A Phylogeographic analysis of *Tetraria triangularis* and *T. ustulata* in the mountains of the south Western Cape

Dissertation in Plant Ecology

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(1) Abstract

Fluctuating altitudinal zonation may fragment the ranges of high altitude species, potentially resulting in high levels of genetic differentiation among populations. This study compares the genetic structure in the populations of two *Tetraria* species: the attitudinally restricted *T. triangularis* and a species of low-mid altitudes, *T. ustulata*. Chloroplast (psbA-trnH) sequence data was investigated, using standard phylogeography methods. Haplotype distributions and AMOVA results reveal significant genetic differentiation between populations and mountain blocks for *T. triangularis*, and not for *T. ustulata*. However results for *T. ustulata* require cautious interpretation due to small sample sizes. There is a refugium in the Limietberg Mountains, with fragmentation in the periphery populations. Additional sampling in the drier Ceres and district is expected to reveal greater genetic differentiation.

(2) Introduction

(1) The framework

The Cape Floristic Region (CFR), situated at the south – western tip of the southern Africa is exceptionally speciose (Goldblatt 1978; Linder, 2003), with ca. 9000 species in an area 90 000km^2 large (Linder, 2003). The majority of the diversity within the CFR is accounted for by Beta and Gamma variation (Linder, 1985; Linder, 1991). In general it has been shown that levels of alpha diversity are not exceptionally high (Bond, 1983). It has long been noted that there is variation at the scale of tens of kilometres for species related in typical Cape Flora Groups (Levyns 1958). At the same time areas of high species richness are often found directly adjacent to areas of low species richness.

Linder (1991) found this diversity is highest in the moister mountain areas dominated by Fynbos vegetation. The topography of the region is dominated by two main sets of mountains, one running parallel to the south coast and the other running parallel to the west coast. These provide a mosaic of alternating areas of high and low altitude. The result is a dissected landscape with many separations between populations. This in combination with barriers to gene flow, such as pollinator specialisation, shifts in flowering times and diversity in abiotic conditions across landscapes, could account for the high levels of speciation in the CFR (Linder 2003).

Range shifts are hypothesised to be driven by species specific physiological thresholds of temperature and precipitation tolerance, in combination with dispersal and reproduction capability (Walther *et al*, 2002). It is suggested that since the last glacial maximum was characterised by cooler and wetter conditions in some parts of the CFR (Meadows and Baxter, 1999), changes from that condition to warmer and drier condition at present, would have driven altitudinal zones up. Work from stable isotopes in glaciers (Petit *et al.*, 1999) implies regular patterns of temperature oscillations in 13 000 year cycles between glacial maxima for the past 40 000 years. This climatic oscillation, overlaid on mosaic of high and low ground, could potentially drive cycles of separation and unification of

altitude-limited species. If the separations are complete enough the separations could result in speciation, resulting in high levels of gamma diversity, perhaps at the scale of mountain blocks, with different species restricted to different blocks (Linder, 2003).

Population contraction associated with the start of warmer, drier conditions will be reduced, resulting in fixation of haplotypes and increased structure which genetic differentiation should reflect. In contrast, I hypothesise that the pairing of peaks with shared single haplotypes will be defined by the highest connection point between those peaks. The lowest differentiation levels will be found between peaks with high altitude connections.

A study conducted using mDNA for the butterfly *Parnassius smintheus* revealed centres of diversity, where groups of refugia (peaks within mountain blocks) shared related haplotypes within groupings (DeChaine & Martin, 2004). The pattern was repeated at a higher hierarchy level, between refugia groupings.

If patterns of structuring are such that many peaks have homogenous haplotypes, then it will be possible to relate peaks with the same haplotype present to a particular climate fluctuation. The size scale at which patterns will be discernable will be determined by the strength of the relationship between bioclimatic envelopes and species' range (Precipitation/Temperature boundaries), and dispersal ability of both pollen and seed.

(2) Aims and Objectives

In this study I seek to examine the distribution of haplotype diversity between a pair of closely related species. One of which, *T. triangularis* is attitudinally limited to putatively isolated mountain peaks. The other is a lowland species; *T. ustulata* whose range is presumed to be continuous across the area where *T. triangularis* is sampled. *T. triangularis* is a low growing (<0.5m) sedge, which is wind pollinated and does not utilise specialised dispersal mechanisms.

This initial study uses a chloroplast marker (psbA-trnH) to avoid the complication of recombination in nuclear DNA. Given evidence for the maternal inheritance mode of plastid DNA this means that this study is essentially tracking seed dispersal and isolation between peaks. Koenig & Ashley (2003) have shown that in Californian Oaks the closest associations in terms of pollen transfer, were between trees less than 60-80 metres apart. If the same holds true in this study, where sites are of the order of tens of kilometres apart, pollen transfer should not play a major role in interbreeding between distinctly separated populations. It is important to heed that single dispersal events have the potential to have big influence on gene flow patterns.

For this reason a plant with a generalist wind pollination syndrome and seeds with no specialised dispersal mechanism is preferred. This will reduce, but not eliminate, the probability of random long distance dispersal events between populations.

In order to distinguish interpopulation differentiation due to mountain block isolation from that of geographic distances, *T. ustulata* was sampled for its continuous range, which overlaps with that of that of *T. triangularis*. If mountain block isolation is important, the prediction is that genetic differentiation in *T. ustulata* should be less than that seen in *T. triangularis.*

The aims of this study are to test the importance of topography in driving genetic differentiation in attitudinally limited species. Furthermore I aim to see if there is any evidence for past climatic fluctuations in the genetic structure of *T. triangularis*.

Analytical Approach

Phylogeographic studies investigate the distribution of allele variation in an explicit geographical context (Schall and Olsen 2000). Phylogeogaphy forms the basis of this study and two forms of analysis were used to explore the genetic pattern: AMOVA and nested clade analysis.

AMOVA analyses of chloroplast DNA sequence data will ascertain the levels at which genetic differentiation is expressed between groups of populations, between populations and within population. Wright's *F* statistics, includes the frequency of diversity amongst populations (F_{ST}), provides a measure of the population genetic differentiation, which varies from the balance between gene flow and genetic drift. The statistics thus give key insight into past histories of populations (Excoffier *et al* 1992). Thus recognizing past patterns (e.g. bottlenecks, gene flow) in genetic partitioning can help to support or refute hypotheses about past (and ongoing) historical events that may affect the structuring of populations.

Nested clade analysis will be used to infer past population events. Nested clade analysis is based on a hierarchical network, based on mutational steps of a variable allele. The assumption is that extant individuals are branch tips in genealogies and that they coalesce back to common ancestor nodes (Avise, 2000).

Templeton and Sing (1993) developed the nested clade analysis (NCA) to test the null hypothesis of no geographical association of haplotypes. The test allows for analysis as to whether significant associations are due to restricted gene flow and identify patterns of significant association that are due to historical events. Nested clade analysis can be used to infer past events such as historical fragmentation, range expansion, restricted gene flow and dispersal that may have influenced the observed population structure of the species (Templeton *et al* 1995).

Caution should be used when considering inferences made by NCA because the inference key (Templeton *et al.*, 1995) uses qualitative assessments and fails to estimate error or statistical examine alternative interpretations possible from the dataset (Knowles and Maddison, 2002).

(3) Methods

(3.1) Study site and Study species

The study is situated in the heart of the Cape Floristic Region, being bounded by 33°16' to the North and 34°16' in the South, 18°55' in the west and 19°40' in the east. This area contains a range of mountain peak heights and more-or-less distinct mountain blocks, which provide a suitable natural experiment to test the effects of topography on genetic structure. Collection sites were guided by the specimen collections housed in the Bolus Herbarium (BOL) at the University of Cape Town, and the Compton Herbarium at the National Botanical Institute (NBI) at Kirstenbosch.

Two species of *Tetraria* were sampled: *T. triangularis*, whose distribution is restricted to higher altitudes, and *T. ustulata*, which occurs across a range of altitudes. Both species share a similar tufted perennial growth form (<1m), seed size and dispersal mode.
The sampling localities of the two species were matched as far as possible to allow for a direct comparison of genetic structure in each.
T. triangularis samples were collected on twelve peaks in three core mountain blocks as defined by low points (<500m) between adjacent high lying areas. *T. ustulata* was collected at 10 sites and nominally assigned to the same mountain block groupings as *T. triangularis*. The Collections sites are divided as described in the table overleaf.

Table 1: Collection localities for *T. triangularis* and *T. ustulata*

Mountain Block	*T. triangularis*	Code	No.	*T. ustulata*	Code	No. seqs
Hex River	Matroosberg	M	5	Martroosberg	M	2
Hex River	Brandwacht	B	1			
Hex River	Perry refuge	P	4			
Hex River	Waaihoek	WH	2	Waaihoek	WH	1
Limietberg	Du Toits	D	8	Du Toit's	D	4
Limietberg	Wemmesrhoek	W	9			
Limietberg	Stettyn's	S	8	Stettyn's	S	1
Limietberg	Blokkop	BK	5	Blokkop	BK	4
Riviersonderend	Jonaskop	J	4	Jonaskop	J	3
Hottentot's Holland	Noordekloof	N	4	Franschoek pas	F	1
				Jonkershoek	JH	5
Hottentot's Holland	Landdroskop	L	5	Viljoen's pas	V	3
Hottentot's Holland	Mount Lebanon	ML	4	Houhoek	H	3
		12	59		9	27

(3.2) Field collection

Specimens were collected at ca.100 stride intervals on transects across and along slopes. This sampling strategy was used to allow for effect of neighbouring plants being closely related and thus skewing the analysis toward populations with homogenous haplotypes. As far as possible 30 individuals were sampled at each site, but time constraints and small population sizes led to smaller sample numbers at some localities (e.g. Brandwacht).

At each study site a herbarium voucher was collected. At some sites (notably Stettyn's, Du Toits and Wemmershoek) more than one form of *T. triangularis* was noted and representative vouchers of both were taken. In future studies it is recommended that a voucher be made of every individual sampled.

In order to avoid contamination by endoparasites, the youngest and healthiest available leaves were collected for DNA studies. Leaf samples were collected in silica gel and stored in small Ziploc bags, to ensure that the material dried out as quickly as possible. The position and altitude of each collection was recorded using an Etrex Global Position

Satellite receiver (GPS). In the laboratory the silica gel was changed to ensure that the material dried out completely.

(3.3) Laboratory Preparation Protocol.

Total genomic DNAs were extracted roughly following the CTAB method as described by Doyle & Doyle (1989), as follows: ca. 60mg of plant material was ground in a ceramic mortar with liquid nitrogen, a small amount of PVP-40 ground sand. The grind was then added to 800µl of preheated hexadectyltrimethylammonium (2*CTAB) extraction buffer and β-mercaptoethanol. This mixture was then incubated for at least 1 hour at 65°C. After incubation 800µl chloroform: isoamyl alcohol (24:1 v/v), samples were then mixed by inversion for 5 minutes and centrifuged for 5 minutes at 12 000rpm.
The aqueous supernatant was then pipetted into a clean labelled microfuge tube, into which an equal volume of ice-cold isopropanol was added. Overnight storage at -10°C allowed the DNA to precipitate. The tubes were then centrifuged at 12 000rpm to recover the DNA The ice-cold isopropanol was then poured off. Microfuge tubes were left to dry off for ca. 15 mins. Once all excess isopropanol had been wiped off the 250µl 75% ethanol was added and the microfuge spun at 12 000 rpm for 2-3mins. The ethanol was then discarded and the tube inverted to allow DNA to dry (2-3 hours), the DNA was then resuspended in 50µl of TE (10mM tris-Cl ph 7.4, 1mM EDTA pH 8.0).

The psba-trnH intergenic spacer region of cpDNA was amplified using the primers psbAR and trnHF (Sedge design courtesy of Dr. Tony Verboom). Polymerase chain reactions (PCR) were carried out using 0.25 units of BIOTAQ™ DNA polymerase (Bioline) in 50µl volumes also containing 5µl 10× NH_4 buffer, 5µl of $MgCl_2$, 2µl dNTP, 1µl of each of the primers, with 1µl of unquantified diluted DNA template. Thermo-cycling was carried out on a Biosystems Hybaid sprint set to the following conditions: initial denaturation at 94°C for 2 mins, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 mins and a final extension step for 7 mins at 72°C

Table 2: Primers used to amplify and sequence the Chloroplast psbA –trnH region

Primer	Inamp	Sequencing	Direction	Sequence(5'-3')
psbAR	Y	Y	Forward	TGCTGTTGAGTTCCTTCTATCG
trnHF	Y	N	Reverse	ACTGCCTTGATCCACTTGGC
psbAAAR	N	Y	Reverse	AAAAAAAAAAGAGAAATACCC
Psb541R	N	Y	Reverse	ATTGGGTATTTCTCTTTC

The amplified DNA was cleaned using GFX™ PCR DNA and Gel Band purification Kits (Amersham Biosciences). The protocol was as for the instructions contained in the kit.

Sequencing was carried out using the psbAR for forward directions and the reverse directions sequenced using psbAAAR and psb541R. These additional internal primers were used to resolve the sequence on the 3' side prominent photopolymer that prevented "clean" sequencing between the psbAR and trhHF sites. The repeating motifs prevalent in this spacer region made the design of additional primers difficult due the number of possible sites where the primer could bind. Sequencing was carried out in 10µl volumes, containing 2µl of BigDye® Terminator v2.0, 1.5µl 5×Sequencing buffer (Applied Biosystems) and a calculated volume of cleaned DNA product based on an estimation of amount of DNA form an acrylamide gel. And the remaining volume made up to 10µl nanopure water.

Cycle sequencing products were resolved with using a Genetic Analyser (ABI PRISM 3100), by the core sequencing facility at the University of Stellenbosch.

(3.4) Compiling sequence data

Forward and reverse strands of DNA sequences were assembled and checked for inaccurate base calling using SeqMan II (LaserGene System Software, DNAStar. Inc.) Consensus sequences were aligned manually using MegAlign (LaserGene System Software, DNAStar. Inc) sequence alignment software. The ends of the alignment were trimmed off data to give all sequences the same length. Poor consensus files were omitted from the data set.

(3.5) Data analysis

(3.5.1) Geographical distribution of haplotypes

In order to evaluate the geographic distributions of the alternative haplotypes, pie charts of each population were made, and then superimposed on a map of the study area.

(3.5.2) Isolation-by-distance

Analysis of Molecular Variation (AMOVA) was carried out using Arlequin 2.0 (Schneider *et al.*, 2000) to test the hierarchical population structure of genetic variation between populations and mountain blocks as defined in Table 1. These analyses are based on differences between haplotypes to calculate genetic distances. In addition, population pairwise F_{ST}'s were calculated and compared to the geographical distances between populations. These results were then compared to physical parameters to see how and if genetic distance related to these physical factors.

Regressions were performed between standardized pairwise populations F_{ST} and geographical distance between populations using Mantel test performed by XLSTAT 7.50. Geographic distance between populations was defined in three ways:

a) Geographical distance: linear distance in kilometres between populations
b) Highest point: Height of highest connection point adjoining two populations. If intermediate populations were separated by one or multiple populations, the highest of those points connecting them was used.
c) Depth joining the lower population to the highest connection point: defined as highest point- altitude of peak with lowest altitudinal boundary.

(3.5.3) Haplotype Networks & nested clade analysis

For the purpose of this analysis gaps were treated as missing and indel characters were coded as absence/presence and coded as separate nucleotide polymorphisms, using simple gap coding. Networks showing relationship between haplotypes were constructed using TCS v1.18 (Clement *et al.* 2000), based on statistical parsimony. The haplotype network was used to identify systems of nested haplotype clades according to rules set out by Templeton & Singh (1993).

Twenty unsampled haplotypes were inferred between those that had been identified in the study. This was issued to infer topology of the relationships among haplotypes the relationship between the inferred phylogeny and geography was implemented using the Geodis software, which implements the Nested Clade analysis (Templeton 1995).

(4) Results

4.1 Sequence data

In *T. triangularis*, a 975-bp length of the alignment of the sequence of the chloroplast psbA-trnH region analysed 59 individuals from 12 populations. The majority of the difference between populations was in the form of indels. Nine haplotypes were identified and the haplotypes sampled more than once were broadly geographically confined.

For T. *ustulata* a 1480-bp length of sequence from the chloroplast psbA-trnH region was aligned for 27 individuals from 9 populations. All of the variation barring one site in three sequences was in the form of indels. Fifteen haplotypes were identified, nine of which were private alleles. The sequences for *T. ustulata* were exceptionally long, in some cases 800bp. Some of the contiging between forward and reverse strands only overlapped on the very ends of the sequence readouts. This coupled with a repeating mosaic patter, resulted in greater than great difficulty in finding the overlap position and

a lack of confidence resulting sequence. The result may be a greater than expected number of haplotype, that only further sampling or sequencing with internal primers, will be able to solve.

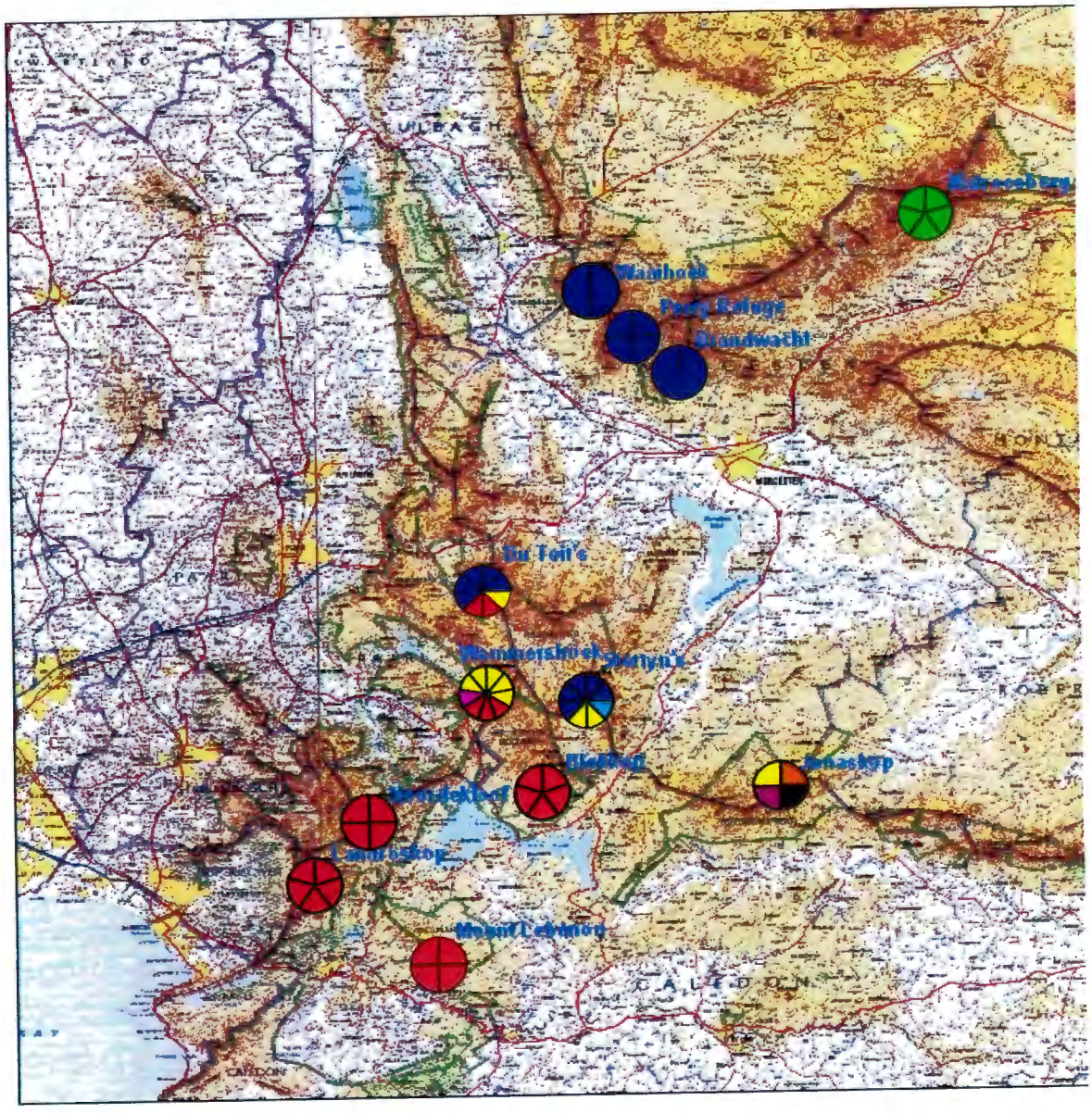

Fig 1: Distribution of the 12 sampled populations of *T. triangularis,* in the fold mountains of the south Western Cape. Colours correspond to that given in the Nested Clade diagram (Fig 2)

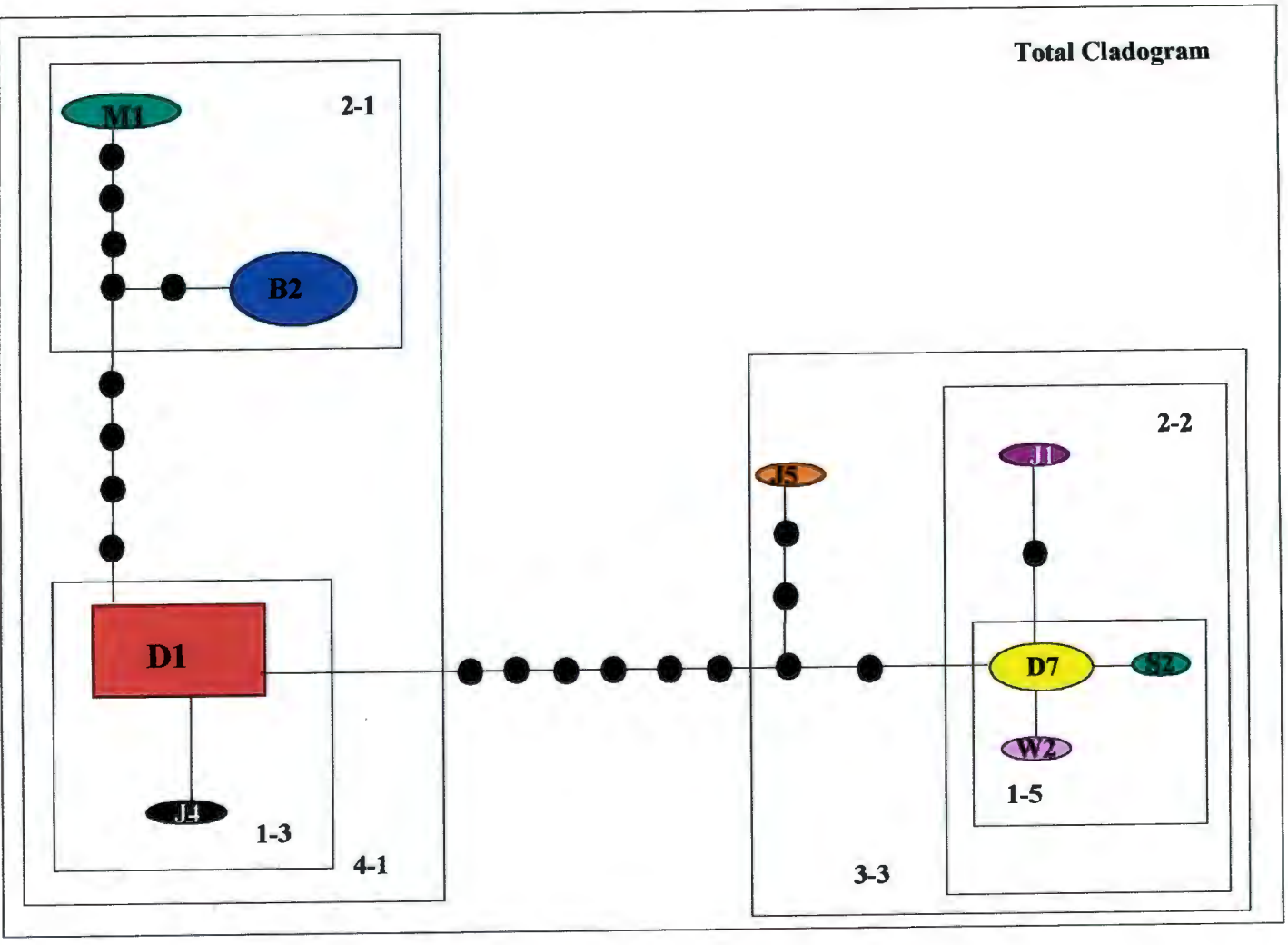

Fig 2: Haplotype Network for Nested Clade analysis. The 9 haplotypes for *T. triangularis* are shown, with only informative clades used in Geodis analysis represented. Black dots indicate inferred haplotypes.

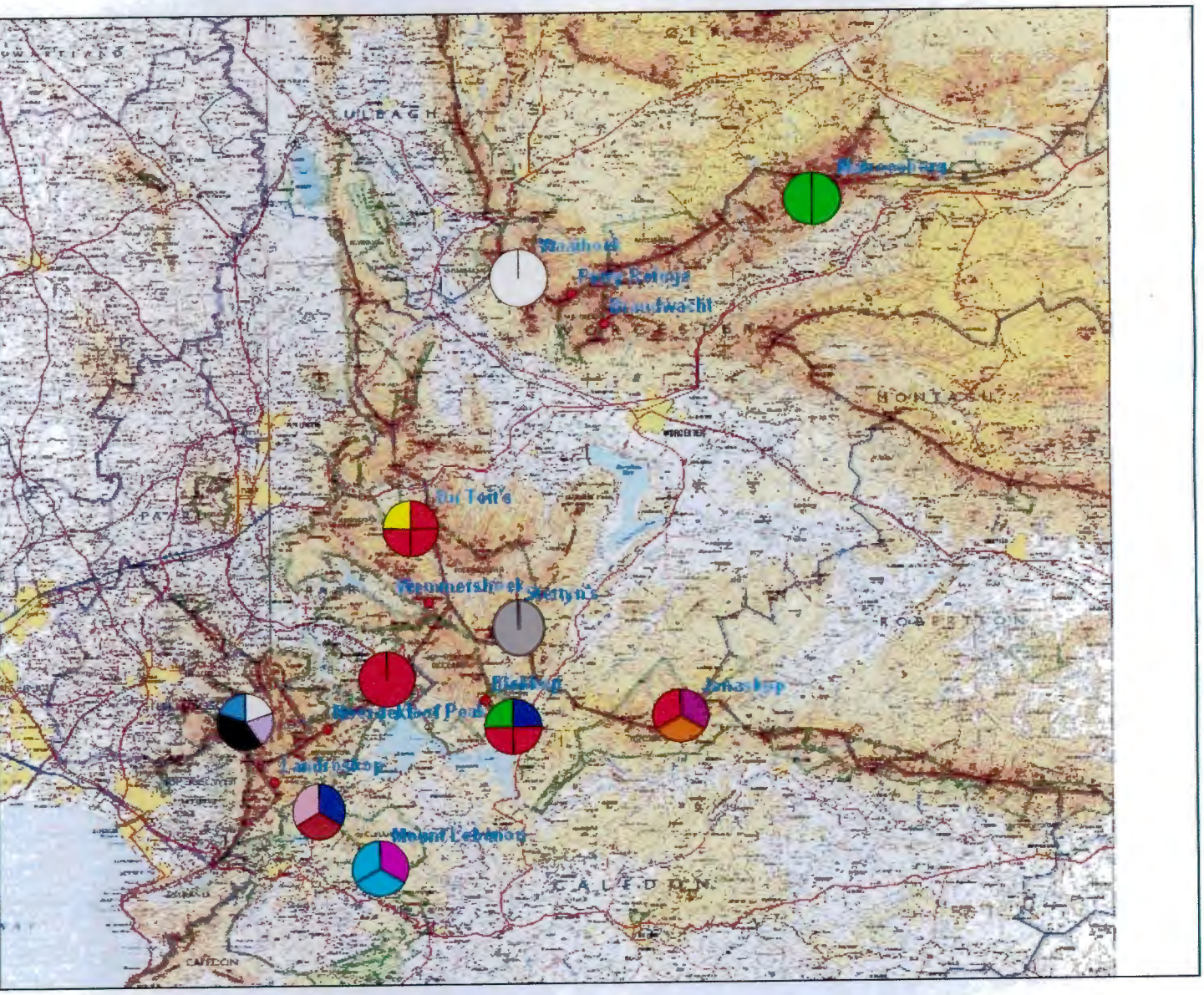

Fig 3: Haplotype distribution of *T. ustulata*, the distribution is comprised of 15 haplotypes 12 of which occur in only 1 population. The three that are shared by more than 1 population are: Red (BKc, occurring at Du Toits, Franshoek, Jonaskop and Blokkop), Navy Blue (BKa, occurring at Landroskop and Blokkop), Green (BKb, occurring at Matroosberg and Blokkop)

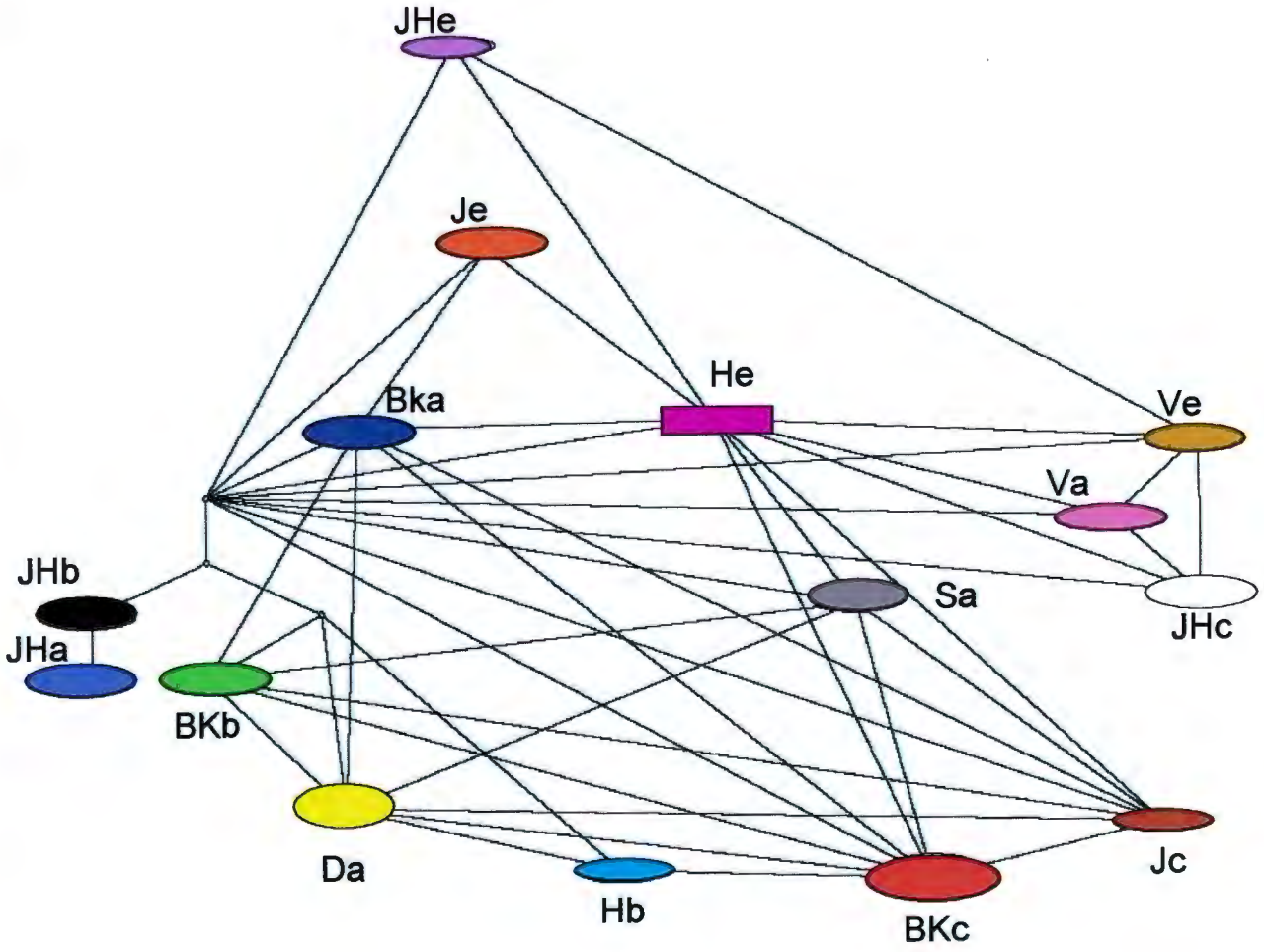

Fig 4: Haplotype network for *T. ustulata*, note that most haplotypes are unresolved colours used correspond with those used for the haplotype distributions. (Fig 3)

(4.2) Distribution of haplotypes between populations

(a) *T. triangularis*

In figure 1, it is evident that there are four haplotypes occurring in more than one population. In green is the Matroosberg haplotype (M1), blue the West Hex River/Limietberg haplotype (B2), in yellow the Limietberg and Riviersonderend haplotype (D7) and in red the Limietberg and Hottentot's Holland haplotype (D1). There is sharing of haplotypes among adjacent populations and no disjunction.

The most common haplotypes are distributed along a north-south gradient; the highest diversity is in the Limietberg block. There is haplotype fixation on 8 of the 12 populations. Five private alleles were recorded, three of which occurred in the Jonaskop population and the other two in the Limietberg, at Stettyn's and Wemmershoek. The geographic distribution of haplotypes suggests a distinct southern (set) population(s) and a region of mixing in the Limietberg and Jonaskop area (Fig. 1). There is a distinct population at Matroosberg and a mix group from the west of the Hex River Block mixed with the northern peaks of the Limietberg (Fig.1).

(b) *T. ustulata*

The arrangement of haplotypes is as less clear for *T. ustulata*. In general the haplotypes are widely spread with little geographic association. The most common haplotype BKc (Red, Fig 3), is confined within a single area (no disjunction). This may suggest association between haplotypes on the basis of geographical distance.
At this sampling scale (numbers of individuals per population), it is interesting that haplotype Bkb and JHc (green and white respectively Fig 3) are distributed over such a wide area, this gives a suggestion that there is more continuous breeding between sample

sites for *T. ustulata* than for *T. triangularis*. So the *T. ustulata* populations are structured such that are associations with distance, but not with the assigned mountain blocks. The sample sizes here are however too small to make statistical meaningful comparisons with patterns in the *T. triangularis*, but indication is that the *T. ustulata* data set would be worth exploring further.

(4.3) Isolation by distance

(a) *T. triangularis*

Table 3: AMOVA results for *T. triangularis* indicating levels genetic subdivision

Source of variation	d. f.	Variance components	Percentage of variation
Among groups	3	39.09584	32.96
Among populations within groups	8	41.28428	34.81
Within populations	47	38.23522	32.23

Analysis of molecular variance (AMOVA) revealed significant ($p < 0.000$) genetic structure at all three hierarchical levels (between mountain blocks, between populations within mountain blocks, within populations) (Table Y). Thirty-three percent of molecular variation was distributed between mountain blocks, 35% of the variation among populations within mountain block and the remaining 32% of the variation within populations. A substantial amount of variation is then distributed among populations, which supports the hypothesis of restricted gene flow and geographic isolation of populations, and mountain blocks. The high level of variation among populations within groups probably reflects the haplotypic diversity within the Limietberg, more than in the other two blocks. If only the Southern groups and northern groups were considered, then the pattern would be more likely to indicate higher % of variation between mountain blocks, low variation between populations within those blocks and lower variation within populations.

A signal of genetic differentiation among populations is positively correlated with genetic distance (F_{ST}), using the Mantell test ($r = 0.580$, $P < 0.001$). F_{ST} correlated negatively with highest point ($r = -0.337$, $P < 0.022$), but not with depth joining the lower population to the highest connection point ($r = 0.130$, $P < 0.211$).

(i) Lowest ranges of population boundaries

In figure 5, altitude is plotted against latitude (analogue to distance from the south coast). The result is a highly significant regression ($p < 0.00$), showing that when populations are collected further north the minimum altitudes at which populations are sampled increases in predictable fashion. The clear exception is the Limietberg block where the pattern is much less clear.

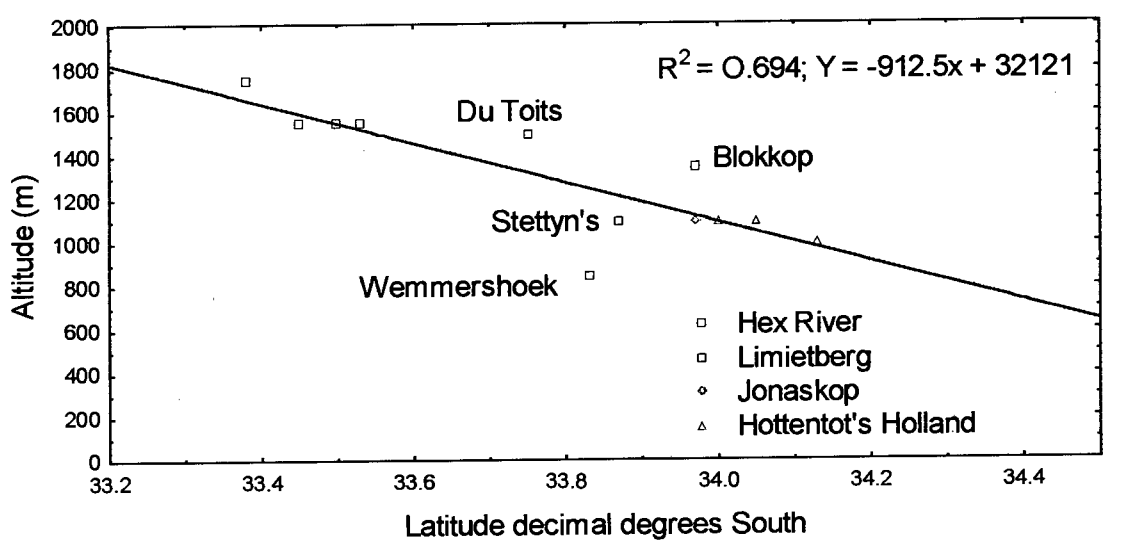

Fig 5: Plot of Altitude of collections at various peaks vs. Latitude($y=-912.5x+32121$; $R^2 = 0.694$ $p=0.0008$). **NOTE**: If Limietberg data is removed from the data set $R^2 = 9787$ ($y= 918.4x + 32336$; $p=0.00003$)

(b) *T. ustulata*

In T. *ustulata* 83% of the variation is found within populations and only 7.45% and 9.48% of the variation is found among mountain blocks and among populations within mountain blocks respectively (Table 4). This suggests that the genetic diversity in *T. ustulata* shows less population structure across the study area than *T. triangularis* is continuous amongst geographically defined mountain blocks. However these results must be viewed with caution, since the results from the AMOVA for *T. ustulata* are all non-significant ($p < 0.05$), most likely due to small sample size effects.

Table 4: AMOVA results for *T. ustulata* indicating levels of genetic subdivision

Source of variation	d. f.	Variance components	Percentage of variation
Among groups	3	11.76752	**7.45**
Among populations within groups	6	14.98497	**9.48**
Within populations	17	131.25000	**83.07**

(4.4) Haplotype networks and nested clade analysis

(a) *T. triangularis*

Haplotype networks

Haplotype D1 (Red, fig B) which is restricted to the southern part of the study area is estimated to have the biggest outgroup probability ($p = 0.3404$). The topology suggests refugia somewhere in the south (Hottentots Holland or Limietberg), with two key branches; the first separates the northern hex river clades M1 (green) and B2 (blue), the second separates the D7 haplotype (Jonaskop and Limietberg)

Nested clade analysis, based on this haplotype tree reveals the nested clade pattern shown in fig2. In figure 2 there are two distinct sub groupings that separate out, Clade 4-1 and clade 3-3. There is a second meaningful division between the ancestral D1 haplotype and

the Hex River. Processes were inferred as described in the latest version of the Templeton inference key downloaded fro the GeoDis Homepage (based on: Templeton, 1995) with results summarised in Table 5. Two possible instances of allopatric fragmentation were inferred in clade 2-1 and 1-3. And an inference was made of continuous range expansion in clade 4-1. All analyses concerning clade with private alleles (particularly Jonaskop haplotypes) were non significant due to small sample size. The strict rules of the analysis were violated to arrive at the two allopatric speciation outcomes, this was deemed acceptable, because of assumed populations (based on simple height correlations between sites) that were unsampled.

A population was assumed at Olifantsberg between Jonaskop and Stettyn's Piek, similarly population was inferred at Milner peak, between Brandwacht and Matroosberg.

Table 2: Results from Geodis analysis for *Tetraria triangularis*

Clade	Permutational CH^2 statistic	Probability	Chain of Inference	Inference
1-3	24.00	0.0410	1,19,(20)	**Allopatric fragmentation (or)**
			1,19,20	Inadequate geographical sampling
1-5	3.37	0.7180	0	Cannot reject Ho
2-1	21.00	0.0000	1,19	**Allopatric fragmentation (or)**
			1,19,20	Inadequate geographical sampling
2-2	5.45	0.2340	0	Cannot reject Ho
3-3	3.61	0.5380	0	Cannot reject Ho
4-1	40.27	0.0000	1,2,11,12	**Contiguous Range expansion**
Total	20.98	0.0010	1,2	**Inconclusive outcome**

(b) *T. ustulata*

He was adjudged to be the haplotype with the biggest outgroup probability (p = 0.1196). This haplotype is sample only once. Sample sizes are too small to resolve most of the loops connecting haplotypes, except that between JHb and JHa (Fig 4).

(6) Discussion

This study aimed to assess the importance of topography in driving genetic differentiation in high altitude species, in contrast to a closely related lowland species. And ask whether there is any evidence for past climatic fluctuations in the genetic structure.

T. triangularis display significant structuring at all three hierarchical levels that were assessed, based on the AMOVA analysis. The patterns of partitioning of variation in each of the hierarchical levels is greater within populations, than expected if sites were structured as distinct non-breeding populations on the scale of individual peaks or mountain blocks. Structuring in *T. ustulata* is partitioned mostly (80%) at the population level, this result is as expected for this species whose range is not explicitly fragmented by areas of unsuitable habitat, as is the case for *T. triangularis*. It must be cautioned however, that the AMOVA results are not significant, probably due to the low samples sizes that were sequenced.

Population F_{ST} values for *T. triangularis* are positively correlated with distance and negatively with depth between populations; this suggests that separation between peaks, is more of a function of distance between peaks and also reflects a gradient of haplotypic diversity across the study area. Perhaps the best predictor of population structure is the size of a valley's (function of depth and distance). This type of analysis was not feasible for the *T. ustulata* data due to inadequate sampling, resulting in non significant AMOVA values.

The inferences made from the NCA analysis, identify possible allopatric fragmentation between the Hex River haplotypes M1 (green) and B2 (blue). This scenario seems plausible considering that the Sanddrif Rivier gorge, divides the Hex River Mountains with a low point of 900m, between the sites where these haplotypes (B2; WH, P, B & M1; M) are represented. The inference for the separation between D1 (red) and J4 (black) is also potentially allopatric fragmentation (or inadequate sampling). This pattern is a little less easy to understand, given the amount of mixing in this area, that said, neither

haplotype was sampled in the same population. I would suggest that further sampling is needed here to resolve the true pattern in this clade.
Between clade 2-1 (M1 & B2) and 1-3, NCA, contiguous range expansion is inferred. This makes sense since the distribution of the two haplotypes B2 and D1 overlaps in the Limietberg block. Seems like there has been some long distance dispersal event that allowed the expansion of the 2-1 clade to the Hex River, without sacrificing its range in the Limietberg (or that populations were more closely contiguous at some point). All inferences relating to the private alleles (J1, J5, W2 and S2), had non significant permutational Chi squared statistic probabilities (due to sample size), and no inference could be made.

Haplotype 1-3 is separated from the other two other major clades, 2-1 and 3-3 by 4 and 6 inferred haplotypes respectively. This suggest that the divergence between them is older than the present pattern of haplotype distributions.

Most of the mixing is accounted for in the Limietberg (and to a lesser degree the Jonaskop), where the high diversity may be as a result of the area acting as a refugium, during a recent range contraction for *T. triangularis.* There is also close proximity between peaks of different heights, such that likelihood of gene flow between peaks, is high, reducing the probability of genetic drift.

This would be consistent with similar trends found for *Eritrichium nanum,* in the central Alps, where areas that had escaped glaciations, contained high haplotypic diversity and areas which had been glaciated displayed dominance of a single haplotype (Stehlik, *et al.* 2002). This same process may be at work in the south of the collection area, as a single haplotype dispersed from the area of high diversity (Limietberg) and rapidly expanded into the Hottentot's Holland mountains, where heights between peaks are low and presently conditions are wet and highly suitable for *T. triangularis* persistence
The highest population densities of *T. triangularis* occur (unquantified personal observation) in the Hottentot's Holland ~~population was~~ continuous between Landdroskop and Noordekloof Piek. On the other hand populations on the Hex River were small and

highly structured. At Brandwacht only two individuals were found and both the Waaihoek and Perry Refuge populations were contained in an area less than 50m × 50m. This suggests that there is a distinction in the types of patterns that will be found in the drier mountains north of the Breë River valley north of the Limietberg. The reasoning for this is that smaller population sizes as suggested by the increasing altitude of collections (when moving north from the south coast) will more easily converge to single haplotypes under genetic drift.

I propose that further collection in the Koue Bokkeveld and Cederburg and surrounding mountains will reveal a more structured pattern than that seen in the centre of the study area. At a later stage, these two areas could be compared to each other to each to see if the patterns hold true.

(7) Conclusions

AMOVA results suggest greater interpopulational differentiation in *T. triangularis*, but the pattern for *T. ustulata* is inadequate and more sampling is required. Pairwise F_{ST}'s are not correlated with the depths of valley between, but rather the distance between peaks is better predictor.

There is evidence for possible fragmentation on the periphery of the study area (Hex River in the north and the Jonaskop in the east), from the NCA analysis. The smaller higher altitude peaks on the Hex River tend higher levels of fixation. It is possible that these effects may be better seen in the drier more northerly range of *T. triangularis*. The expected differentiation is not clear in the centre of the study area because the study site dominated by a central refugium. So at the scale at which the question was asked may be too fine scale to see the kinds of genetic differentiation that was expected.

The high haplotypic diversity in the central block, may be due to long-term stable large population sizes, with frequent connections (dispersal or range expansion), between peaks. This supported by the long periods of isolation required for the accumulation of alleles and the presence of the ancestral haplotype here.

Alternatively the evolution/existence of a reproductively isolated flowering form of *T. triangularis* within the Limietberg, would serve to maintain existence of alternative haplotypes in the same populations, despite small population sizes.

(8) Acknowledgements

This project would in no way have been a reality without the hard work dedication, insights drive and enthusiasm of my supervisor Dr. Tony Verboom. In fact there would a great number of taller *T. triangularis* plants out there if it was not for him. Not to mention the late hours in the lab and at computers, sorting out my issues.

I wish to thank the landowners who were so gracious as to allow us to collect on their properties. Without their custodianship and enthusiasm there would be no interesting mountain refuges explore looking for thought provoking patterns.

The Molecular Laboratory can be a lonely place, thank to Tracey Nowell, Nicola Bergh, Ryan de Roo, Graeme Oatley and all the other 'usual' suspects for knowing the answers to my stupid questions and for listening to talk radio.

A huge thank you to Susan Botha for always being there, even when she didn't want to.

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