VARIATION IN *Thamnocortus insignis* – GENETICALLY AND ENVIRONMENTALLY PLASTIC?

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Abstract

Environmental variation of *Thamnocortus insignis* Mast. (Restionaceae) is presented, by using average dry weight measurements of six populations of this species found along the coast of the Cape Province. The dry weight measurements were used to detect variation in the growth rates of the six populations, which in turn indicates the environmental variation among the populations. The results were showed no significant difference between all six populations and this was due to the relatively very small sizes of the seedlings when the measurements were taken. Genetic variation was investigated by using isozyme electrophoresis of the EST locus. One population (Puntjie) showed significant deviation from the Hardy-Weinberg equilibrium, whilst all the others agreed with the assumptions of the model except for the Superthatch-extra tall population, which was homologous. Estimation of allelic diversity among the populations produced a Fst of 0.098 that implies that there is a high level of gene flow among the six populations of the species. The allelic diversity estimate within populations was also very low (Fis = 0.079) implying that the genetic neighbourhoods are relatively large and there is a high incidence of gene flow within the populations. Both estimation results support the predicted results for dioecious wind-pollinated species such as *T. insignis*.

CONTENTS

1] INTRODUCTION

1.1) Biology of the Restionaceae

The Restionaceae is a remarkable family of Southern Hemisphere evergreen, wind-pollinated, rush-like plants (Linder, 1991). Most of the Restionaceae species occur on poor sandy or lime soils, and where there's winter rainfall. The distribution of this family's genera and species is very uneven and it is only in Southern Africa that its members dominate almost all vegetation types. About 400 species in 40 genera are known, and 318 of these are found in Africa. Of the 318 species, all but four occur in the Cape Floristic Region making up 4 – 5 % of the total flora of the region. The Restionaceae have a relatively simple basic growth-form. This consists of either a rhizome or a basal node with a rhizome-like anatomy, which is the perennial part of the plant. The rhizome that is usually covered by overlapping shiny brown or white papery scales produces aerial culms which are highly variable and adventitious roots.

The Restionaceae and Anarthriceae families are very closely related, and have been placed in the order Restionales with the Ecdeiocoleaceae, Centrolepidaceae, Joinvilleaceae and Flagellariaceae. Moreover, according to Linder (1985) the Restionaceae and Anarthriaceae are phylogenetically clearly sister groups.

Thamnocortus (Berg.) is the second largest southern African genus in the Restionaceae family, found in the Cape Floristic Region. The genus has a plant habit dependent on the variability of the culms produced by the rhizomes. The habit of *Thamnocortus* is a particularly common one in the genus, and this is in the form of tussocks. These tussocks are sometimes tall and slender with the rhizome well developed, or sometimes short. The culms of the genus are simple very rarely branching, and often with clusters of sterile shoots at the nodes the year after flowering. The genus is made up of 33 species, one of which was investigated for this paper. *Thamnocortus insignis* (Mast.) a member of the genus *Thamnocortus* is a thatching reed belonging to the dioecious, wind-pollinated Restionaceae family. Its range is widespread in the Cape Floristic Region, along the coastal plain of Bredasdorp in the west to Riversdale in the east.

This species is the only restio that is widely exploited economically by the thatching industry. Plants of this species are made up of two meters tall, heavily lignified culms that are ideal for thatching.

Previous studies on this reed indicate great morphological variability between the populations found from west to east of the distribution range. The variation is such that populations from the west (i.e. Bredasdorp) have short culms and are not good for use as thatching. Populations from the east (i.e. Riversdale) have long culms and are ideal for use of thatching. The morphological variation is associated with the variation in environmental conditions of the different areas, but is there a genetic basis to such a variation?

1.2) Variation in *T. insignis*

Population genetics is the study of how Mendel's Laws and other genetic principles apply to entire populations (Hart and Clark, 1990). Such a study is essential to a proper understanding of evolution because, fundamentally, evolution is the result of progressive change in the genetic composition of a population.

Studying a population's genetics seeks to understand and predict effects on populations such as segregation, recombination, transposition and mutation. At the same time it takes into account ecological and evolutionary factors like population size, patterns of mating, geographic distribution of individuals, migration, genetic drift and natural selection. These genetic, ecological and evolutionary factors that influence populations, also interact upon each other. Gaining an understanding for these complex interactions requires a variety of approaches. These include careful descriptive observations, interpretation of statistical patterns, and controlled manipulative experiments.

The field of population genetics has as its tasks, the determination of how much genetic variation exists in natural populations, and the explaining of that variation in terms of its origin, maintenance and evolutionary importance. Genetic variation, in the form of multiple alleles for many individual loci, exists in many natural populations. Various methods can be used to measure such genetic variation in populations by studying their genetic structure. However, all the methods are limited to the study of a certain number of genes, usually a small number compared to the total number of genes in the organism.

Extrapolation of the results for a small number of genes or isozymes in the case of this study is thus questionable because of the uncertainty as to whether the isozyme studied is truly representative of the genotype/genome. Avise (1989) however, stated that clues of genome architecture within species (i.e. populations) can be done, but are usually limited primarily to comparisons of allele frequencies such as determined by electrophoresis.

For much of the past 30 years, isozymes have been the markers of choice for studies in molecular phylogeography. Recent years have witnessed the development of a group of molecular tools that can be used to assess DNA variation directly, such as microsatellites, RAPD's and direct DNA measures (i.e. DNA fingerprinting). Most notable of these is the Polymerase Chain Reaction (PCR) technique, which requires sequencing the DNA genome. Population genetics theory suggests that population differentiation should be greater in organellar genes: those encoded by chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) than for nuclear genes. Phylogenetic studies using mtDNA have worked remarkable in animals however, in plants the technique has been less useful. This is because of the relatively low rate of nucleotide substitution in the molecule, and its ability to strive for overall structural re-arrangement. As a result, assessing cpDNA, which is considered a single unit of inheritance not subject to recombination, has done most phylogeographic variation studies in plants. However, intraspecific cpDNA variation is generally very low due to its slow rate of evolution therefore, isozymes were the markers of choice for investigating genetic variation in *Thamnocortus insignis*.

The genetic structure, which refers to the non-random distribution of alleles and genotypes, of *Thamnocortus insignis* populations, will be evaluated, with respect to the effects of some of the genetic and ecological complex interactions. Isozyme analysis of a single locus, namely Esterase (EST), will be used to test for the effects of such genetic, ecological and evolutionary interactions within these populations.
The aim of this paper is to investigate the geographical variation between populations of *Thamnocortus insignis*, and to explain whether the variation (if present) is due to:

1. environmental factors such as rainfall and soil chemistry or,
2. genotypic factors.

Environmental factors may be influencing the variability in the populations, whilst the populations are genetically uniform. On the other hand, genetic factors may be responsible for the variation, whilst the environmental factors have minimum effect. Such variation will be investigated by looking at the differences in the growth rates of the seedlings making up the different the populations; then electrophoretic analyses will be used to look at genotypic variation between the populations. Since an isozyme is being used to investigate the relationship of the populations of *T. insignis*, mutation, migration and genetic drift will be the principle factors shaping the genetic structure of the species. However, it should be kept in mind that the use of a single locus to evaluate genetic structure in populations is limiting, as knowledge of genetic structure based on many loci produces more accurate results and interpretations (Stebbins, 1989).

Understanding the origin and maintenance of geographic variation represents an important task in evolution biology. Recent studies of geographic variation have emphasised molecular methods, which reveal the geography of genetic variation (Thorpe et al., 1995). Further support of the use of molecular methods to provide information suitable for identifying geographic variation and reconstructing phylogenetic relationships among populations, was provided by Ellsworth et al. (1994). Geographic patterns of genetic variation lead to insights into evolutionary patterns of population fragmentation and the role of gene flow in structuring populations (Avise et al., 1987). Plant breeding systems on the whole have been identified as major factors influencing gene structure (Loveless and Hamrick, 1984), obligate outcrossing as in *T. insignis* would be expected to increase genetic neighbourhoods and reduce population subdivisions. Moreover, incompatibility mechanisms could cause pollen dispersal and genetic neighbourhoods to become larger.

Since the species under study in this paper is wind pollinated, it is important to note that wind pollination is generally associated with low genetic variation among populations with large genetic neighbourhoods within populations (Loveless and Hamrick, 1984). This idea was later supported by Honig et al. (1992), in their study on the pollen movement of *Staberoha banksii* (Restionaceae), and they concluded that

genetic neighbourhoods were large, so that differentiation within populations would be kept to a minimum.

Godt and Hamrick (1999) noted that widespread species are usually comprised of more individuals than endemic species. Moreover, population sizes tend to be larger in widespread species. The larger numbers should facilitate the maintenance of a greater allelic diversity at neutral or nearly neutral loci. Reviews of plant allozyme literature support this theoretical prediction; species with larger geographic ranges maintain more genetic diversity not only at species level, but also within populations (Godt and Hamrick, 1999). Further, Hamrick and Godt (1990) showed evidence that outcrossing wind-pollinating plants (e.g. *T. insignis*) have a low proportion of total genetic diversity distributed among populations, a relatively high genetic diversity within populations, but a low total genetic diversity compared to species with other breeding systems. Therefore, results similar to those of Hamrick and Godt (1990), supporting high genetic diversity within populations than amongst populations are expected for *T. insignis*.

1.3) Implications

Results of the investigation will have tremendous implications on the potential cultivation of *T. insignis*. If the variation between the populations is genetically based then the area from which the plants are collected will be crucial (i.e. should be collected from area producing the tallest plants). Alternatively, if the variation is due to environmental factors, then the plants can be collected from anywhere along the distribution range but the growth conditions must be similar to those in the habitat of the plants producing the best thatching reeds.

2] MATERIALS AND METHODS

2.1) Materials

Thamnocortus insignis seeds collected from ten different populations were planted at Kisternbosch. Of the ten, only six collections germinated into seedlings that were collected for growth rate and genetic analyses. The successfully germinated populations were from: Pontjie, Takkies, Canca, Klipfontein, and Superthatch (extra tall) Superthatch (see Figure 1).

2.2) Growth rate

Thirty seedling samples were collected after 2 and 4 months of germination, and the wet and dry weights of each of the seedlings from the six populations measured each time. Statistical analyses (two-way ANOVA) were done on the dry weight results to test for significant differences between the six populations, in terms of growth rate and the differences between the dry weights measured after one and two months since germination.

2.3) Enzyme extraction

Seedling samples were kept in a freezer at about –10 degrees prior to extraction. 10 representative seedlings from each of the six populations were used for the extraction of the enzymes. Each of the 10 seedlings of one population was cut up into small pieces into an eppendorf, a small amount of liquid nitrogen added and then quickly ground up. The powder was further grounded using the 4 to 5 drops of 'vegetative extraction buffer1' described in Table 1 (Cheliak and Pitel, 1984) in the Appendix. The pH was adjusted to 6.7 with Tris and 0.66 ml 2-mercaptoethanol for 100 buffer was added. Also because PVP (one of the buffer ingredients) does not dissolve easily, a pinch of the powder is added when grinding. This was repeated for each of the populations. Six wicks (made up of filter paper cuts) per population were soaked in the grindate mixture, giving an overall of 60 wicks (10 for each of the 6 populations). The wicks were kept in small plastic tubes in the –70 degrees freezer until the electrophoresis 'run'.

2.4) Electrophoretic procedure

2.4.1) Gel preparation

Two 12.5 % starch gels were prepared with 56.25g starch in 450ml-gel buffer using the following recipe. 300ml and150 ml gel buffers were measured separately. The 300ml-gel buffer was heated to boiling in a 1000ml long neck volumetric flask. Whilst the solution was heating, 56.225g of starch was suspended in the cold 150ml of gel buffer in a 2000ml thick-walled vacuum flask and mixed thoroughly to remove all lumps. The boiling buffer was then poured into the 2000ml flask and the mixture swirled for about 10 to 15 seconds. A stopper was then placed on the flask and a vacuum applied to de-gas the hot starch solution until large bubbles appeared. The vacuum was released slowly and the starch immediately poured into clean glass molds.

2.4.2) Gel and electrode buffer preparation

The gel and electrode buffer 7 system by Soltis et al. (1983) was used to prepare the buffers. The ingredients are listed in Table 2 & 3 in the Appendix. The Tris Citrate buffer was prepared by adding 5.45g of Tris, 1.28g Citric acid and the volume brought to 900ml. 100ml electrode buffer solution was added to give a 1:9 ratio, and 1.0 M NaOH used to adjust the pH.

2.4.3) Electrophoretic 'run'

The starch gels were allowed to cool for about an hour, after which the gels were cut length-wise (single slit) about 6cm from their base right through the thickness. The wicks with the enzymes were removed from the freezer and inserted evenly (about 2 or 3 mm apart) along the length of the gels. An overall number of 30 wicks were inserted into each of the gels, 5 wicks for each of the 6 populations in both gels. Two 'marker' wicks stained in Bromophenol blue were placed at the beginning and end of each of the 30 wicks per gel.

Cling wrap was placed over the gels with the wicks and the gels taken into the 0 degrees cold-room where the electrophoresis run took place. The electrode buffer (same as the gel buffer 7) were used for the run, and were poured into two elongated trays per gel. Thin dishtowels were used to connect the cathodes, and an electric current produced between the gels and the anodes. The runs were conducted at 70 mA with a mean voltage of about 160 V. After about30 to 45 minutes, depending on how

far off the marker stains had travelled in the gels, the wicks were removed from the gels. The gels were run for approximately 7 hours, where the enzyme markers had almost reached the top of the gels.

2.5) Staining procedure

The Esterase (Est) enzyme was stained using the substances listed in Table 4 of the Appendix (Calorimetric; EC 3.1.1). When the electrophoresis runs were complete, the gels were removed from the electrode connections and the cold-room. In the laboratory, the gels were thinly sliced and a slice from each of the two gels placed in a clean glass dish. The stain (Table 4 in Appendix) was then poured into both containers over the gels, and the staining was done at room temperature. The 1M Phosphate buffer used as one of the staining agents was made up with 60g NaH_2PO_4 in 300ml water. The pH was adjusted by adding 1M NaOH (approximately 170ml for pH 6.0), and the total volume was brought up to 500ml.

The gels were left in the stains for about 30 minutes when the bands started showing clearly. It is important to fox the stains at this stage t prevent the bands from becoming too dark and difficult to make out. The gels were fixed by pouring out the staining solutions and adding the fixing solution made up of HCL mixed in water (1: 5 ratio). The gels were then scanned onto the computer and photographed, as some clarity of the gels is lost with fixation and storage.

2.6) Data analysis

Counting the number of alleles (represented by the bands on the starch gel) for each of the 10 plants in each population scored the gels. The BIOSYS-1 computer programme (Swofford and Selander, 1989) was used to calculate the following for all the populations:

- allele frequencies,
- observed and expected genotype frequencies with Chi-square tests to measure the deviations from the Hardy-Weinberg equilibrium (Levene, 1949),
- coefficients for heterozygote deficiency or excess (fixation tests, Wright 1965) where D= (Hobs/ Hexp)- 1 as a measure of inbreeding,
- coefficients of population differentiation using F-statistics (Wright, 1965, 1978; Nei, 1977; Weir and Cockerham, 1984) to measure the decreases in the proportions of heterozygous genotypes.

3] RESULTS

3.1) Growth Rate (environmental variation)

Table 5 shows the average dry weights (measure of growth) for the plant samples from each of the six populations, collected after one and two months since the germination of the seeds, to monitor the growth rates of the populations.

Table 5: The average growth rates of *T. insignis* populations measured one and two months after germination.

Populations	**1 month after germination**	**2 months after germination**
Puntjie	0.0010	0.0030
Takkiesfontein	0.0010	0.0031
Canca	0.0009	0.0024
Klipfontein	0.0010	0.0029
Superthatch(e.t)	0.0012	0.0028
Superthatch	0.0011	0.0035
Puntjie Comparisons	**Takkies Comparisons**	**Canca Comparisons**
P vs T p = 0.476	T vs P p = 0.476	C vs P p = 0.400
P vs C p = 0.400	T vs C p = 0.296	C vs T p = 0.296
P vs K p = 0.918	T vs K p = 0.449	C vs K p = 0.318
P vs S(extra tall) p = 0.979	T vs Se p = 0.405	C vs Se p = 0.124
P vs S p = 0.410	T vs S 0.614	C vs S p = 0.404
Klipfontein Comparisons	**Superthatch(e.t) Comparisons**	**Superthatch Comparisons**
K vs P p = 0.918	Se vs P p = 0.979	S vs P p = 0.410
K vs T p = 0.449	Se vs T p = 0.405	S vs T 0.614
K vs C p = 0.318	Se vs C p = 0.124	S vs C p = 0.404
K vs Se p = 0.986	Se vs K p = 0.986	S vs K p = 0.500
K vs S p = 0.500	Se vs S p = 0.625	S vs Se p = 0.625

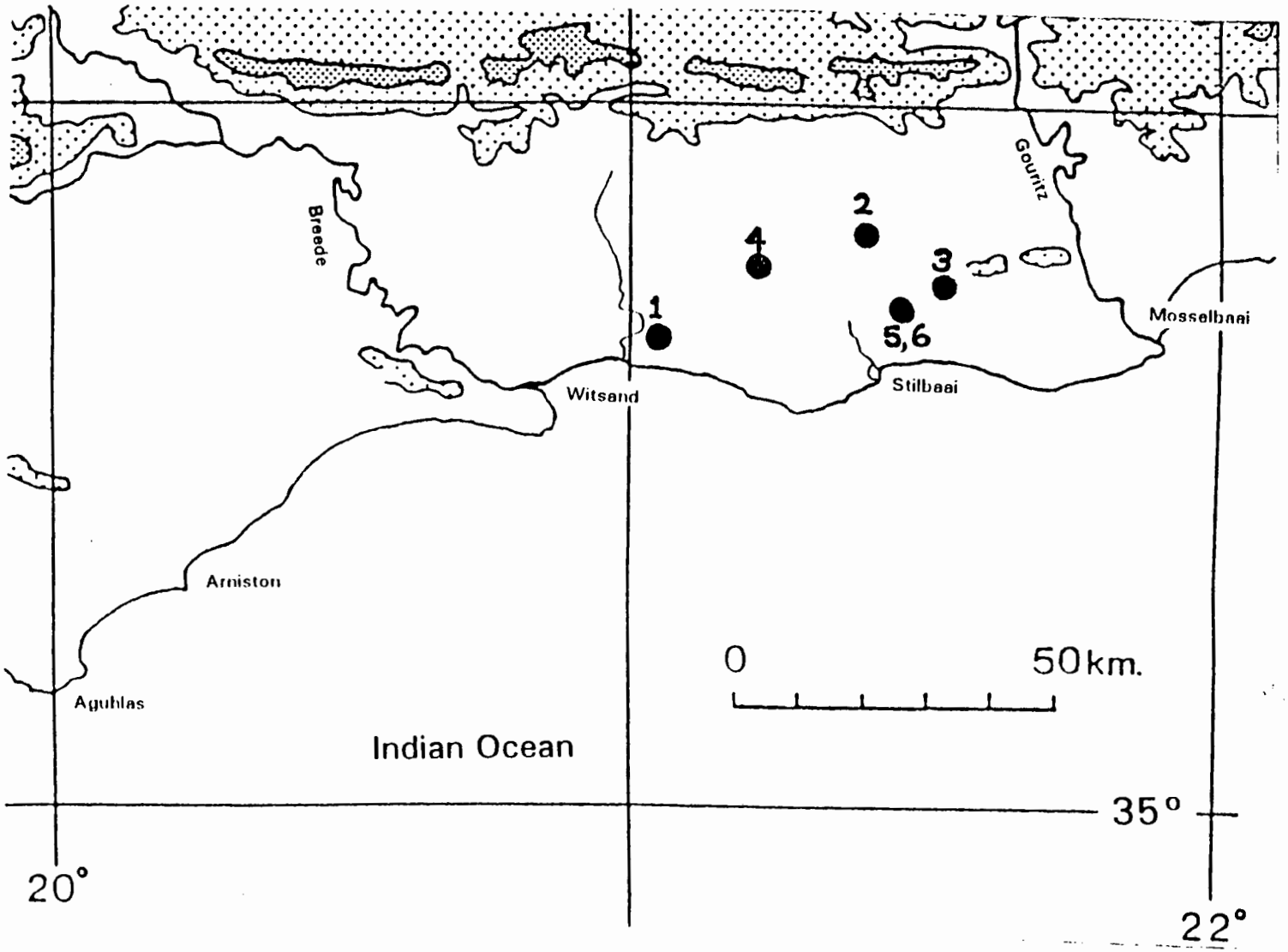

Figure 1 Caption ?

(6) Superthatch	1	1.000	-0.053	0.000

Table 14: Matrix of single-locus (EST) genetic similarity or distance coefficients (Nei, 1972).

Population	**1**	**2**	**3**	**4**	**5**	**6**
(1) Puntjie	*****					
(2) Takkieskraal	1.000	*****				
(3) Canca	0.965	0.995	*****			
(4) Klipfontein	1.000	1.000	0.965	*****		
(5) Super-tall	0.964	0.994	1.000	0.964	*****	
(6) Superthatch	0.982	1.000	1.000	0.982	1.000	*****

Table 15: The Fis(ik) statistic for each allele of the EST locus, with the weighed average of Fis for all alleles at the EST locus in the i(th) population.

	Subpopulation					
Allele	**1**	**2**	**3**	**4**	**5**	**6**
A	0.733	-0.176	-0.053	-0.333	------	-0.053
B	0.733	-0.176	------	-0.333	------	-0.053
C	------	------	-0.053	------	------	------
Mean	0.733	-0.176	-0.053	-0.333	------	-0.053

Table 16: The Fis(k), Tit(k) and Fst(k)-statistics for each allele of the EST locus. [Fis(k) values are the weighed average of Fis(ik) across subpopulations]

Allele	Fis(k)	Fit(k)	Fst(k)
A	0.079	0.162	0.090
B	0.091	0.191	0.111
C	-0.053	-0.008	0.098
Mean	0.079	0.169	0.098

3.2) Isozyme electrophoresis (genetic variation)

Geographic variation of gene frequencies has been documented for decades (Sumner, 1930; Wright and Dobzhansky, 1942), but it was not until after the development of gel electrophoresis (Smithies, 1955, 1959) and ways of adequately interpreting the bands (Lewontin and Hubby, 1966; Markert and Moller, 1959; Shaw, 1965), that intensive attention was given to the study of variation in gene frequencies. Electrophoretic techniques have ernomously increased the ability to obtain genotypic data without the work and time required to do direct mating tests. Protein electrophoresis, the migration of proteins or their functional forms (e.g. isozymes) under the influence of an electric field, is the most useful procedure developed for revealing genetic variation. This technique involves the migration of protein molecules (enzymes) from samples (plants or animals) through the starch gel in response to an electric current. The different alleles in a locus of the enzyme (or isozyme) are represented as dark bands after staining and these bands can then be scored to give allele frequencies. This procedure is thus very important as genetic variation can be easily quantified using the concept of allele frequencies.

The differences in the band patterns are due to the differences in the electrophoretic mobilities of the enzymes or isozymes. Enzymes with faster mobilities will form bands towards the top of the gels and slower ones will have bands showing closer to the base of the gels. The differences in the electrophoretic mobilities are due to the differences in shape, size and charge of the enzymes/isozymes. Single dark bands on a stained gel represent homozygosity for that particular allele of the enzyme being tested; similarly two or more bands are indicative of heterozygosity for the elleles of the enzyme studied.

The different band patterns of the isozyme system for Esterase (EST) for *T. insignis* populations are illustrated in Figures 2 and 3. The figures show the scans of the starch gels for the samples tested for the EST isozyme, and the band interpretations from the bands on the gels. Activity in the single locus of *T. insignis* was found in three areas representing three different alleles (A, B and C), all in the positive (cathode) part of the gels. The most common allele (found in all the population samples) had medium speed compared to the other two remaining alleles and this was named 'A'.

The next common allele in the populations had the fastest mobility and thus was nearest the top of the gel and it was called 'B'; and the very rare allele found in only one population (Canca or population 3) had the slowest speed and was called 'C'. Populations from Puntjie (1), Takkieskraal (2), Klipfontein (4) and Superthatch (6), all possess the two alleles A and B. The Canca population (3) only has allele A and the rare allele C found only in this population. The other population, Superthatch-extra tall (5) has only the commonly occurring allele in all the populations, allele A. The allele frequencies of the six populations are shown in Table 6.

Tables 7 to 12, show the results of the chi-square analyses for the deviations from the Hardy-Weinberg equilibrium model expectations. These results show that only the Puntjie population (1) deviates from the Hardy-Weinberg equilibrium model, but the deviation is not that convincing ($p=0.046$ which when rounded off is 0.05) considering the entire population is being represented by only 10 samples in the investigation. Four of the remaining populations investigated are not significantly different to the Hardy-Weinberg equilibrium model because their p-values are greater than 0.05, at the 95%

significance level. The last population (Superthatch-extra tall) didn't have results for the deviations because there were no polymorphic alleles in the population samples. Coefficients of heterozygote deficiency are given in Table 13, and these consist of the weighted average inbreeding coefficient (Fis) for the alleles at the EST locus. Also D (Hobs./Hexp. – 1, Wright, 1965) is given in that table. D is a similar measure as the inbreeding coefficient (Fis), but it indicates the deficiency of heterozygotes as a ratio of alleles observed over those expected, with a minus sign. These results are consistent with the Hardy-Weinberg deviation results in that only the samples from the Puntjie population (1) show a deficiency in heterozygotes. This means in this population there were fewer heterozygotes than what was expected. The other four populations (excluding Superthatch-extra tall, which has no polymorphisms) had either more or equal numbers of heterozygotes as what was expected. Therefore, there were no deficiencies in heterozygosity in these populations.

Measures of genetic identity (Nei, 1972) are represented in Table 14, and these show which populations are more similar genetically for the particular locus, than the others. Populations that share all alleles have the value 1 and those not sharing any alleles would have the value for zero for their relationships. The genetic structure of the species, summarised in Tables 15 and 16, show that there is a high amount of gene flow among and within populations of this species. This implies that there is relatively low amount of diversity among and within the populations.

4] DISCUSSION

4.1) Environmental variation

The results obtained from this part of the investigation of *T. insignis*, showed that there is a high amount of variability in the growth rates of the six populations of this species. This variability is not suprising as the seeds of the different populations were collected at different locations each with their own environmental conditions. The samples of seeds collected from population closer to each other (e.g. Takkiesfontein and Klipfontein; both Superthatch populations and Canca), had dry weight measures that were almost similar after two months. The first measurements of dry weights showed no particular growth patterns and things can be attributed to the relatively small size of the seedlings at that stage.

Statistical analyses (Two-way ANOVA's) however, indicated that even though there was a significant difference between the growth rates measured after one month since germination and after two months ($p = 0.006$), there still was no significant difference in growth between the six populations sampled ($p = 0.350$). This result was not suprising because even after five months since germination the seedlings were still considerably small. The average dry weights of the populations after 2 months also indicate that at that stage the seedlings were still very small and the potential growth rates of the population members not yet visible. Results obtained from adult plants of each of the six populations will provide better indications of the growth rates, and the population with the fastest growth rate (tallest plants) will be the ideal one for use in thatching.

4.2) Deviations from the H-W equilibrium model

Predicting genotype frequencies from knowledge of allele frequencies is quite easy with the computer programme BIOSYS-1. Measures of deviation from the Hardy-Weinberg (H-W) equilibrium are based on deviations from the eight assumptions stated by the H-W model. The assumptions which were made to predict genotype frequencies by the model, result in particular relationships between genes and genotype frequencies. Deviations from the assumptions of this model suggest that some amount of inbreeding is taking place in the species (Table 7 to 12). Such deviations from the assumptions are also represented as heterozygote deficiencies (Table 13).

In this study heterozygote deficiency was only found in the Puntjie population (D= -0.747), which is the same population that showed evidence of deviation from the H-W equilibrium. One of the populations (Superthatch-extra tall) didn't provide any results for deviations from the H-W equilibrium and the heterozygote deficiency, because it is homozygous for the 'A' allele represented for the EST locus.

Since the species is dioecious, selfing or inbreeding cannot be the reason for the deviation from the H-W equilibrium or the deficiency in heterozygotes in the population from Puntjie (1). A possible alternative explanation for such results is either that the genotype frequencies are not representative of the entire genome of the population, or that some other evolutionary factor (i.e. mutation) is shaping the population. Moreover, homozygote selective advantage (due to natural selection) may have occurred and this would not only explain the results of Puntjie, but those of Superthatch-extra tall as well.

4.3) Population differentiation

Different parameters can be used to estimate population differentiation, but F-statistics (Wright, 1965) are best because they are suited to small data sets. As 10 samples per population were used for the genetic investigation of *T. insignis*, the F-statistics (Fis, Fit and Fst) are suitable measures for such variation in the species. The fixation index (Fst) in the populations investigated refers to the proportion of total allelic diversity among populations (Table 16).

This fixation index was quite low (0.098) in the three alleles present in the EST locus of the populations. This result is what is expected of a dioecious wind pollinated species such as *T. insignis*. Gst (Fst equivalent) values of 0.099 on average are found for outcrossing wind-pollinated taxa (Hamrick and Godt, 1990). The low fixation index results imply that there is a considerably high level of gene flow among the six populations studied, especially for the four populations (Takkiesfontein, Klipfontein, Canca and Superthatch) that show evidence of high allele heterozygosity for the EST locus (Tables 7 to 13). There is a general indication of genetic interaction between the populations of the species. Also the low average Fst value indicates that there are low levels of genetic drift in the populations studied, especially the four previously mentioned populations.

The allelic diversity found within the populations is represented by the inbreeding coefficient (Fis) in Table 16. The average result of this F-statistic is also quite low (0.079) and it means the genetic neighbourhood sizes are relatively large, with lots of gene flow within the populations. This result supports the prediction from work by Honig et al. (1992) on *Stéberola banksii* for which large neighbourhoods were also

found. The suggested explanation for the high level of gene flow in species like *T. insignis* that are dioecious and the male and female plants are considerably separated, is that there may be a high amount of airborne pollen mixing from different males (Honig et al., 1992). The genetic distance within populations is very small (about 5 to 10 meters apart) and therefore, the low F (is) value is not suprising. However, the distance among the populations is kilometres apart instead of meters, and this reduces the level of gene flow in comparison to that within populations. Since the species is wind-pollinated a lot of the restriction of gene flow by distance is eliminated hence, the low F(st) value.

Generally, results of the population differentiation in *T. insignis,* show that the species has high incidences of gene flow among and within populations. This means the individuals within populations are quite similar and that there is little genetic variation among populations of this species. This is especially evident in populations from Takkiesfontein (2), Canca (3),Klipfontein (4), and Superthatch (6). However, a rare allele 'C' was found to occur in the Canca population and this implies that there could be a genetic barrier between this population and the other populations. Therefore, of all the populations investigated, the Canca (3) population shows the most genetic variability because if has sufficient numbers of heterozygotes in it, supports the H-W equilibrium model and has a unique allele for the EST locus being investigated.

At this stage it is important to note that the sample size used in this investigation of genetic variability was small (N=10) therefore, the results obtained in this study may

not be representative of whole populations. Moreover, a single locus was tested and it is known that most isozyme studies require investigations of many loci to represent average allelic diversity (Hamrick and Godt, 1990).

However, a similar study was attempted by Hooks (unpublished honours project, 1989) where he was looking for genetic variation among the *T. insignis* populations using the SDH locus. He looked at five populations, of which one (Klipfontein) is the same as in this study, the other populations Hectorskraal and Witbakenkop) are very close to the other populations investigated in this study (Takkiesfontein and the Superthatch populations, respectively). In his study, Hooks had problems interpreting the allele bands and as a result carried out a conservative and liberal estimate of allelic diversity.

In the liberal estimation, he found significant deviations from the Hardy-Weinberg equilibrium in three of the populations he studied and these included Klipfontein. Such a result contradicted what is expected of wind-pollinated dioecoius species. Further, results of allelic diversity among populations implied that there was very little gene flow (Fst = 0.242) between the Springfield and De Hoop populations (far West) and between those two populations and the other populations from the east. He suggested distance and different flowering times as reasons for the isolations to explain the results. On the other hand, the conservative estimate of allelic diversity produced results similar to those of this study. The allelic diversity among populations (Fst) was 0.038, and implied high levels of gene flow among the populations, as is predicted for dioecoius wind-pollinated species.

It seems then that the conservative estimate of genetic diversity is more accurate because it produced results similar to the ones obtained in this study of the EST locus. This conclusion is reached because the studies were done independently but produced similar results working of the same plant species and almost the same populations. As mentioned before, isozyme studies require investigations of many loci to represent average allelic diversity, and for *T. insignis* already two loci have been investigated and these produced similar results. The species is a step closer to having accurate evidence of allelic diversity.

The results available to date suggest that there is a small genetic difference between the populations (high gene flow) and therefore, the differences in growth rates that are seen in this species are not genetically based. This implies that the suitability of these reeds to thatching is environmentally related and is not due to genetic variation. Therefore, potential thatching reeds can be collected from any of the locations but need to be grown under similar environmental conditions as those present in the locations producing the best (tallest) reeds.

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6] APPENDIX

Table 1: The 'vegetative extraction buffer 1' used for grinding (after Cheliak and Pitel, 1984)

8 % PVP (added when grinding)	8.00 g
0.3 M Sucrose	10.27g
0.5 mM EDTA	38.02 mg
1mM Dithiothreitol	15.43 mg
1 mM Ascorbic acid	17.73 mg
0.1 % Bovine Serum Albumin	100.00 mg
0.4 mM NAD	26.54 mg
0.3 mM NADP	22.96 mg
0.2 mM Pyridoxal 5' Phosphate	4.94 mg
Distilled water	100.00 ml

Table 2: The electrode buffer 7 recipe (taken from Soltis et al., 1983)

0.038 M LiOH
0.188 M Boric acid, pH 8.6
1.60g LiOH.H2
11.60g Boric acid, pH adjusted with dry ingredients

Table 3: The gel buffer 7 ingredients used (taken from Soltis et al., 1983)

0.045 M Tris
0.007 M Citric acid
0.004 M LiOH
0.019 M Boric acid, pH 8.3

Table 4: The staining recipe for the Esterase enzyme

α- naphthyl acetate	40mg
β- naphthyl acetate (dissolved in 2ml acetone)	40mg
1.0 M phosphate buffer, pH 6.0	10ml
Water	90ml
Fast Blue RR salt	100mg