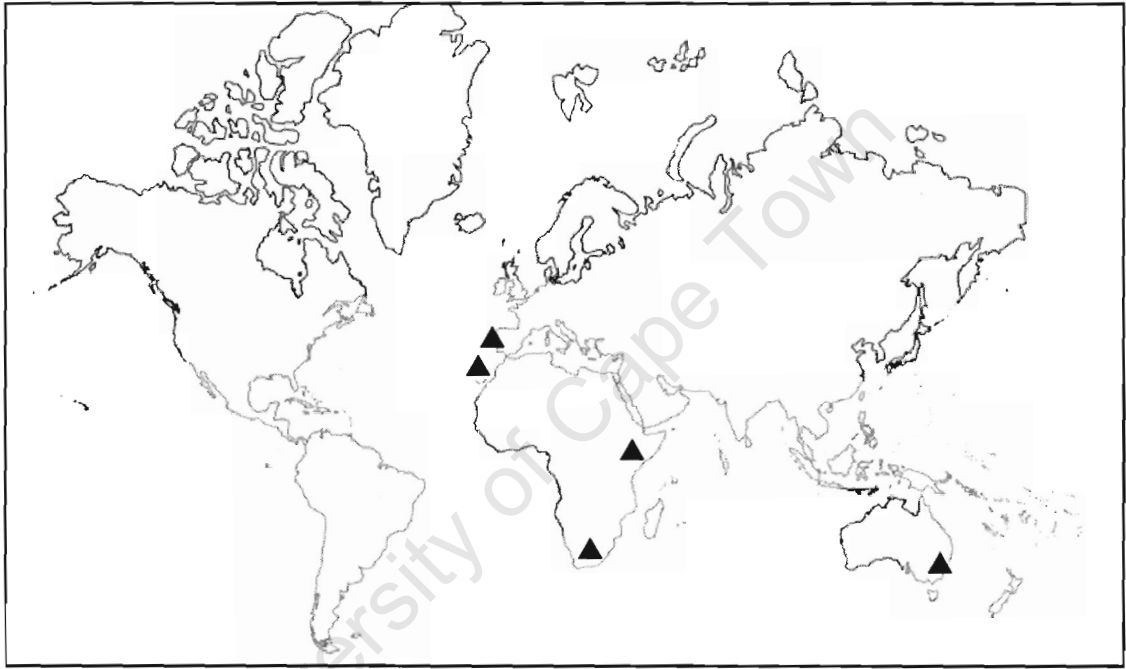


Figure 3: Distribution (shaded grey) of *Leptodon smithii* (from Magill and van Rooy 1998, Scott *et al.* 1976, Sainsbury 1955), with worldwide sampling localities (triangles).

*Neckera valentiniana* (Hedw.)

*N. valentiniana* is found in wet forests, forming large mats on trees and rocks. Its leaves are conspicuously undulate, and the stems and branches are flattened. The plants are autoicous. It is found from Limpopo in the north, down in a broad band along the east coast to the Cape Peninsula (Figure 4) (Magill and van Rooy 1998).

*Aerobryopsis capensis* (C. Müll.) Fleisch

*A. capensis* is a glossy yellow-green plant, with leaves that are widely spreading when wet and dry. It has an irregular branching pattern. Its primary branches grow flattened on the substrate (usually trees), with pendant secondary branches. The plants are autoicous. It is found from Limpopo to Mpumalanga, with a gap in northern KwaZulu-Natal, then a continuous distribution from southern KwaZulu-Natal to the southern Cape and a few isolated patches in the Western Cape (Figure 5) (Magill and van Rooy 1998).

*Pyrrobryum spiniforme* (Hedw.) Mitt

*P. spiniforme* is found in wet forests on rocks, ground or trees (Gradstein *et al.* 2001). Its stem has a lower naked section, and a densely leaved non-branched upper section. Its leaves are long and narrow with a denticulate margin. They are crisped when dry, spreading when wet. The perichaetia are born at the base of the stems, and the plants are classified as acrocarpous. The plants are synoicous. It is found in patches along a narrow coastal band from Limpopo to the Cape Peninsula (Figure 6) (Magill 1987).

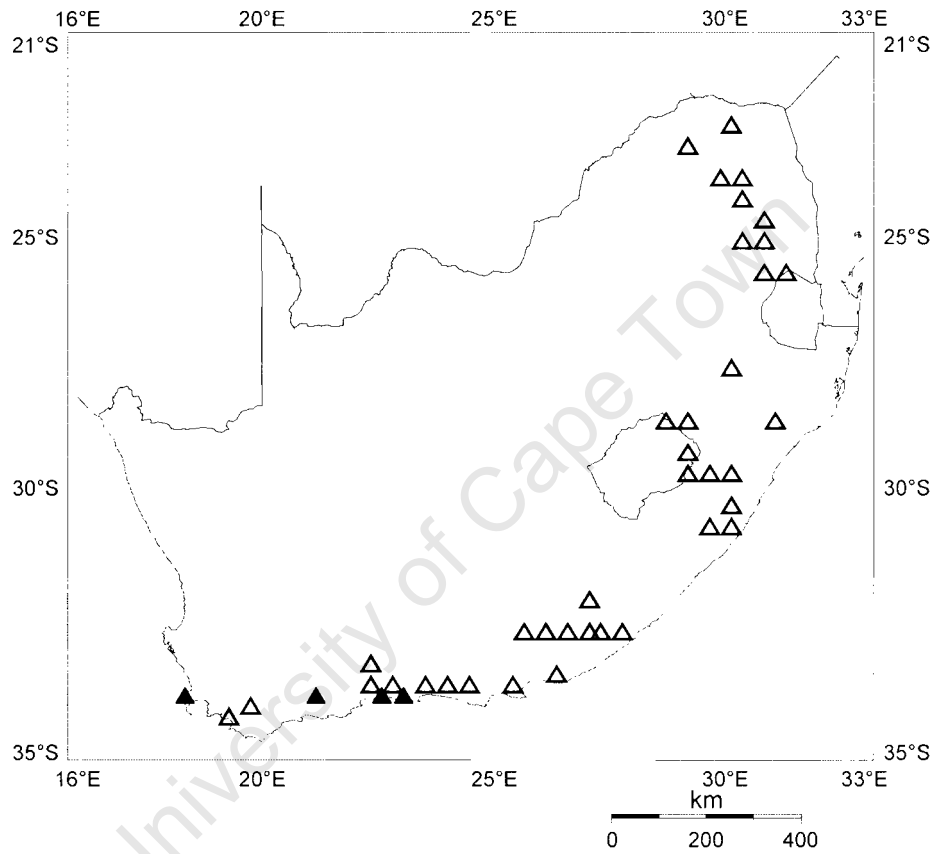


Figure 4: Distribution of *Neckera valentiniana* in South Africa (from Magill and van Rooy 1998). Solid triangles are sampling localities for this study, and open triangles are other sites where it has been found in South Africa.

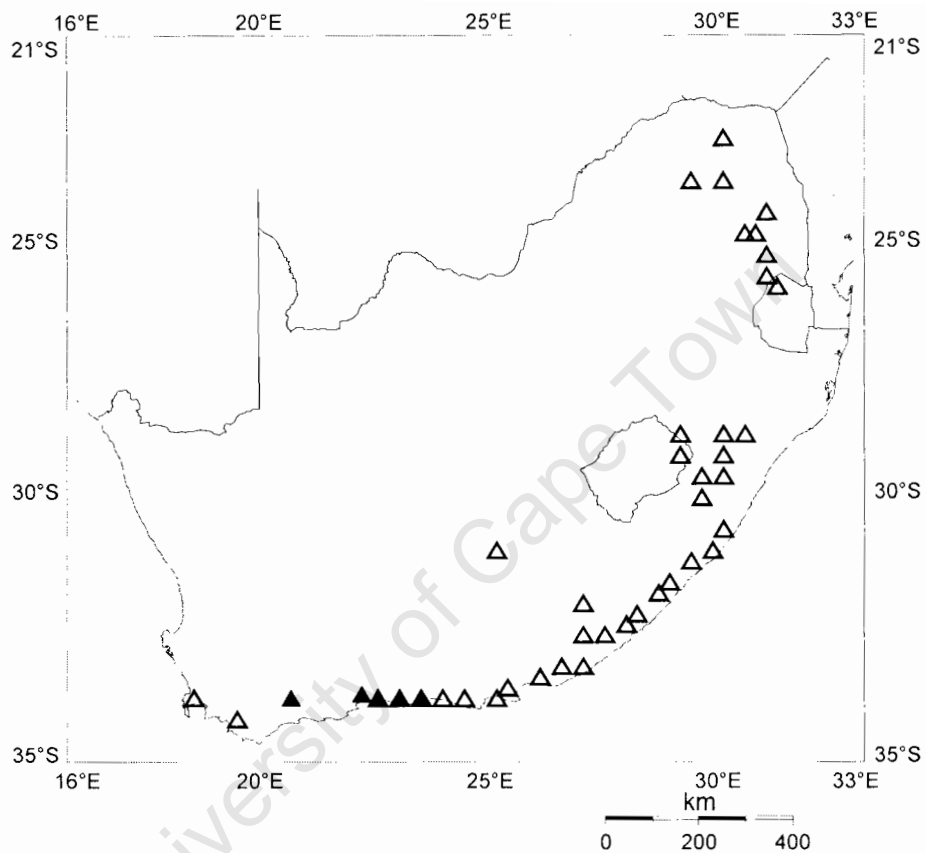


Figure 5: Distribution of *Aerobryopsis capensis* in South Africa (from Magill and van Rooy 1998). Solid triangles are sampling localities for this study, and open triangles are other sites where it has been found in South Africa.

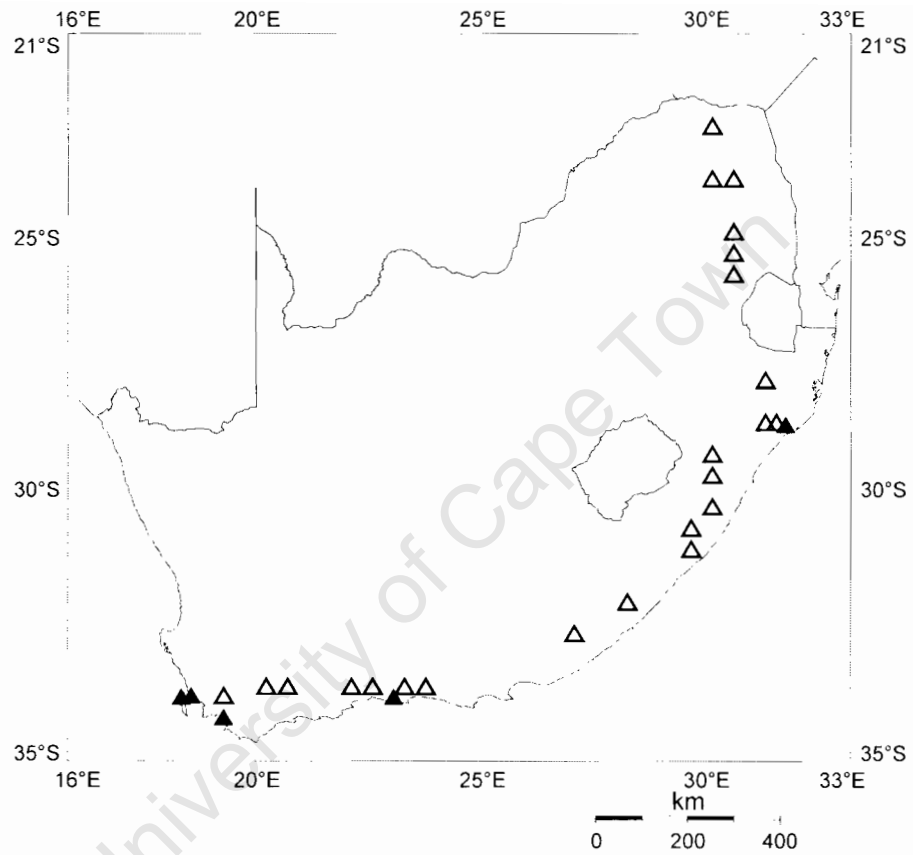


Figure 6: Distribution of *Pyrrhobryum spiniforme* in South Africa (from Magill 1987). Solid triangles are sampling localities for this study, and open triangles are other sites where it has been found in South Africa.

## 2.2 DNA extraction, amplification and sequencing

DNA was extracted using a CTAB method modified from Doyle and Doyle (1987). About 20 mg of fresh or dried material was placed in a 1.5 µl microcentrifuge tube. The plant tissue was flash frozen using liquid nitrogen and finely ground with a plastic pestle in the microcentrifuge tube. 700 µl of pre-heated 2X CTAB buffer and 1 µl of β-mercaptoethanol were used for each sample. The ground tissue and buffer were vortexed and the tubes were placed in a 65°C water bath for at least 1 h. Alternatively the plant material was ground in a mortar and pestle, with 700 µl CTAB buffer, 1 µl β-mercaptoethanol and a spatula tip of PVP (Polyvinyl pyrrolidone).

After incubation, 600 µl of chloroform: isoamyl alcohol (24:1 v/v) was added to each sample. The tubes were centrifuged for 5 min at 12 000 rpm. The supernatant was transferred to a clean 1 µl microcentrifuge tube, and an equal volume of ice-cold isopropanol was added. The samples were left overnight in a -20°C freezer, for the DNA to precipitate. When the samples were removed from the freezer they were centrifuged for 5 min at 12000 rpm. The pellet containing the DNA was rinsed three times using 75% ethanol and dried for 1 h in a desiccator. The DNA pellet was resuspended in 50 µl of elution buffer. Extracted DNA was visualised on a 1% agarose gel. Depending on the apparent concentration of the DNA extraction, the stock was diluted to either  $10^{-1}$  or  $10^{-2}$ .

Initial amplification of the target regions was performed using 0.75 units of *Taq* polymerase (Bioline, Biotaq™). Each 30 µl reaction tube also contained 1X NH<sub>4</sub> buffer, 5 mM MgCl<sub>2</sub> buffer, dNTPs (0.1 mM of each nucleotide, Promega), and 0.3 mM each of the forward and reverse primer (Table 1). 3 µl of diluted DNA template were used. The thermal cycle used for amplification was: 94°C for 2 min, 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min, then 72 °C for 7 min. PCR products were checked on a 1% gel, using ethidium bromide to visualize them. PCR products were cleaned using Qiaquick® purification columns (QIAGEN) or GFX™ PCR DNA and Gel band Purification Kit (Amersham Biosciences). The DNA was eluted for at least 10 min, using 30 µl of elution buffer. Concentration of PCR products was estimated from a 1% agarose gel with a 40 and 60 ng PGEM (Applied Biosystems) standard.

Cycle sequencing was performed in a 10  $\mu$ l reaction volume. Between 1 and 3  $\mu$ l of cleaned product was used for each reaction, with the balance made up of distilled purified water. The cycle sequencing reaction mixture contained 1.84  $\mu$ l of H<sub>2</sub>O, 2  $\mu$ l Terminator Ready Reaction mix, 0.16  $\mu$ l 10 mM primer and 1  $\mu$ l 5X sequencing buffer. The Terminator Ready Reaction and sequencing buffer were from the ABI PRISM® Big Dye™ kit (Applied Biosystems, Inc. Palo Alto, CA). The cycle sequencing thermal profile used was 25 cycles of: 30 s at 96°C, 15 s at 50°C and 4 min at 60°C. Initial amplification and cycle sequencing were done on either a GeneAmp® PCR system (Applied Biosystems Inc., Palo Alto, CA or a Hybaid PCR Sprint Temperature Cycling System, Ashford, Middlesex TW15 1XB, UK). Final products from the cycle sequencing reaction were cleaned in spin columns and resolved on an ABI 3900 autosequencer.

Table 1: Details of the two primers used in DNA amplification and sequencing

	<b>Forward</b>	<b>Reference</b>	<b>Reverse</b>	<b>Reference</b>
ITS1 (nuclear)	18KRC: GCA CGC GCG CTA CAC TGA	Hedderson and Nowell (2005)	ITS2: GCT GCG TTC TTC ATC GAT GC	Baldwin 1992
<i>trnL</i> -F (chloroplast)	<i>trnC</i> : CGA AAT CGG TAG ACG CTA CG	Taberlet 1991	<i>trnF</i> : ATT TGA ACT GGT GAC ACG AG	Taberlet 1991

Forward and reverse sequences were assembled using Seqman (LaserGene System Software, DNASTar Inc.). The consensus sequence was saved as a single file, and assembled sequences were aligned manually in MegAlign (LaserGene System Software, DNASTar Inc). All sites that showed variation were re-checked in Seqman to verify that variation was unambiguous. Each indel, irrespective of its length, was treated as a single character coded by adding DNA symbols to the end of the sequence matrix.

### 2.3 Data analyses

A combination of different analyses was used as they can be complementary; therefore a more thorough assessment of the processes affecting the distribution of genetic variation can be

made (Arbogast and Kenagy 2001, Cruzan and Templeton 2000, Bermingham and Moritz 1998).

Haplotype trees were estimated in TCS 1.13 (Clement *et al.* 2000). The haplotype tree represents the evolutionary steps that connect the observed haplotypes. The tree is based on a 95% set of plausible solutions since the probability of parsimony is calculated for pairwise differences until the probability exceeds 0.95. The trees that portray linkages among haplotypes that have a high probability ( $\geq 0.95$ ) of being true are included. Therefore the tree documents the extent of uncertainty in the data Templeton *et al.* (1992).

As there was too much length variation in the *P. spiniforme* sequences a haplotype tree could not be estimated, so a neighbour joining tree was calculated in PAUP, version 4.0b10 (Swofford 2002), and using total character difference as the distance option.

#### *Analyses performed with Leptodon smithii population samples only*

A population was defined as having four or more samples; samples were only included if they had both ITS and *trnL-F* data. Population samples are listed in Table 2. Only population samples were used in the following analyses: mismatch distribution, AMOVA, NCA and haplotype frequencies.

Loops in the haplotype tree might indicate recombination, homoplasy (reverse parallel mutations), or alternatively parsimonious connections. Recombination was inferred from the haplotype tree (Figure 9), if by removing the haplotype, two or more homoplasies were resolved, or when a single recombination event resolved a single homoplasy involving a mutation regarded as evolving in a completely parsimonious fashion (Templeton *et al.* 1992). For all population analyses the recombinants were removed, as the analyses rely on using non-recombining data. Of course, this also requires removal of any descendants of recombinant haplotypes.

TCS was used to define the nested clades for the nested clade analysis (NCA) (Templeton 1992). NCA requires a haplotype tree to 'build' the nested design on. The nesting design inherently incorporates historical information. Nested clade analysis allows the spatial

distribution of genetic variation to be studied. The significance of association between genetic variation and geographic distribution (Templeton 1995) was calculated in GeoDis (Posada *et al.* 2000). Non-random association of haplotypes with geographic location can result from restricted gene flow or historical events (e.g. fragmentation, range expansion and colonisation). The different causes yield quantitatively different patterns in the distance measurements, which can be analysed statistically. Any statistically significant patterns were evaluated using an inference key (Templeton *et al.* 1995, updated 2003). The inference procedure can find the best combination of factors that explains the genetic variation (Templeton 1998).

All population genetics analyses were performed using the program Arlequin (Schneider, Roessli and Excoffier 2000). AMOVA (Analysis of Molecular Variance) estimates the partitioning of genetic variation within and among populations. This method uses variation in gene frequencies among populations, but also takes into account the number of mutations between haplotypes. Population pairwise F statistics were calculated to test for differences between populations. The mismatch distribution shows the number of differences between pairs of sequences. From the distribution one can potentially evaluate demographic models (Schneider and Excoffier 1999). A unimodal distribution shows that demographic events in the past have forced coalescent events into a narrow time window (Slatkin and Hudson 1991). Arlequin fits the mismatch distribution to a sudden expansion model, which has a unimodal distribution.

Table 2: Locality and collection details of the samples of *L. smithii* used in the population analyses. The asterisked samples are recombinants and were only included in the haplotype frequencies analysis.

Sample	Locality	Province	Collector
N1	Nursery Ravine, Table Mountain	Western Cape	Mwafongo
N2	Nursery Ravine, Table Mountain	Western Cape	Mwafongo
N3	Nursery Ravine, Table Mountain	Western Cape	Mwafongo
N4	Nursery Ravine, Table Mountain	Western Cape	Mwafongo
N5	Nursery Ravine, Table Mountain	Western Cape	Mc Grath
N6	Nursery Ravine, Table Mountain	Western Cape	Mc Grath
N7	Nursery Ravine, Table Mountain	Western Cape	Mc Grath
N8 *	Nursery Ravine, Table Mountain	Western Cape	Mc Grath
N9	Nursery Ravine, Table Mountain	Western Cape	Mc Grath
58L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo
J1	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J1b	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J2	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J3	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J4 *	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J5	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J6	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J7	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J8	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
J9	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
56L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
57L *	Jonkershoek, Stellenbosch	Western Cape	Mwafongo
SF1	Groenvlei, Sedgfield	Western Cape	Hedderson
SF2	Groenvlei, Sedgfield	Western Cape	Hedderson
SF3	Groenvlei, Sedgfield	Western Cape	Hedderson
SF4	Groenvlei, Sedgfield	Western Cape	Hedderson
SF5	Groenvlei, Sedgfield	Western Cape	Hedderson
SF6	Groenvlei, Sedgfield	Western Cape	Hedderson
SF7	Groenvlei, Sedgfield	Western Cape	Hedderson
SF8	Groenvlei, Sedgfield	Western Cape	Hedderson
FK3	Fernkloof, Hermanus	Western Cape	Mc Grath
FK4 *	Fernkloof, Hermanus	Western Cape	Mc Grath
FK5	Fernkloof, Hermanus	Western Cape	Mc Grath
FK7	Fernkloof, Hermanus	Western Cape	Mc Grath
FK8	Fernkloof, Hermanus	Western Cape	Mc Grath
LS23 *	Fernkloof, Hermanus	Western Cape	Mc Grath
LS24	Fernkloof, Hermanus	Western Cape	Mc Grath
LS25	Fernkloof, Hermanus	Western Cape	Mc Grath
LS26	Fernkloof, Hermanus	Western Cape	Mc Grath
LS27 *	Fernkloof, Hermanus	Western Cape	Mc Grath
LS15	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS16	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS17 *	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS18 *	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS19 *	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS6 *	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath
LS34	Blouberg	Limpopo	Hedderson
LS35	Blouberg	Limpopo	Hedderson
LS36	Blouberg	Limpopo	Hedderson
LS37	Blouberg	Limpopo	Hedderson

## Chapter 3

### Results

#### 3.1 *Leptodon smithii*

##### *All samples*

There are 89 ITS1 samples, with 23 haplotypes and 14 singletons (Figure 7, Table 3). The final length of the region used in the analysis was 550 bp. Haplotype B has the highest frequency (25), the most connections (7) with the rest of the network and is widespread throughout South Africa; it is also found in Madeira and Australia. Fifteen samples have Haplotype D, which has six connections, and is also quite widespread, with samples from Kenya and Portugal. Fourteen samples have Haplotype A. Apart from one sample from the Orange Free State, it is restricted to the Western Cape. Six samples, found only at Jonkershoek and Nursery Ravine, have Haplotype C. Haplotype S, which is very different from the rest of the samples, is from Buffels Nek Forestry Station, Knysna.

The *trnL-F* region had 79 individuals, with six haplotypes (Figure 8, Table 3). 61 samples, widespread throughout South Africa (with one sample from Kenya, and two from Australia), have haplotype 1. Twelve samples, restricted to Jonkershoek and Nursery Ravine, have haplotype 2. Only one sample from the Eastern Cape has haplotype 3. The samples from Madeira form a separate clade (haplotypes 4, 5 and 6), separated by four mutations.

Table 3: Location of the *Leptodon smithii* samples with their corresponding haplotype allocation obtained from the TCS analysis. The first 20 entries are located in South Africa; the countries of the remaining four are indicated in the Table. Corresponding numbers/letters indicate identical haplotypes.

<b>Locality</b>	<b>trnL-F</b>	<b>ITS1</b>
Table Mountain, Cape Town	1, 2	A, B, C, D, K
Jonkershoek, Stellenbosch	1, 2	A, C, H, L, M
Groenvlei, Sedgefield	1	A, B, F, O
Fernkloof, Hermanus	1	B, E, G, I
Grootvadersbosch, Heidelberg	1	B, D, K, N
Krantzberg	1	B, T
Blouberg	1	B, U
Montague's Pass, George	1	A
Grootwinterhoek, Tulbagh	1	B, Q
Fernkloof, Grahamstown	1	B, D
Helderberg, Somerset West	1, 2	B
Knysna	1	B, D, S, Z
Natal, South Africa	1	B
Drakensberg	1	B, D
Uitvlugt farm, Paulpietersburg	1	
Graskop mts, Transvaal	1	
Mlanlane Forest station		R
de Kelders		G
Euefeeskloof, Zastron	1	A
Bosberge Forest, Somerset East	3	B
Kenya	1	D, J
New South Wales, Australia	1	B
Madeira	4, 5, 6	B, R, X, Y
Coimbra, Portugal	5	D

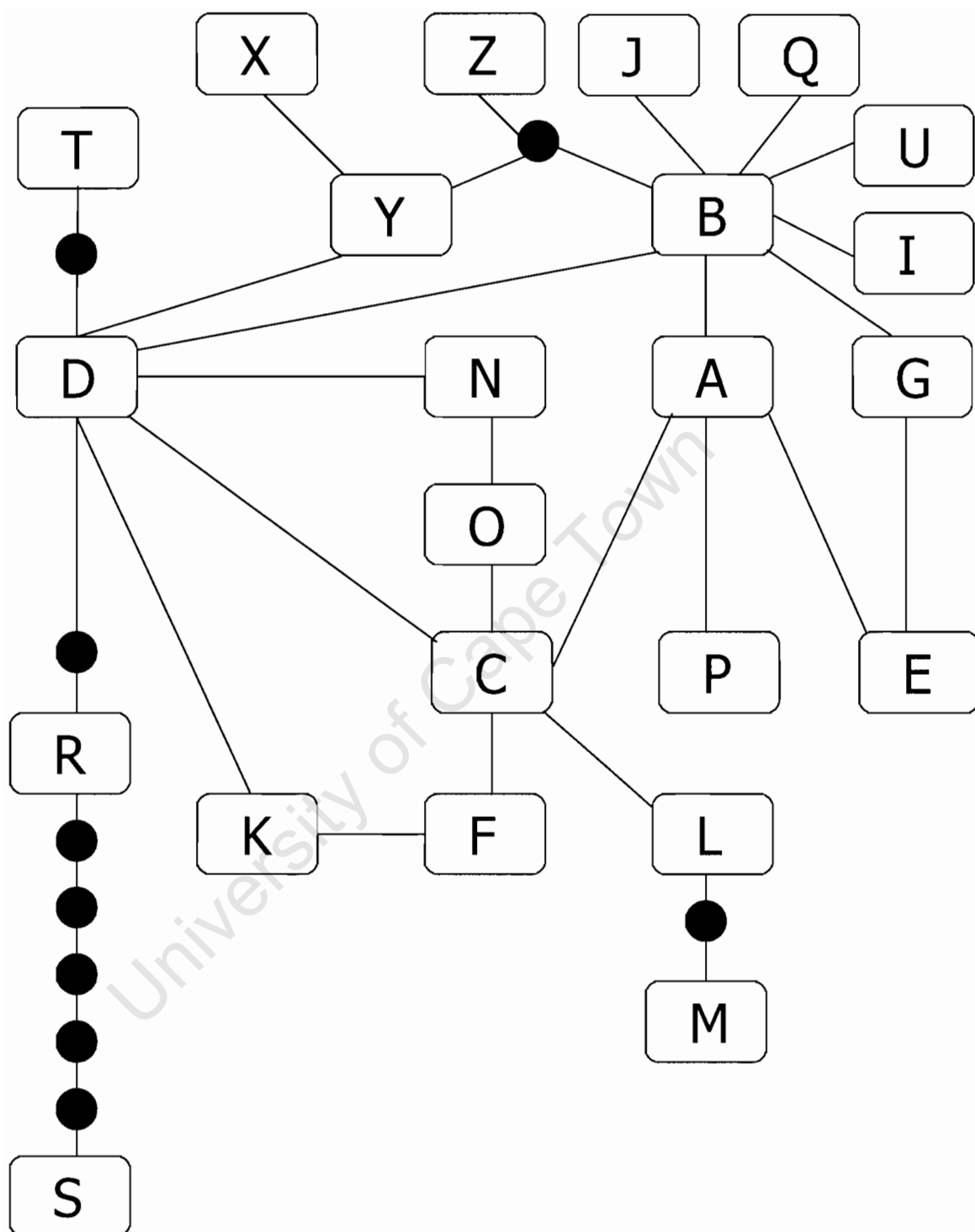


Figure 7: *Leptodon smithii* ITS1 haplotype tree obtained from the TCS analysis with all samples included. Letters denote haplotypes, a line between one haplotype indicates one mutation, and a filled black circle indicates a missing haplotype or a mutation.

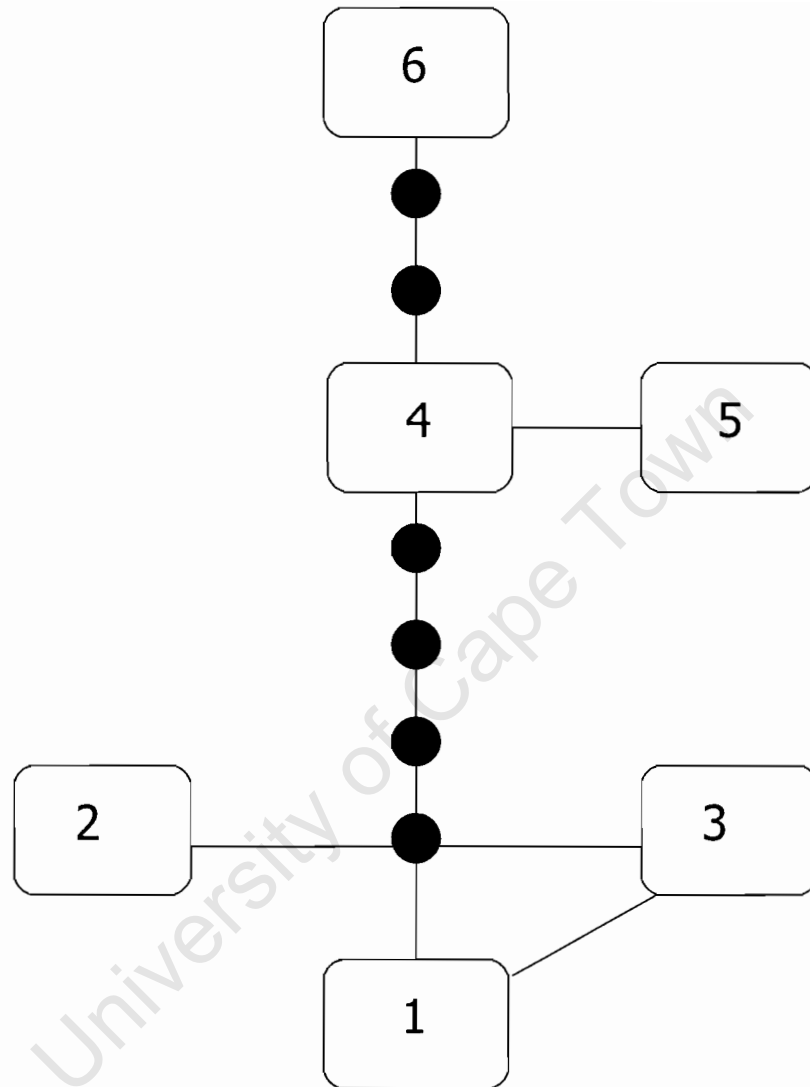


Figure 8: *Leptodon smithii* trnL-F haplotype tree obtained from the TCS analysis with all samples included. Letters denote haplotypes, a line between one haplotype indicates one mutation, and a filled black circle indicates a missing haplotype or a mutation.

### *Population level sampling*

The ITS1 region has 49 samples, with 14 haplotypes, six of which are singletons (Figure 9). Haplotype D and C both have five connections, B has four connections and A has three. The only haplotype found in all populations is hapB. HapA is found in three populations (Nursery Ravine, Jonkershoek and Sedgefield), but there appears to be a gap, as it was not found at Grootvadersbosch or Fernkloof. HapD is widespread but is not found at Sedgefield or Fernkloof (Figure 10).

All populations except Nursery Ravine have unique haplotypes. Nursery Ravine has only interior haplotypes, which have higher frequencies than in the other populations. Fernkloof has a high proportion of haplotypes that are unique to it (more than half). HapF is only found at Sedgefield, it is also at a high frequency in the population. Apart from Nursery Ravine the interior (ancestral) haplotypes are at generally low frequencies in the populations.

The interior haplotypes (A, B and D) are more widespread, although haplotype C is restricted to Jonkershoek and Nursery Ravine. The tip haplotypes have not yet spread (Figures 9 and 10).

HapE arises from hapA, but is not found in the same population as hapA. HapO and hapF are found at Sedgefield, but their ancestral haplotype is hapC, which is only found at Jonkershoek and Nursery Ravine.

There are multiple loops in the haplotype network (Figure 9). With all recombinant haplotypes removed there are 39 samples. Table 4 shows the inferred recombinants.

### *Nested clade analysis*

The nesting design is shown in Figure 11. Three clades have significant associations of genetic variation with geographical distribution. The results are summarized in Table 5. For clade 1-2 the inferred reason for a significant association is past fragmentation followed by range expansion or long distance colonization. For clade 1-3 the inference key suggested that there was inadequate geographical sampling to infer what processes caused the significant association, and for clade 2-2 there was contiguous range expansion.

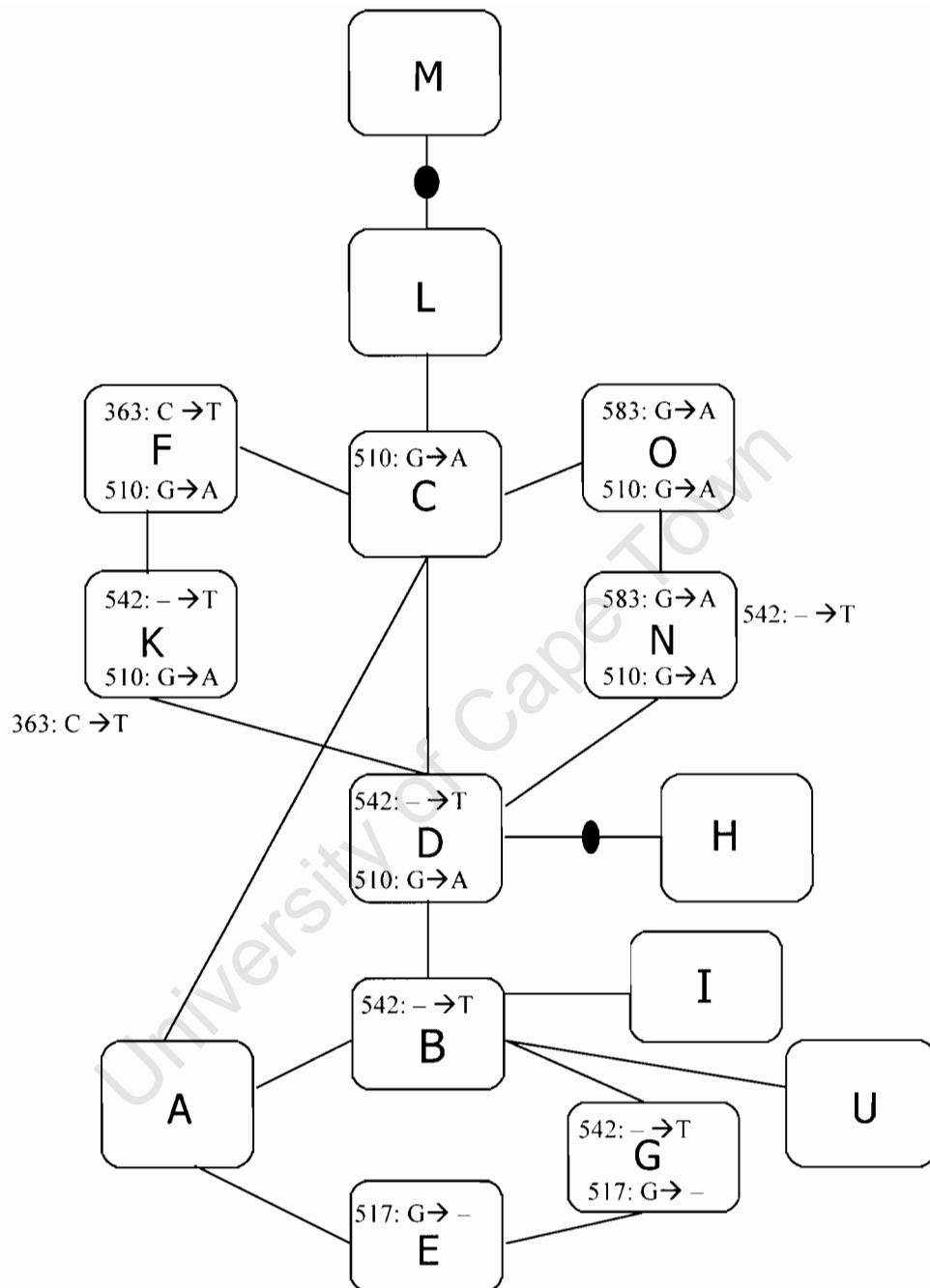


Figure 9: *Leptodon smithii* haplotype tree using only ITS1 population samples from the TCS analysis. Above each haplotype code the sequence position and type of mutation are shown, e.g. for haplotype E, a deletion of G occurred at position 517. This information is shown only for haplotypes involved in recombination.

Table 4: Inferred recombinants from *Leptodon smithii* from the ITS haplotype tree obtained from the TCS analysis (Figure 9). For each recombinant, the parent haplotypes and respective localities are given.

<b>Recombinant</b>	<b>Locality</b>	<b>Parent 1</b>	<b>Locality</b>	<b>Parent 2</b>	<b>Locality</b>
HapD	widespread	HapB	Widespread	HapC	Jonkershoek, Nursery Ravine
HapG	Fernkloof	HapB	Widespread	HapE	Fernkloof
HapN	Grootvadersbosch	SF5 HapO	Grootvadersbosch	HapD	widespread
HapK	Grootvadersbosch, Nursery Ravine	HapF	Sedgefield	HapD	widespread

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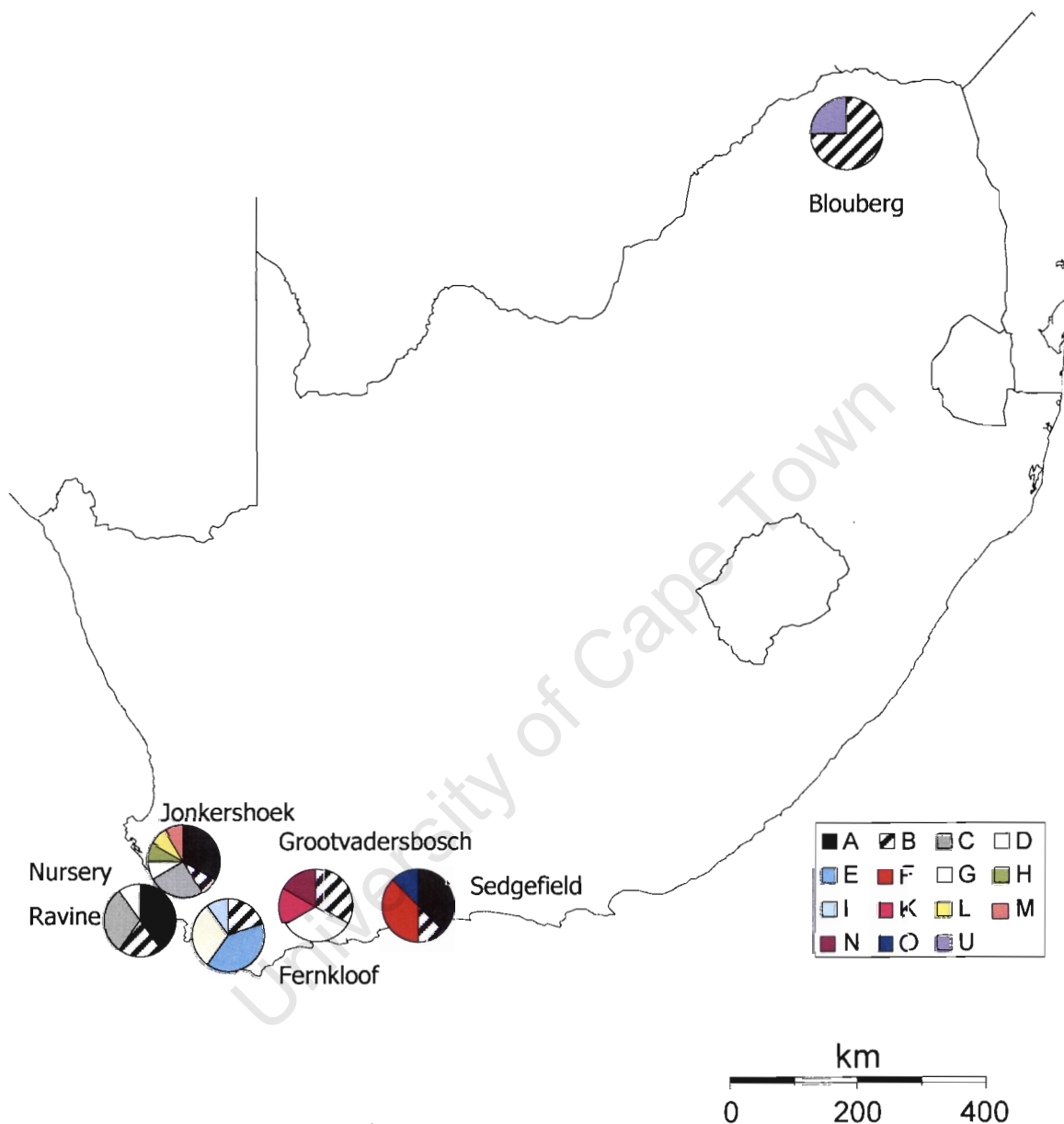


Figure 10: Map of the distribution of the ITS haplotypes for the South African *Leptodon smithii* population samples. The pie charts show the frequencies for each haplotype, obtained from Figure 9, in each population. Each haplotype is indicated by a unique colour, identified in the key.

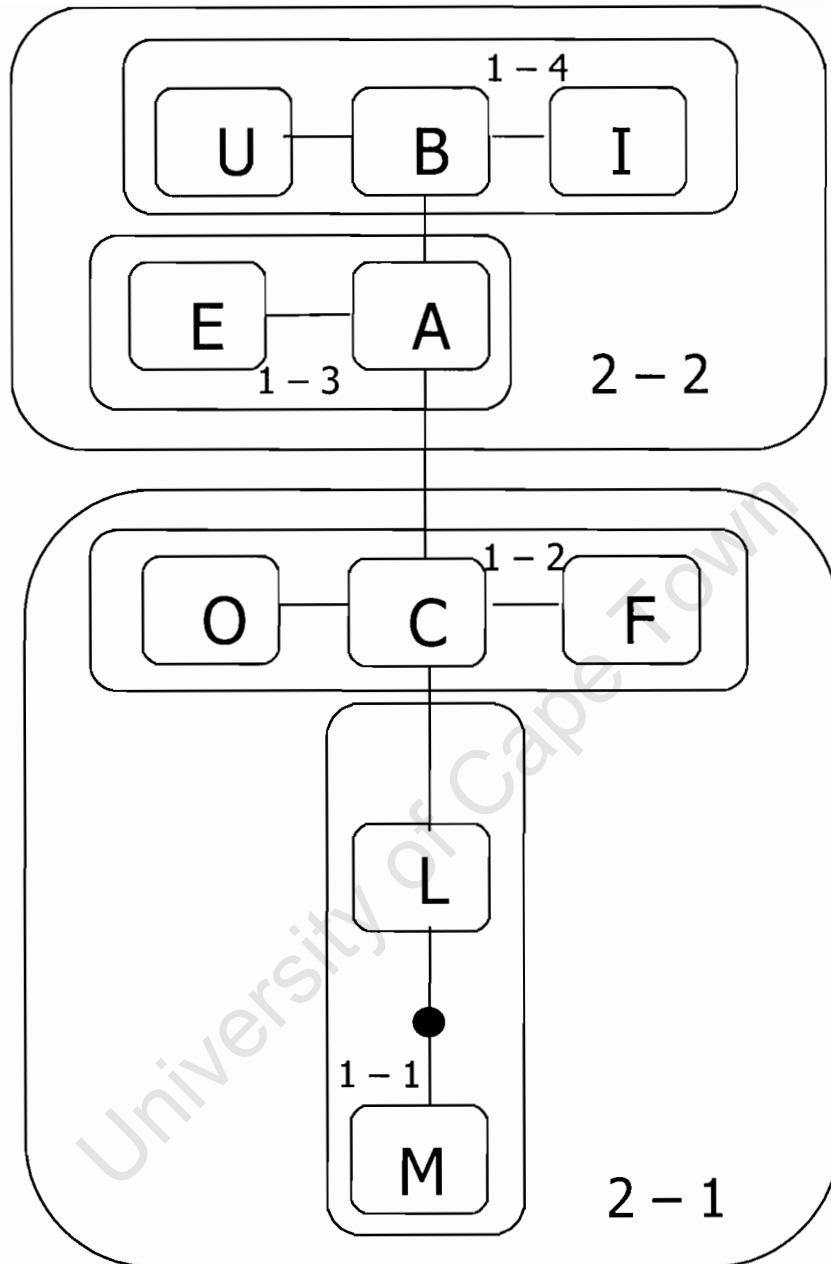


Figure 11: Nested clade design for the *Leptodon smithii* ITS1 population samples (with recombinants removed) as defined in the TCS analysis. Numbers indicate the clade allocations; letters indicate haplotypes. Details of the significance values for each nested clade are given in Table 5.

Table 5: Significance of the association of genetic variation and geographic distribution for the clades, obtained from the Geodis analysis on the *Leptodon smithii* ITS population samples, with all recombinants removed. The clade allocation is given in Figure 11. Dc = clade distance, Dn = nested clade distance, NS= Not significant, small = significantly small, large = significantly large

<b>Clade</b>	<b>Distribution</b>	<b>Significance</b>
Clade 1-2		
HapC (interior)	Nursery Ravine, Jonkershoek	Dc small, Dn small
HapF (tip)	Sedgefield	Dc small, Dn large
HapO (tip)	Sedgefield	Dc NS, Dn NS
Tip- interior		Dc NS, Dn small
Inference key: 1 – 2 – 11 – 12 – 13		
Clade 1-3		
HapA (interior)	Nursery Ravine Jonkershoek, Sedgefield	Dc large, Dn large
HapE (tip)	Fernkloof	Dc small, Dn small
Tip-interior		Dc large, Dn large
Inference key: 1 – 19 – 20		
Clade 2-2		
Clade 1-3	Nursery Ravine, Fernkloof, Sedgefield, Jonkershoek	Dc small, Dn small
Clade 1-4	Nursery Ravine, Fernkloof, Sedgefield, Jonkershoek, Blouberg, Grootvadersbosch	Dc large, Dn large
Tip-interior		Dc small, Dn small
Inference key: 1 – 2 – 11 – 12		

*Population genetics*

For both ITS and *trnL-F* the percentage of variation found within populations is higher than the percentage of variation found among populations (Table 6). The ITS *Fst* is significantly greater than the observed value.

Table 6: Summary of results from AMOVA for *Leptodon smithii* population samples for ITS1 and *trnL-F* markers.

		<b>ITS1</b>	<b><i>trnL-F</i></b>
<b>percentage of variation</b>	<b>among populations</b>	15.78	37.99
	<b>within populations</b>	84.22	62.01
	<b>Fst</b>	0.15782	0.3799
	<b>P value</b>	0.00098	0.0000

Table 7: Pairwise population F-statistics for *Leptodon smithii* population samples, significant results are asterisked ( $p = 0.05$ ), above the diagonal are from the ITS region, and below the line are from the *trnL-F* region.

	<b>Jonkershoek</b>	<b>Nursery Ravine</b>	<b>Fernkloof</b>	<b>Sedgefield</b>	<b>Blouberg</b>
<b>Jonkershoek</b>		-0.088	0.239*	0.053	0.257*
<b>Nursery Ravine</b>	0.148		0.257*	0.065	0.237*
<b>Fernkloof</b>	0.167	0.588*		0.245*	0.240
<b>Sedgefield</b>	0.187	0.608*	0.000		0.258*
<b>Blouberg</b>	0.076	0.510	0.000	0.000	

The mismatch distribution for ITS1 is unimodal (Figure 12). The observed and model distributions are almost the same. Most pairwise differences occur at one, and then gradually decline. There are also a number of pairs that have no differences. The highest number of differences a pair of samples can have is seven. The *trnL-F* mismatch distribution shows that most sequences have no differences between them.

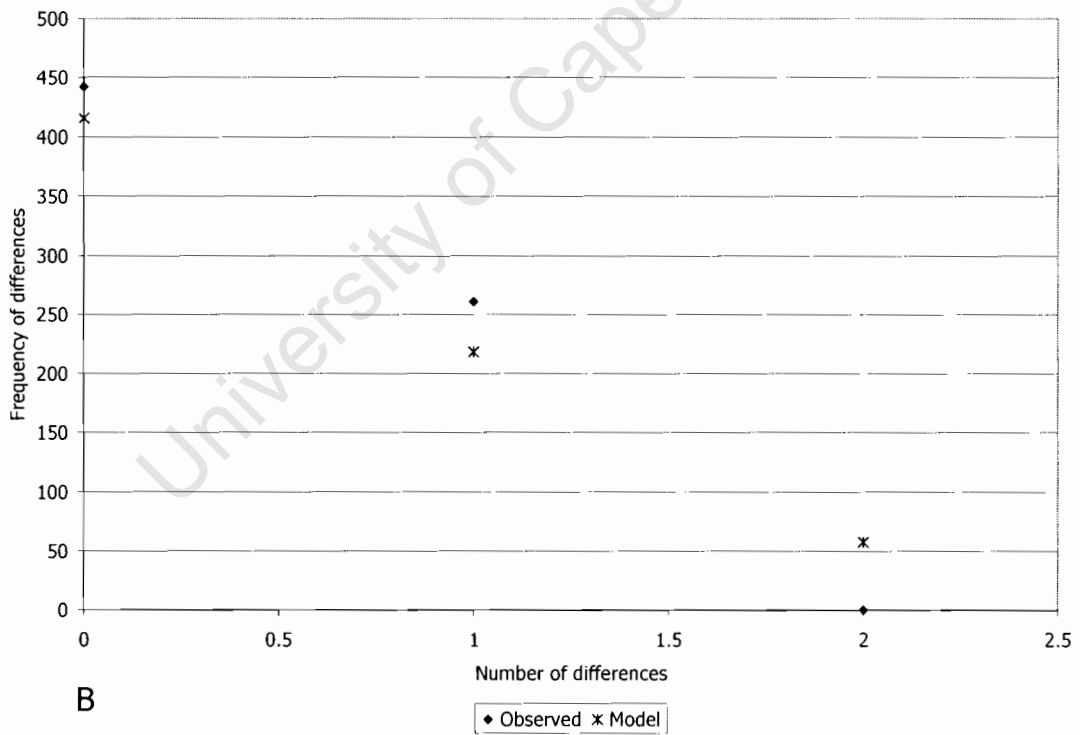
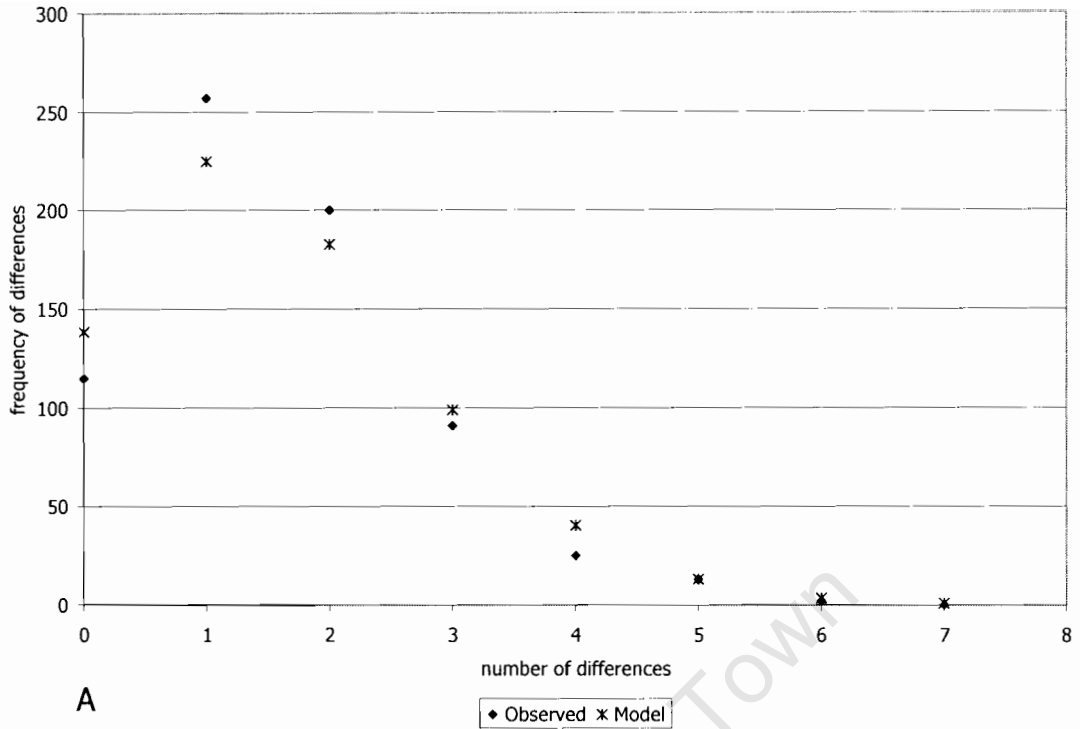


Figure 12: Observed and modelled mismatch distributions for the *Leptodon smithii* population samples from the A) ITS1 and B) *trnL-F* regions.

### 3.2 *Neckera valentiniana*

There was no variation in ITS1, therefore no further sampling or analyses were performed.

### 3.3 *Aerobryopsis capensis*

There are five ITS and two *trnL-F* haplotypes (Table 8). Unfortunately I could not sample any more, due to time constraints. *trnL-F* Hap1 is found in all the populations. The ITS1 haplotype tree forms two clades, separated by three steps (Figure 13).

Table 8: Haplotype allocation (from TCS analysis) for each *Aerobryopsis capensis* sampling locality in South Africa for both marker regions.

Locality	<i>trnL-F</i>	ITS1
Natures valley	1	B, E
Sedgefield	1	D
Montague's Pass, George	1	C
Knysna	1, 2	A
Grootvadersbosch	1	A

### 3.4 *Pyrrhobryum spiniforme*

There are three haplotypes for *trnL-F* (Table 9). Hap1 is found in all the populations. The ITS sequences vary in length between 214 bp to 911 bp. As the lengths were so different it was not possible to compare between sequences and determine haplotypes. Hence a neighbour joining tree (Figure 14) was prepared to show the variation in the sequences. There is very little variation within group A. Group A has long sequences, between 840 bp and 911 bp, except PS27, which has 540 bp. Group B has much more variation, in length and genetic differences. Group B varies in length between 214 bp and 764bp. Samples from Group A are found in all the populations, while Group B samples are restricted to the Western Cape.

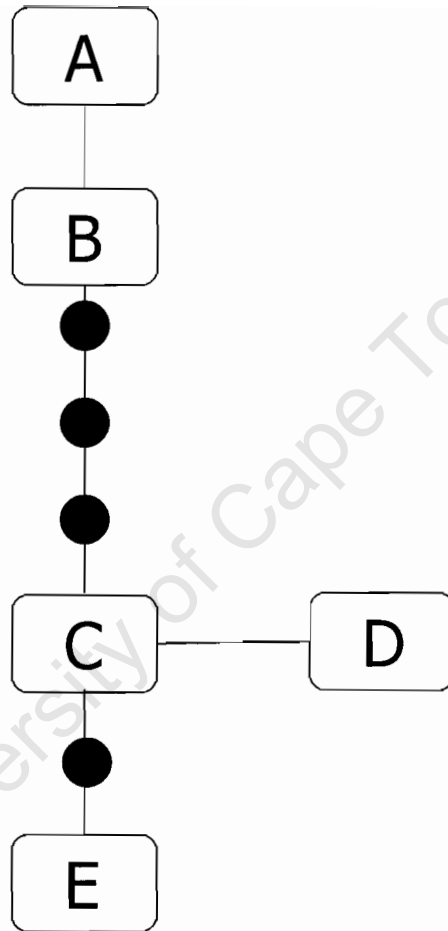


Figure 13: *Aerobryopsis capensis* ITS1 haplotype tree obtained from the TCS analysis. Letters denote haplotypes, a line between one haplotype indicates one mutation, and a filled black circle indicates a missing haplotype or a mutation.

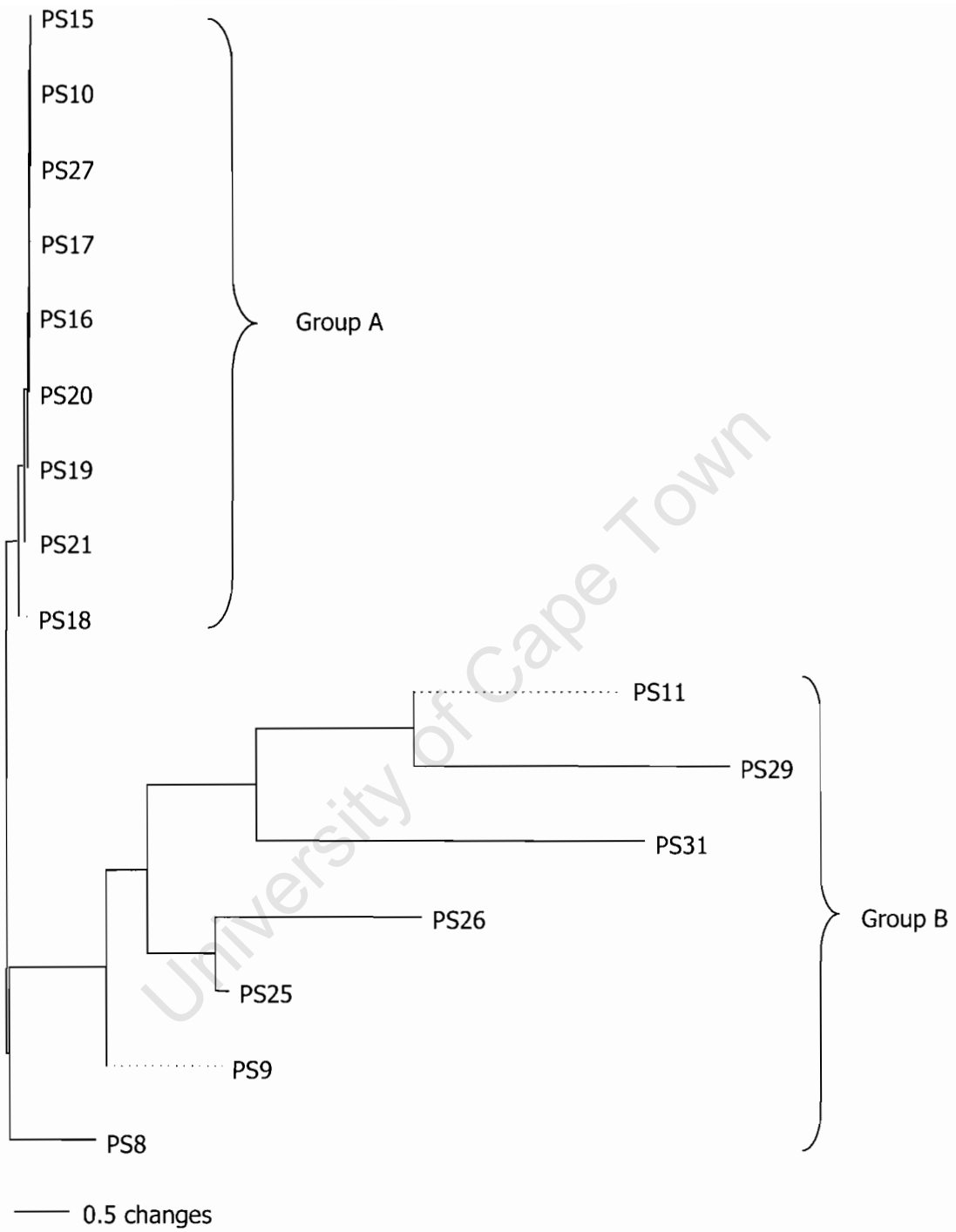


Figure 14: *Pyrrhobryum spiniforme* ITS1 neighbour-joining tree. Reference codes indicate the samples which are detailed Table 13. Branch length bar indicates number of changes along a branch.

Table 9: Haplotype allocation (*trnL-F*) and neighbour joining tree groups illustrated in Figure 14 (ITS1) for each *Pyrrhobryum spiniforme* sampling locality in South Africa.

<b>Locality</b>	<b><i>trnL-F</i></b>	<b>ITS1</b>
Diepwalle, Knysna	1, 2	A, B
Eshowe	1	A
Jonkershoek, Stellenbosch	1	A, B
Nursery Ravine, Table Mountain	1, 3	A, B

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## Chapter 4

### Discussion

It is evident from this study that forest-faithful moss species show levels and patterns of genetic variation that are likely to be highly informative with respect to the history of afro-montane forests. In most of the species examined, levels of haplotypic diversity are high. For example, *L. smithii* and *A. capensis* show 29 haplotypes in 94 samples and seven haplotypes in sixteen samples respectively over the sampled geographic range. These levels of variation compare favourably to other phylogeographic studies of angiosperms and bryophytes. For example, across Spain Gutierrez Larena *et al.* (2002) found 15 *trnL-F* haplotypes within 105 samples of the angiosperm *Armeria*, while Hedderson and Nowell (2005) found 22 ITS1 haplotypes among 93 individuals of the moss *Homalothecium sericeum*.

In addition, there appears to be a high degree of geographic structure in the variation, particularly with *L. smithii*, which is discussed below. For the two other mosses that had variation it is likely that further sampling would reveal more geographical structuring. In both *A. capensis* and *P. spiniforme* there is one widespread *trnL-F* haplotype, and a few unique haplotypes, similar to the situation found in *L. smithii*.

#### 4.1 The markers

As noted before the markers provided high levels of phylogeographic signal. They were both easy to amplify, which was expected, as they are both universal markers. For all the species showing variation, ITS1 generally showed more variation than *trnL-F*. This corresponds with what is known about chloroplast and nuclear mutation rates, as the nuclear genome mutates faster than the chloroplast genome (Parker *et al.* 1998, Ennos *et al.* 1999). To compensate for the low levels of variation in *trnL-F* future studies could include more chloroplast regions, such as the *atpB-rbcL* spacer, and *rps4* gene. As cpDNA is generally inherited without recombination, the entire genome should have a single history, so the data could be combined (Schaal *et al.* 1998, McDaniel and Shaw 2003). However recent studies have shown that plastid DNA can undergo recombination (reviewed in Wolfe and Randall 2004). This has only been reported in a few cases, and most likely would not be problematic at the below species level, but it does need to be taken into consideration.

Therefore the separate data sets would need to be tested for incongruence first, before being combined (Wolfe and Randall 2004).

The observed ITS variation in *P. spiniforme* is mostly from length variation (sequence length varied between 214 and 911 bp, whereas for the other three mosses the length varied between 560 and 670 bp). Length variation has also been found in other acrocarpous mosses (sporophyte growing terminally on main stem), such as *Pohlia* and *Pyrrhobryum mnioides* (Shaw and McDaniel 2002). As ITS occurs as a multicopy gene family it is possible to have hundreds to thousands of copies of the same sequence (Schaal *et al.* 1998, Alvarez and Wendel 2003). Usually this is not problematic, because of the process of concerted evolution, which results in the copies evolving in unison (Alvarez and Wendel 2003). However concerted evolution does not always occur in angiosperm and gymnosperm ITS sequences (reviewed in Alvarez and Wendel 2003, Bailey *et al.* 2003). Therefore it is possible that amplification of ITS will produce a suite of fragments, some of which may be pseudogenes and non-homologous copies (Schaal *et al.* 1998). Pseudogenes are DNA sequences that are under no functional constraints, and so evolve differently and at independent rates (Bailey *et al.* 2003, Alvarez and Wendel 2003). Methods for detection are outlined in Bailey *et al.* (2003) and Alvarez and Wendel (2003). The length variation in *P. spiniforme* could be due to non-homologous sequences or pseudogenes. If the length variation is from having multiple ITS copies, the different copies could be cloned and sequenced. Future analyses of this species using ITS would therefore require detailed examination of the ITS region to determine whether truly homologous regions are being compared. The possibility of finding pseudogenes and non-homologous copies further shows how important it is to include markers from different genomes.

Only one of the studied species, *N. valentiniana*, showed no variation across the sampled range. As discussed below, there may be good historical explanations for this observation, but differences in levels of genetic variation could also be due to intrinsic differences among species (even if they have shared the same history). DNA could mutate at different rates, which is not unusual within bryophytes, particularly with ITS (Shaw and McDaniel 2002). Longton and Hedderson (2002), in a study of *Bryum argenteum* and other Bryaceae found that most of the variation occurred within *Bryum argenteum*. A study of *Mielichhoferia elongata* found morphologically

indistinguishable but phylogenetically distant populations a few meters apart at one site, while a Swedish population was closely related genetically to populations in the Rocky Mountains (Shaw 2000). Potentially, DNA variation could be influenced by life history, habitat preference and breeding system. There do not seem to be any trends with respect to breeding system among the species studied here as both *N. valentiniana* (no variation) and *A. capensis* (substantial variation) are autoicous. There may be a trend with respect to habitat as *L. smithii* (substantial variation) can tolerate drier forests, whilst *N. valentiniana* (no variation) is mostly found in wet forests. However *P. spiniforme* (intermediate/unknown levels) is also found in wet forests. As DNA levels of variation do have the potential to differ between species, studies need to include as many species as possible.

#### 4.2 Phylogeography of *Leptodon smithii*

##### *Global scale*

*L. smithii* is similar to many other mosses in that it has a widespread distribution, which spans multiple continents. Whether these distributions are caused by fragmentation of ancient distribution, or repeated intercontinental dispersal is still controversial (Shaw 2001).

The South African samples show considerably higher levels of variation than those from any of the other areas, which suggests that *L. smithii* originated in Africa, or at least that the other sampled populations are derived from Africa. However, this could be a sampling artifact. Sampling density is higher in South Africa than for the other regions, so there is a possibility that more variation exists in the other areas that was not sampled. However, given the extent of sampling in east Africa and Australia, such additional haplotypes must be rare.

The *trnL-F* samples from Madeira and Portugal form a separate clade from the other samples. It is therefore likely that those populations have been separated from the other sampled populations for a long time. Meanwhile the Kenyan and Australian samples have the most frequent, widespread haplotypes from South Africa, for both markers. This could either be because of recent dispersal, or as a result of retention of ancestral lineages. However if the populations had been separated for a long time (and fragmentation explanations would require many millions of years of separation) we would expect them to show more divergence; only one of the seven haplotypes

from these two areas is unique, and even then it is not distantly separated. Whilst the current data are strongly consistent with a dispersalist explanation, there is insufficient sampling from these areas to be fully confident of what has actually happened.

#### *South African scale*

The population genetic analyses show significant structuring among populations (Table 6 and 7). Therefore there has been restricted gene flow between populations. Particular populations show patterns of isolation through haplotype distribution and frequencies. The Jonkershoek and Nursery Ravine populations have been separated from the other forests for a long time, as both hapC and hap2 are restricted to them. Fernkloof has also been separated from the other populations, as there is a gap around it with hapA and D and it has a high number of unique haplotypes. More sampling is needed around Fernkloof, as the nested clade analysis, while showing that there are significant geographical associations, does not show fragmentation or restricted gene flow. It is likely that the rare haplotypes are more widespread than the population sampling shows, as the sample from de Kelders (16L) did have a rare haplotype.

The interior (older) ITS haplotypes are at lower frequencies than expected from coalescent theory in some populations, which suggests isolation (ancestors removed as a result of stochastic processes). Alternatively the missing haplotypes may not have been sampled, but this is unlikely since the sampling of approximately 10 sequences per population should have been high enough to detect the uncommon haplotypes (Cruzan and Templeton 2000).

There are shared haplotypes in all the forest patches, which is either because of dispersal or because of ancestral retention from a previously widespread distribution. As there is population structuring (i.e. limited dispersal), it is likely that the shared haplotypes are from a previously more widespread distribution. This is supported by palynological and biogeographical studies (Lawes 1990, DeBusk 1998), and evidence of range expansion from the data. The contiguous range expansion inferred from Clade 2-2 (Table 5) would only have been possible if forests were previously more widespread, as Blouberg is so far away from the other populations. The mismatch distribution (Figure 12) also depicts a range expansion. The timing of the range

expansion corresponds to between 18 000 and 45 000 years ago, i.e. the range expansion occurred before the Last Glacial Maximum. This corresponds to other studies that show that before the LGM forests were widespread (Lawes 1990, Scott *et al.* 1997). The timing was estimated from a broad survey of green algae and land plants (Bakker *et al.* 1995) that reported that sequence divergence in the ITS region ranges from 0.8-2.0%/Ma. Although this is only a rough estimate of timing, other methods for dating such as using fossils or a geomorphological occurrence (e.g., the formation of the Atacama Desert was used by McDaniel and Shaw (2003)), could not be applied since appropriate calibrations were not available for this study. It is not possible to find out from these data if the range expansions correspond to each other, but they both suggest that forests were once widespread.

Although there is evidence of previously widespread forests there is also evidence of dispersal. The recombinant haplotypes show that sexual reproduction is happening, and therefore dispersal through spores is very likely (Longton 1997). This can be seen with hapD (a recombinant haplotype), which is now found throughout South Africa, and in Portugal and Kenya.

The inference key result for Clade 1-2 suggests either long distance colonization or past fragmentation followed by range expansion. For both scenarios we can assume that HapC originated in either Jonkershoek or Nursery Ravine, as it is found there at relatively high frequencies. Either scenario is possible. Past fragmentation suggests that forests were previously widespread, which would have allowed hapC to spread. Upon fragmentation, new variants derived from hapC became common in the Sedgefield localities and through random processes the ancestral haplotype was lost. Fragmentation would also reduce the probability of the new haplotypes spreading as well as recolonisation by the ancestral form. Alternatively, long distance colonization suggests that hapC spread to Sedgefield via a long distance colonization event, where it produced two new haplotypes. As hapC came to Sedgefield via random dispersal, it is not present there at high frequencies, or has been lost due to drift, and so was not sampled.

Although there is population structuring with *L. smithii*, there is also evidence for dispersal and widespread forests. It is not clear from the results to what extent dispersal does still happen or if it is necessary for forests to be widespread for

dispersal to occur. It should be possible to estimate the immigration rate per generation between populations, using the program LAMARC (Beerli and Felsenstein 1999). This program uses a likelihood approach to integrate the parameter estimates over all possible genealogies and migration events among populations (Beerli and Felsenstein 1999). However when the analyses were attempted on this data set, they did not work, most likely because of low levels of variation. The results do give an indication of the complex history of *L. smithii*, which includes processes such as fragmentation, range expansion and dispersal.

#### 4.3 A better understanding of Cape forest history: toward a comparative statistical phylogeographic approach

*L. smithii*, *A. capensis* and *P. spiniforme* show substantial levels of variation, and for each there are strong indications that this variation is geographically structured. The structure must be related to forest history as the mosses are found only in forests. For example, the results from *L. smithii* show that there is structuring among populations. If the forest patches have become established only recently, there would be no geographic component to haplotype variation among the populations; for the area studied, therefore, recent invasion is not a likely scenario.

The present study is largely exploratory, and therefore much of the phylogeographical analysis is effectively descriptive. While it is possible to infer hypotheses about restricted gene flow and range expansion with descriptive phylogeography, it is not possible to distinguish between alternative hypotheses, nor is it possible to attach confidence limits to the hypotheses (Knowles and Maddison 2002). Furthermore the descriptive approach takes no account of the stochastic nature of the coalescent process (Knowles 2004, Carstens *et al.* 2005). It is historical processes such as range expansion, long distance colonization and fragmentation that actually form the genetic structure, but descriptive approaches do not always provide appropriate tests between alternative processes. It is also possible with descriptive approaches to over interpret the data, which can be extremely misleading (Knowles and Maddison 2002).

Descriptive approaches are based on *ad hoc* explanations of genetic variation, but with statistical phylogeographic approaches it is possible to test hypotheses by performing coalescent simulations of population models that are defined *a priori*

(Knowles 2001, Knowles 2004). The models can incorporate a wide range of different histories, and so can account for the complexity of forest history (Knowles 2004). However the models should be able to distinguish between alternative hypotheses, and be simple enough to be tested (Knowles and Madson 2002, Knowles 2004, Carstens *et al.* 2004a). In order to use these methods it would be necessary to do more sampling.

Future progress in understanding the history of South Africa's forests will rely on incorporating explicit tests of historico-demographic hypotheses. The hypotheses (from literature) of why forests have a disjunct distribution can be tested through a gene tree/population tree approach (Knowles 2001) and coalescent simulations. The hypothesis of fragmentation of a previously widespread forest (Acocks 1953, Levyns 1964) would have each tip on the population tree represented by an individual population, while the alternative hypothesis of multiple refugia (Midgley *et al.* 1997) would have each tip represented by regional groupings of populations, e.g. Knysna forest, and Jonkershoek and Nursery Ravine. This study found shared haplotypes among all the populations, which can either be explained by interpopulation dispersal or incomplete lineage sorting of ancestral polymorphisms: the alternative hypotheses could be tested using a similar approach (Carstens *et al.* 2004b). Another hypothesis to test would be that geographically dry areas, such as the Sundays and Fish River valleys (Geldenhuys 1989, Lawes 1990), are a barrier to gene flow. Incorporating external data from previous palynological and biogeographical studies makes the tests more robust (Knowles 2004). Other methods apart from gene tree/ population tree method are available for testing hypotheses e.g. parametric bootstrap and Bayesian analyses (Carstens *et al.* 2004a). Which method to use depends on the question being asked (Knowles 2004).

Of the species sampled, only *N. valentiniana* showed no variation at the sampled locus. While absence of variation is not directly useful for phylogeographic reconstruction, (i.e. it is not possible to do phylogeographic analyses with no variation) it can still be informative. The absence of variation strongly suggests that the species experienced a genetic bottleneck (due to decreased population size from being restricted to refugia), with subsequent recent dispersal. Since *N. valentiniana* prefers wet forests, this suggests that they were rarer (i.e. extremely small fragments) in the past. It might be useful to try using rapidly evolving markers such

as ISSRs or AFLPs with *N. valentiniana*. Although they lack the historical aspect of sequence data, the patterns of variation might reveal where refugia for *N. valentiniana* would have been located.

The only way to distinguish a particular species' history from broader landscape history is to incorporate multiple species in a study. In this study the differences in genetic variation highlight the value of comparing multiple species. At the same locus some species had substantial variation while one had none. If only one species had been studied, the information about either phylogeographic patterns or recent dispersal would not have been known. Absence of variation might have been seen to be just an artifact of that marker, and not related to recent dispersal. Conversely, phylogeographic congruence indicates shared history (Arbogast and Kenagy 2001), for example, congruent patterns of phylogeography in vertebrates in Australian rainforests have confirmed previous theory about historical barriers predicted by climatic modelling (Moritz 1996).

Diverse organisms such as bryophytes, vascular plants, invertebrates, mammals and birds have different histories because of different habitat preferences and levels of dispersal. They also occupy different trophic levels and have different life history patterns (Cruzan and Templeton 2000). Therefore, to fully elucidate forest history it would be necessary to study a range of forest faithful organisms. The best way to synthesize data from comparative studies would be through testing independent *a priori* hypotheses (Carstens *et al.* 2004a).

#### 4.4 Conclusions

With reference to the particular aims of this study:

- 1) The phylogeographic patterns for *Leptodon smithii* show that recombination does occur in ITS, and that the genetic variation for both markers is geographically structured. From genetic parameters and phylogeographic patterns there is evidence for population structuring, previously widespread forests and dispersal. Therefore the history of *L. smithii* is complex, and so it was not possible to detect its' history comprehensively.
- 2) *Aerobryopsis capensis* has sufficient levels of variation for it to be used in phylogeographic analyses; it just needs more sampling.

- 3) There is variation in *Pyrrhobryum spiniforme*, but before it is studied further the length variation within the ITS sequences needs to be resolved.

There is potential for elucidating forest history with bryophyte phylogeography. As Chiang and Schaal 1999 propose, "mosses are ideal candidates for studies that examine broad patterns of biogeographical differentiation." To get better resolution of forest history it would be necessary to increase sampling, within the species studied, and to include more diverse species, and to incorporate statistical phylogeographic methods. Phylogeographic methods are conducive to widespread sampling, which is particularly important in an African context, as there is such a rarity of paleoecological sites.

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## Appendix one

Sample numbers, names, localities and collector for all accessions used in this study. *trnL-F* and ITS1 refer to the haplotype allocation from the TCS analysis. I sequenced all the samples, except *Leptodon smithii* (numbers 61 – 85; and 91 – 95), which were from obtained a previous study (Mwafongo, 2002).

Table 10: Collection details and haplotype allocations for the *Leptodon smithii* samples

Number	Sample name	Locality	Province	Collector	<i>trnL-F</i>	ITS1
South African samples						
1	N1	Nursery Ravine, Table Mountain	Western Cape	Mwafongo	2	A
2	N2	Nursery Ravine, Table Mountain	Western Cape	Mwafongo	1	C
3	N3	Nursery Ravine, Table Mountain	Western Cape	Mwafongo	2	C
4	N4	Nursery Ravine, Table Mountain	Western Cape	Mwafongo	1	C
5	N5	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	2	A
6	N6	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	2	A
7	N7	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	2	B
8	N8	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	D
9	N9	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	2	A
10	N10	Nursery Ravine, Table Mountain	Western Cape	Mc Grath		A
11	J1	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	M
12	J1b	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	2	C
13	J2	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	A
14	J3	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	A
15	J4	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	H
16	J5	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	A
17	J6	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	A
18	J7	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	2	L
19	J8	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	C
20	J9	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	2	C
21	SF1	Groenvlei, Sedgefield	Western Cape	Hedderson 14725	1	B
22	SF2	Groenvlei, Sedgefield	Western Cape	Hedderson 14730	1	A
23	SF3	Groenvlei, Sedgefield	Western Cape	Hedderson 14729	1	A
24	SF4	Groenvlei, Sedgefield	Western Cape	Hedderson 14728	1	F
25	SF5	Groenvlei, Sedgefield	Western Cape	Hedderson 14724	1	O
26	SF6	Groenvlei, Sedgefield	Western Cape	Hedderson 14727	1	F
27	SF7	Groenvlei, Sedgefield	Western Cape	Hedderson 14726	1	A
28	SF8	Groenvlei, Sedgefield	Western Cape	Hedderson 14727	1	F
29	FK3	Fernkloof, Hermanus	Western Cape	Mc Grath	1	E
30	FK4	Fernkloof, Hermanus	Western Cape	Mc Grath	1	G
31	FK5	Fernkloof, Hermanus	Western Cape	Mc Grath	1	E
32	FK7	Fernkloof, Hermanus	Western Cape	Mc Grath	1	E
33	FK8	Fernkloof, Hermanus	Western Cape	Mc Grath	1	E
34	LS23	Fernkloof, Hermanus	Western Cape	Mc Grath	1	G
35	LS24	Fernkloof, Hermanus	Western Cape	Mc Grath	1	B
36	LS25	Fernkloof, Hermanus	Western Cape	Mc Grath	1	B
37	LS26	Fernkloof, Hermanus	Western Cape	Mc Grath	1	I
38	LS27	Fernkloof, Hermanus	Western Cape	Mc Grath	1	G
39	LS15	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	B
40	LS16	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	B
41	LS17	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	D
42	LS18	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	K
43	LS19	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	D

Number	Sample name	Locality	Province	Collector	trnL-F	ITS1
44	LS6	Grootvadersbosch, Heidelberg	Western Cape	Mc Grath	1	N
45	LS32	Krantzberg	Limpopo	Hedderson 15739		T
46	LS33	Krantzberg	Limpopo	Hedderson 15750	1	B
47	LS34	Blouberg	Limpopo	Hedderson 15703	1	U
48	LS35	Blouberg	Limpopo	Hedderson 15701	1	B
49	LS36	Blouberg	Limpopo	Hedderson 15700	1	B
50	LS37	Blouberg	Limpopo	Hedderson 15698	1	B
51	WF	Montague's Pass, George	Western Cape	Hedderson 14692	1	A
52	LS3	Grootwinterhoek, Tulbagh	Western Cape	Hedderson 14230	1	Q
53	LS4	Grootwinterhoek, Tulbagh	Western Cape	Hedderson 14262	1	B
54	LS7	Fernkloof, Grahamstown	Western Cape	Hedderson 14883	1	D
55	LS8	Fernkloof, Grahamstown	Western Cape	Hedderson 14894	1	B
56	LS9	Helderberg, Somerset West	Western Cape	Chase	2	B
57	LS10	Helderberg, Somerset West	Western Cape	Chase	1	B
58	LS11	Diepwalle, Knysna	Western Cape	Mc Grath	1	D
59	LS12	Diepwalle, Knysna	Western Cape	Mc Grath	1	B
60	LS13	Diepwalle, Knysna	Western Cape	Mc Grath	1	B
61	6L	Natal, South Africa	Kwa-Zulu Natal	Magill 5130	1	B
62	41L	Above Gudu Falls,Drakensberg	Kwa-Zulu Natal	Magill 6754	1	D
63	42L	Above Gudu Falls,Drakensberg	Kwa-Zulu Natal	Magill 6749	1	
64	18L	Uitvlugt farm, Paulpietersburg	Kwa-Zulu Natal	Glen 2287	1	
65	67L	Oliviershoek, Drakensberg	Kwa-Zulu Natal	Magill 6759	1	B
66	9L	Buffels Nek, Knysna	Western Cape	Magill 5961		Z
67	10L	Buffels Nek, Knysna	Western Cape	Magill 5985		S
68	14L	Graskop mts, Transvaal	Mpumalanga	Stirton 9928	1	
69	15L	Mianlane Forest station	Eastern Cape	van Rooy 2271		R
70	16L	de Kelders	Western Cape	Taylor 10397		G
71	20L	Euefeeskloof, Zastron	Orange Free State	van Rooy 2561	1	A
72	47L	Orange Kloof, Table Mountain	Western Cape	Hylander 10823		B
73	49L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo		D
74	51L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	
75	52L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	2	
76	53L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo		D
77	54L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo		D
78	56L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	1	B
79	57L	Jonkershoek, Stellenbosch	Western Cape	Mwafongo	2	D
80	58L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo	1	B
81	59L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo		D
82	60L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo		D
83	61L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo		K
84	62L	Nursery Ravine, Table Mountain	Western Cape	Mwafongo		D
85	22L	Bosberge Forest, Somerset East	Eastern Cape	Smook 3957	3	B
Non South African samples						
86	LS27A	Kenya		Chuah-Petiot	1	D
87	LS29	Kenya		Chuah-Petiot		J
88	LS30	New South Wales, Australia		Stajsic and Klazenga VS2854	1	B
89	LS31	New South Wales, Australia		Stajsic and Klazenga VS2828	1	B
90	1L	Madeira, Ribeira da pont do solo		Fontinha, Nobrega, Hedenäs	6	R
91	2L	Madeira, Ribeira da pont do solo		Fontinha, Nobrega, Hedenäs	4	X
92	3L	Madeira, Cidrao		Hedenäs B9717	4	Y
93	4L	Madeira, Siera Margarita		Een	5	B
94	5L	Portugal, Coimbra		Hedenäs B44578	5	D

Table 11: Collection details and haplotype allocations for the *Neckera valentiniana* samples

Sample name	Locality	Province	Collector	trnL-F	ITS1
1-1	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	-	A
1-3	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	-	A
2-3	Window Gorge, Table Mountain	Western Cape	Mc Grath	-	A
G1	Grootvadersbosch	Western Cape	Mc Grath	-	A
G2	Grootvadersbosch	Western Cape	Mc Grath	-	A
G3	Grootvadersbosch	Western Cape	Mc Grath	-	A
W1	Wilderness	Western Cape	Hedderson	-	A

Table 12: Collection details and haplotype allocations for the *Aerobryopsis capensis* samples

Sample name	Locality	Province	Collector	trnL-F	ITS1
AC1	Natures valley	Western Cape	Hedderson 15009	1	E
AC2	Sedgefield	Western Cape	Hedderson 15033	1	D
AC3	Sedgefield	Western Cape	Hedderson 15047	1	
AC4	Montague's Pass, George	Western Cape	Hedderson 14675	1	C
AC5	Natures valley	Western Cape	Hedderson 15004		B
AC8	Knysna	Western Cape	Mc Grath	1	A
AC9	Diepwalle, Knysna	Western Cape	Mc Grath	2	A
AC10	Diepwalle, Knysna	Western Cape	Mc Grath	1	
AC11	Diepwalle, Knysna	Western Cape	Mc Grath	1	A
AC12	Diepwalle, Knysna	Western Cape	Mc Grath	1	A
AC14	Garden of Eden, Knysna	Western Cape	Mc Grath	1	B
AC15	Garden of Eden, Knysna	Western Cape	Mc Grath	1	A
AC16	Garden of Eden, Knysna	Western Cape	Mc Grath		A
AC17	Grootvadersbosch	Western Cape	Mc Grath	1	A
AC18	Grootvadersbosch	Western Cape	Mc Grath		A
AC21	Grootvadersbosch	Western Cape	Mc Grath	1	A

Table 12: Collection details and haplotype allocations for the *Pyrrhobryum spiniforme* samples

Sample name	Locality	Province	Collector	trnL-F	ITS1
PS8	Diepwalle, Knysna	Western Cape	Mc Grath	1	B
PS9	Diepwalle, Knysna	Western Cape	Mc Grath		B
PS10	Diepwalle, Knysna	Western Cape	Mc Grath	1	A
PS11	Diepwalle, Knysna	Western Cape	Mc Grath	1	B
PS13	Diepwalle, Knysna	Western Cape	Mc Grath	1	
PS15	Diepwalle, Knysna	Western Cape	Mc Grath	1	A
PS16	Diepwalle, Knysna	Western Cape	Mc Grath	2	A
PS17	Diepwalle, Knysna	Western Cape	Mc Grath	1	A
PS18	Eshowe	KwaZulu Natal	Hedderson 14796	1	A
PS19	Eshowe	KwaZulu Natal	Hedderson 14801 1\2	1	A
PS20	Eshowe	KwaZulu Natal	Hedderson 14801 2\2	1	A
PS21	Jonkershoek, Stellenbosch	Western Cape	Mc Grath	1	A
PS25	Jonkershoek, Stellenbosch	Western Cape	Mc Grath	1	B
PS26	Jonkershoek, Stellenbosch	Western Cape	Mc Grath		B
PS27	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	A
PS28	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	3	
PS29	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	B
PS30	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	
PS31	Nursery Ravine, Table Mountain	Western Cape	Mc Grath		B
PS32	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	
PS33	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	
PS34	Nursery Ravine, Table Mountain	Western Cape	Mc Grath	1	