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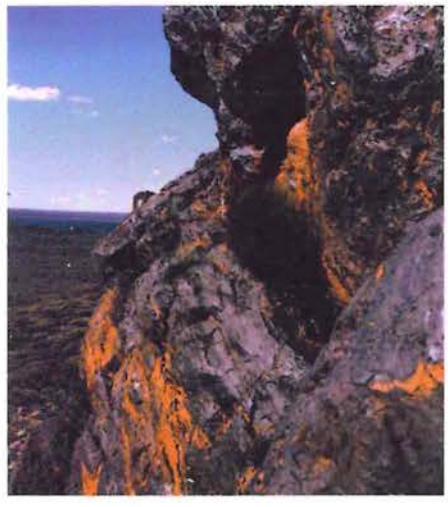
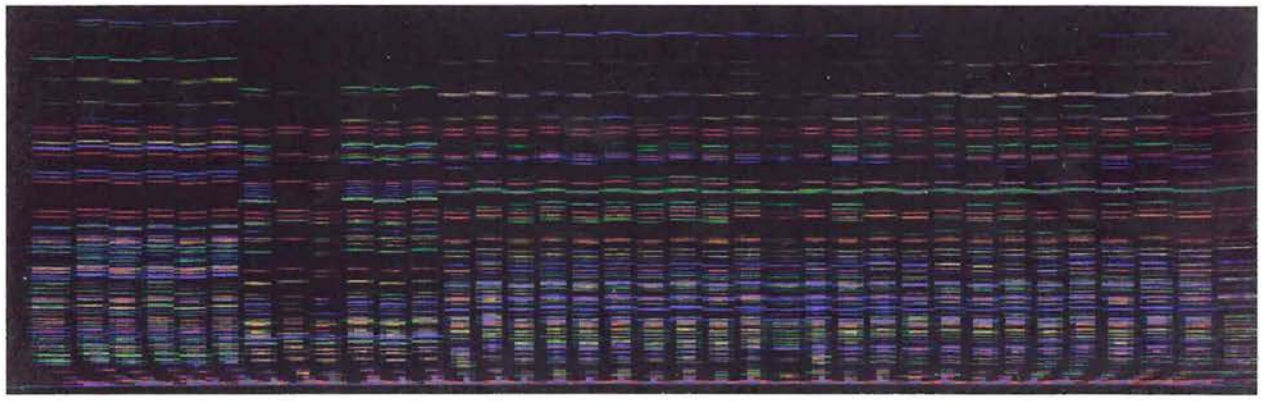
Genetic differentiation, soil stored seed bank and propagation of the DRF *Tetratheca paynterae* and *T. "Die Hardy"* (Tremandaceae)

a report commissioned by

Portman Iron Ore Ltd

August 2002





Genetic differentiation, soil stored seed bank and propagation of the DRF *Tetratheca paynterae* and *T."Die Hardy"* (Tremandaceae).

Written by

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5 August 2002

Commissioned by

Portman Iron Ore Limited

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ACKNOWLEDGMENTS

We thank Bob Dixon, Russell Barrett, Katie Biggs (BGPA), Ryonen Butcher (CALM), Jerome Bull (Ecologia) and Daniel Seivers for assistance with fieldwork and sample collection, Eric Bunn and Shane Turner (BGPA) for assistance with propagation from cuttings, Anle Tieu (BGPA) for assistance with seed germination, and Robyn Taylor (BGPA) for assistance with genetic analysis.

Summary

In April 2002, Portman Iron Ore Limited commissioned the Science Laboratory, Kings Park and Botanic Garden to undertake *(i)* an assessment of genetic variation within and among populations of *Tetratheca paynterae* and *T. "Die-Hardy"* using the DNA fingerprinting technique AFLP, (ii) an assessment of the soil stored seed bank of *T. paynterae* including viability and germination tests, *(iii)* propagation trials of *T. paynterae* and *T. "Die Hardy"* from stem cuttings. This report presents the results of these studies as of 31 July 2002, and addresses the taxonomic status of the *T. "Die Hardy"* populations.

Genetic variation was assessed for 27 plants from the W3 and W5 sites at Windarling and 10 plants from two populations of the Die Hardy Range by scoring 45 AFLP markers, of which 35 (77.8%) were polymorphic. An ordination of these genetic data showed no significant genetic differentiation within the *T. paynterae* population at Windarling. Two populations of *T. "Die Hardy"* showed genetic differentiation, although this was not statistically significant due to small sample sizes. There was very marked genetic differentiation between *T. paynterae* and *T. "Die Hardy"* ($P<0.001$), with 15 AFLP markers (33.3% of the total markers scored) demonstrating fixed or highly significant marker frequency differences. The strength of genetic differentiation between these populations strongly supports a formal taxonomic recognition of *Tetratheca "Die Hardy"* as a taxon distinct from *T. paynterae,* and, given the high number of highly significant genetic differences, most appropriately at species rank.

A large soil stored seed bank was found within some targeted rock crevices below plants at Windarling (45,133 seeds/1m^3). Of these, 72% were found to be viable by a cut test. Seed trials resulted in significant levels of germination for all treatments (including controls), but greater germination rates were observed for various combinations of giberellic acid, nicking and/or smoking treatments. Mean germination rates of viable seed varied from 20% (for controls) to 50% (giberellic acid and nicking).

Propagation from cuttings for both *T. paynterae* and *T. "Die-Hardy"* showed a marked level of variation among hormone treatments and genotypes, although all were successful to some extent. Overall, 10% of cuttings produced roots and shoots up to 110 days post-treatment. Ultimately, the quality of the source material appears to be the single major factor limiting success of propagation from cuttings. However, more detailed research is required to optimise the success and benefits of propagation and germination.

PART 1: GENETIC ANALYSIS OF *TETRATHECA PAYNTERAE* FROM THE WINDARLING RANGE AND *TETRATHECA* "DIE HARDY RANGE".

Introduction

In April 2002, Portman Iron Ore Limited commissioned the Science Laboratory, Kings Park and Botanic Garden, to undertake genetic testing of *Tetratheca paynterae* from the Windarling Range. The initial objective was to assess the levels of genetic variation within the population of the W3 deposit of the Windarling Range and to determine the extent of genetic differentiation among sub-populations.

Tetratheca paynterae is an erect or decumbent, leafless shrub growing to between 10 to 50 cm high. The flowers are a distinctive pink/purple colour with flowering occurring between April and November (Alford, 1995). *Tetratheca paynterae* is confined to the Windarling Range with the majority of the species in the rocky outcrops in the proposed W3 deposit. The proposed expansion of iron ore mining into the W3 deposit of the Windarling Range will directly impact up to 89% of the existing population of *T.paynterae* (ecologia environment, 2002). This initial genetic study was commissioned to address the possibility of genetic differentiation between the plants to be removed and the remaining 11%.

Tetratheca "Die Hardy" is endemic to rocky outcrops in the Die Hardy Range, 11km NE of the Windarling Range. A second objective of this genetic study was to assess the level of genetic differentiation between *T. paynterae* and *T. "Die Hardy"*, and subsequently to assess the taxonomic status of *T. "Die Hardy"*. These genetic studies complement the current detailed morphometric and DNA sequence studies of Butcher *et al.* 2002.

Methods

Sample Collection

Stem material collected from the Windarling Range (Figure 1) and from the Die Hardy Range (Figure 2) was stored in the field either as desiccated tissue in silica gel or as fresh tissue wrapped in moist paper and stored in the lab at 4°C until DNA extraction. Note that sampling at the Windarling Range included plants across the entire distribution on W3, as well as a new population discovered on W5 (Figure 1). In the course of our sampling, we also determined that the two previously identified plants on W4 (ecologia environment 2000) were misidentified as *T. paynterae*, and are in fact *Mirbelia sp.* We found no *T. paynterae* on W4.

DNA Extraction

DNA was extracted from either fresh or silica dried stem material using the QIAGEN Plant extraction minikit. Modifications were made to the recommended protocol to accommodate for the larger mass of tissue required for successful DNA extraction of *Tetratheca* (see appendix 1).

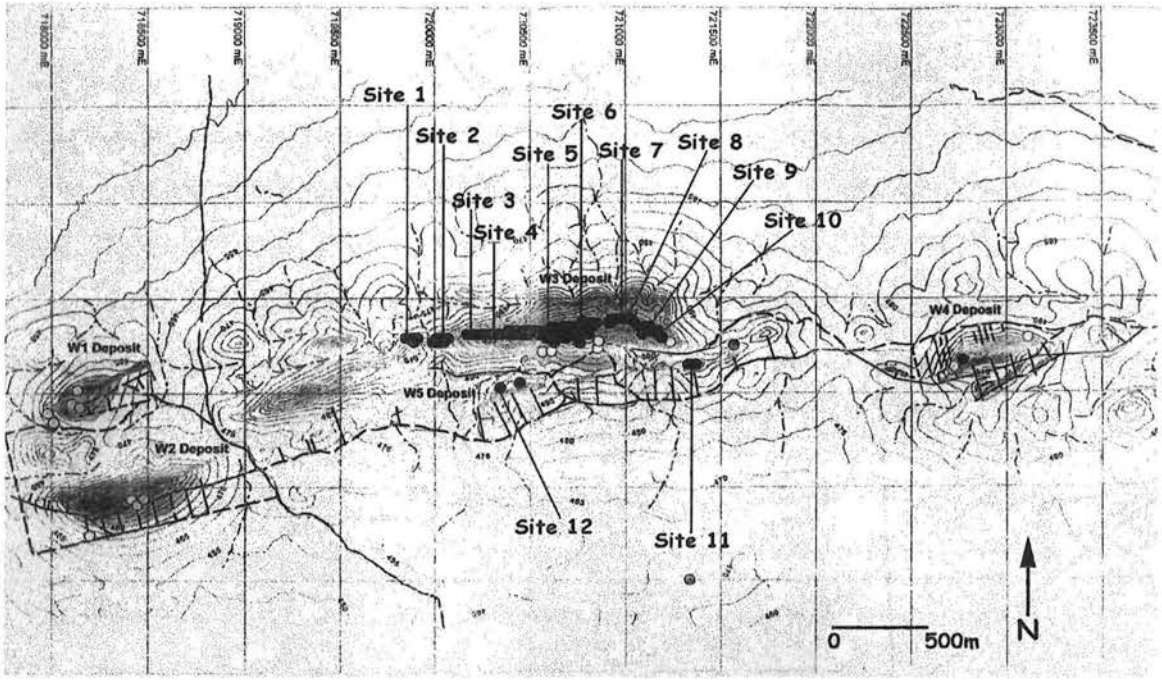


Figure 1. Map of the Windarling Range showing collection sites of *Tetratheca paynterae*.

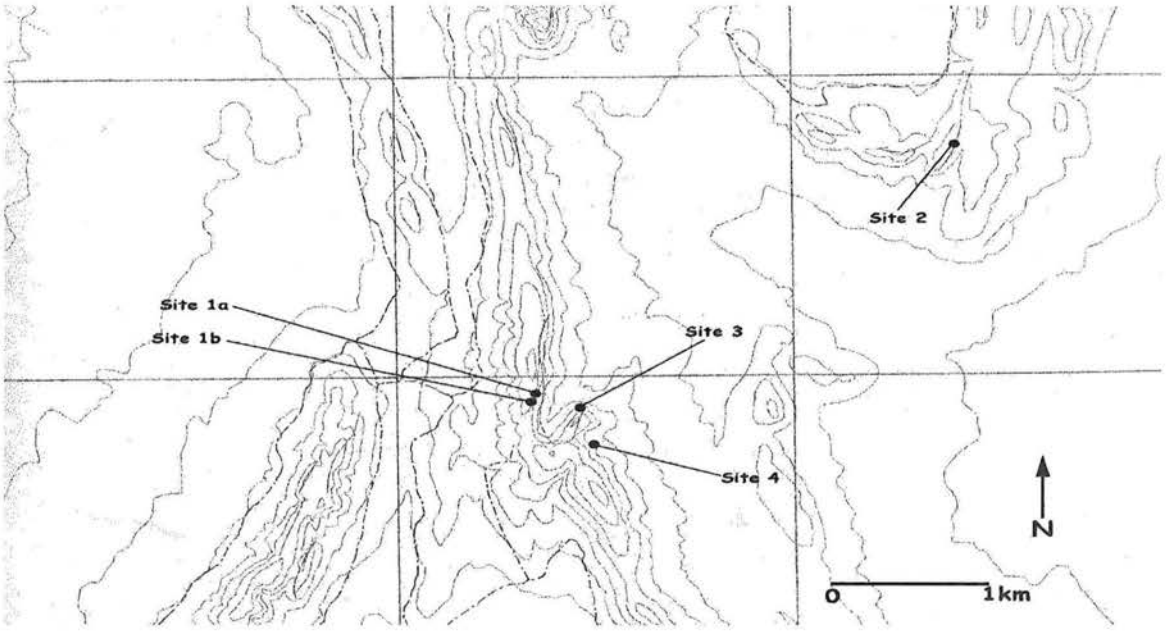


Figure 2. Map of the Die Hardy Range showing collection sites of *Tetratheca* "Die Hardy Range".

Genetic Analysis

Following extraction of the DNA from stem material, AFLP fingerprints were generated for *T.paynterae* and *T. "Die Hardy"* (see appendix 2). The AFLP fragments were scored for their presence or absence (i.e. 1 or 0) for each sample with the aid of ABI Prism Genescan software. Only consistent and unambiguous fragments were scored (see appendix 3). The resultant AFLP genotypes were analysed to estimate the genetic diversity within populations, the partitioning of the genetic variance and the levels of genetic differentiation between populations.

The levels of genetic diversity within each population were estimated by the proportion of polymorphic loci and the expected heterozygosity (H_e) using the program AFLP-SURV 1.0 (Vekemans, 2002). The expected heterozygosity is an estimate of genetic diversity based on an 'ideal' population displaying random mating and non-overlapping generations. Although many natural populations may not display these attributes, the expected heterozygosity does provide an unbiased estimate of genetic diversity relatively independent of sampling size.

In addition the partitioning of genetic variance was estimated by performing an Analysis of Molecular Variance (AMOVA) on the AFLP genotypes using the program GenAlEx (Peakall and Smouse, 2001). The AMOVA analysis generates an estimate of the proportion of the total genetic variation lying both within and among populations. It thereby gives an indication of the levels of genetic diversity within populations and differentiation amongst all populations and regions.

The genetic distance between individuals was calculated in STATISTICA (StatSoft, Inc., 1995) using a Manhattan distance measure. A multi-dimensional scaling (MDS) plot was generated to visually represent the degree of genetic similarity between individuals. The MDS ordination assigns individuals to a position within a two-dimensional matrix

according to their genetic distance. The closer two points (individuals) are in the ordination the more genetically similar they are.

Ellipses were drawn around the cluster of points for each population to represent the total genetic variance of the population. Tightly clustered populations will have smaller ellipses suggesting less genetic variability than those populations with larger ellipses. In addition, the MDS ordination gives a visual representation of the degree of differentiation between populations. Distinct, non-overlapping ellipses suggest significant genetic differentiation, whereas overlapping ellipses suggest little or no differentiation between the respective populations.

Pairwise Fisher's exact tests (Raymond and Rousset, 1995) were used to determine the statistical significance of genetic differentiation between all pairs of populations using the program Tools For Population Genetic Analysis (TFPGA; Miller, 1997). The Fisher's exact test is based on the frequency of the AFLP markers to generate probability values between each population. Probability values >0.05 suggest there is no significant genetic differentiation between populations, whereas P values < 0.05 suggest significant genetic differentiation. The "exact test" was performed on each locus, as well as over all loci, for each pair of populations, and between regions.

Results

In total, 45 markers (bands) were scored from DNA fingerprints generated by the AFLP primer pair m-cac/e-act (Figure 3, appendix 3). Of these 45 markers, 35 (77.8%) were polymorphic, where a band was identified as polymorphic when absent in one or more individuals. There was markedly less genetic variation in the Die Hardy populations compared to the Windarling population, however this is largely due to the smaller sample sizes (Table 1).

Table 1: Summary of genetic diversity statistics for *Tetratheca paynterae* and *T. "Die Hardy"*.

Population	n	%polymorphism	H_e	SE
Windarling	27	66.7	0.24	0.02
Die Hardy Site 1	4	13.3	0.12	0.02
Die Hardy Site 2	6	8.9	0.07	0.02

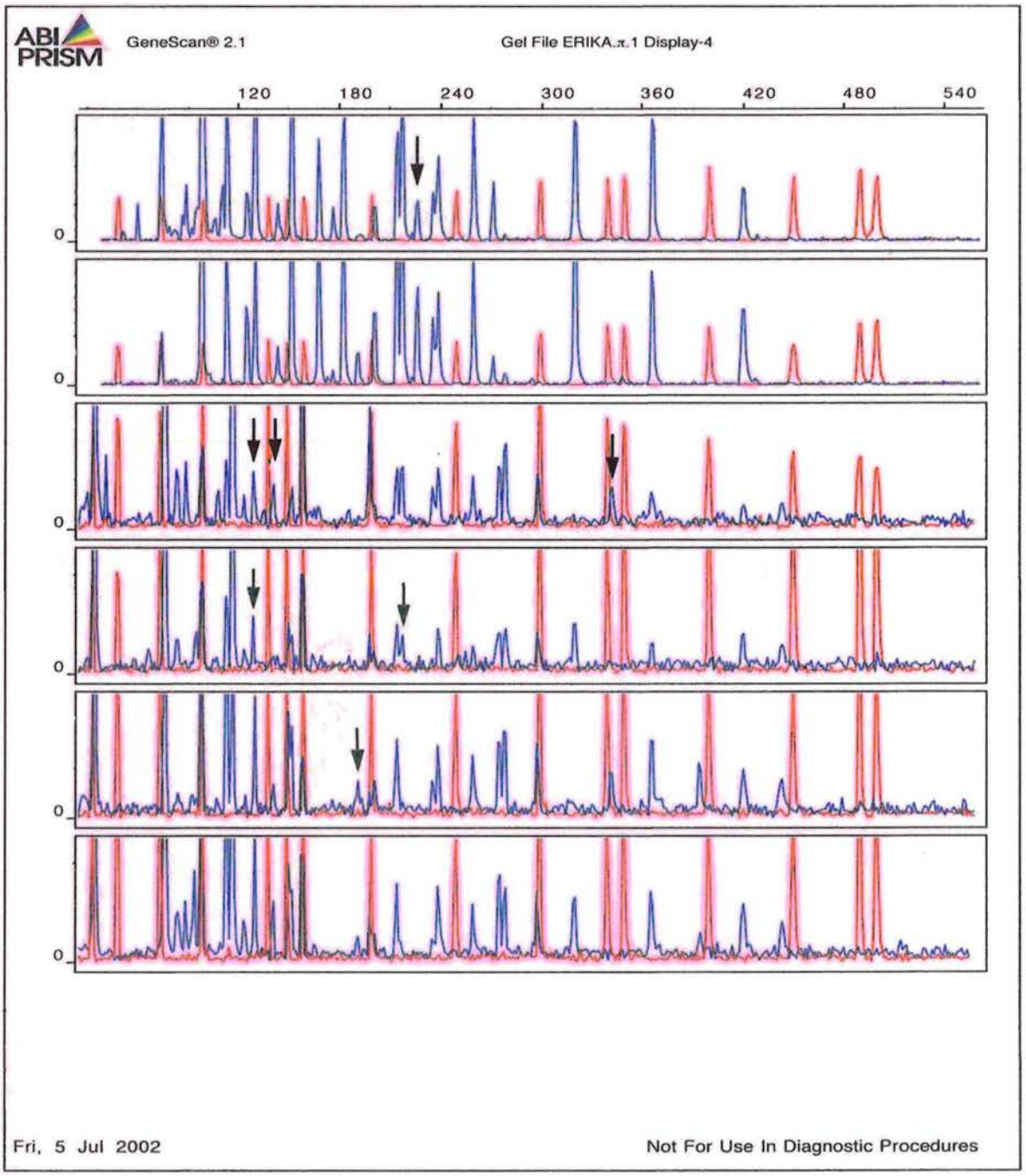
n = population sample size.

% Polymorphism = the proportion of markers (bands) which are polymorphic in each population (i.e. there is variability in the presence and absence state of markers amongst individuals within the population).

H_e = expected heterozygosity, a measure of genetic diversity within populations.

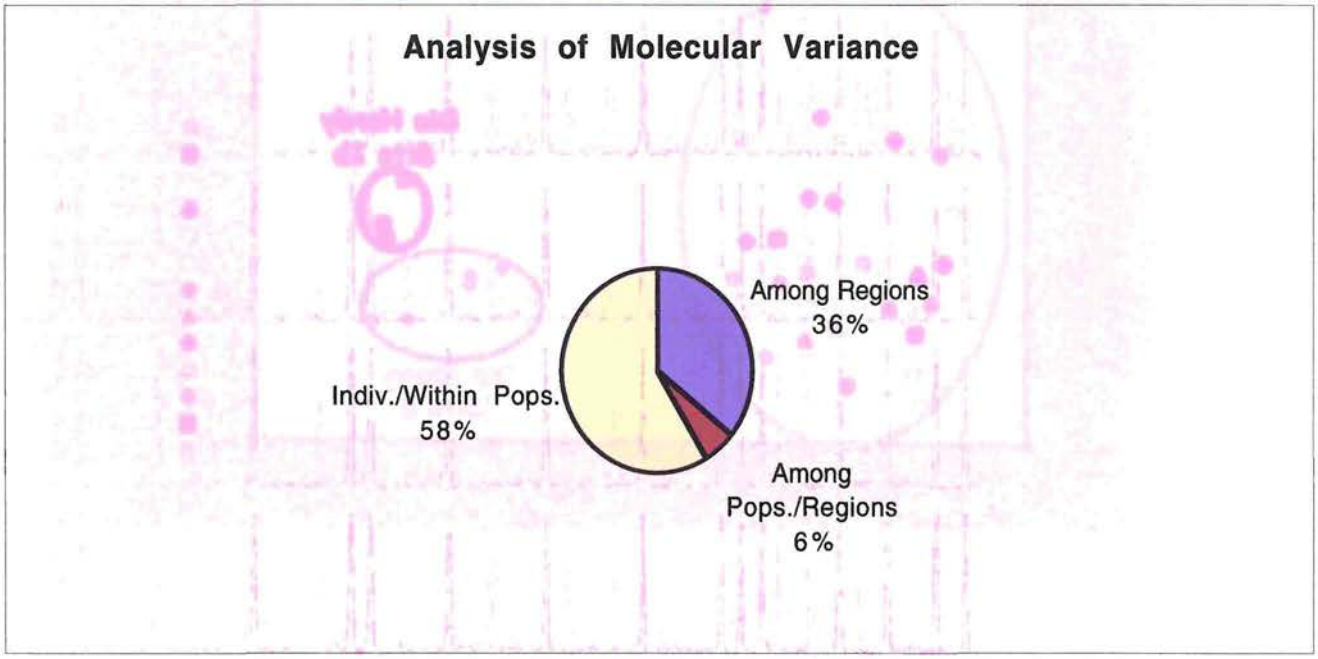
SE = standard error of H_e.

Figure 3: DNA fingerprints generated by the AFLP primer pair m-cac/e-act for *Tetratheca paynterae* and *T. "Die Hardy"*. The first two fingerprints are from the Windarling Range plants, the next two are plants from Site 1b of the Die Hardy Range, and the last two fingerprints are plants from Site 2 of the Die Hardy Range. Note the four fixed genetic differences between the Windarling and Die Hardy plants (black arrows), and the three fixed differences between the two Die Hardy populations (green arrows).



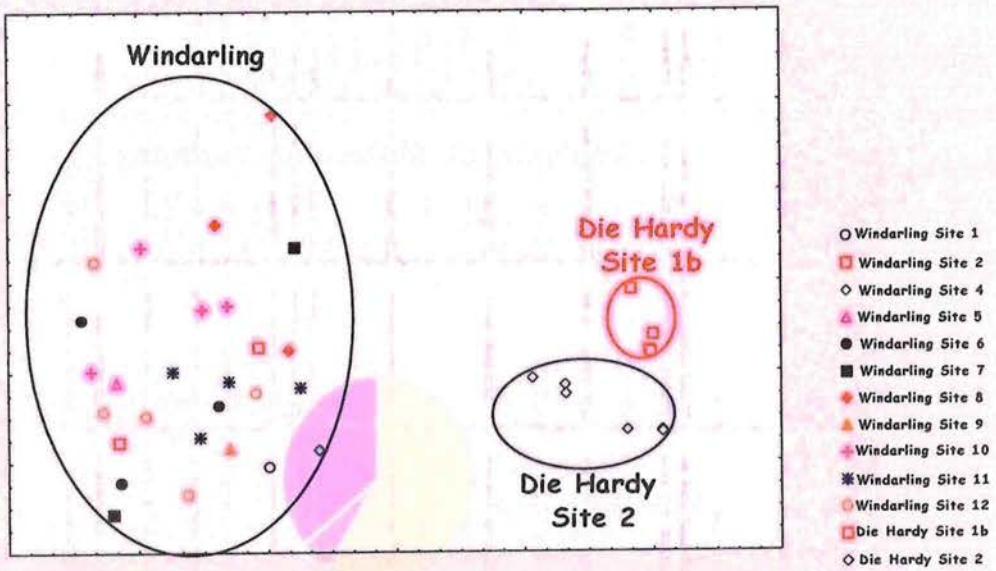
A hierarchical analysis of molecular variance (AMOVA) partitioned 36% of the total variance between the two regions, Windarling Range and Die Hardy Range (Figure 4). This result indicates strong genetic differentiation between the Windarling and Die Hardy regions. A large proportion of the remaining variance (58%) was partitioned among individuals within populations, with only 6% among populations within regions. This suggests little genetic differentiation among populations within the Windarling and Die Hardy regions (Figure 4).

Figure 4: Partitioning of the total genetic variance from 45 AFLP makers in *Tetratheca* spp. by an Analysis of Molecular Variance (AMOVA). The two regions were the Windarling Range and Die Hardy Range respectively. The populations within regions were the 10 Windarling sites and two Die Hardy sites from which *T. paynterae* and *T. "Die Hardy"* respectively *was* sampled (see Figures 1 and 2).



The MDS ordination of these genetic data visually demonstrates little to no genetic differentiation between subpopulations sampled within the Windarling region (ie little clustering of points for each site) (Fig. 5). In contrast the ellipses of the two Die Hardy sites are non-overlapping, indicating genetic differentiation between these two populations. The MDS ordination clearly indicates very marked genetic differentiation between the Windarling and Die Hardy regions (Figure 5).

Figure 5: MDS ordination of genetic variation generated by 45 AFLP markers for *Tetratheca paynterae* of the Windarling Range and *T.* "*Die Hardy*". Non-overlapping ellipses indicate genetic differentiation. The site locations are detailed in Figures 1 and 2.



Tests of statistical significance of genetic differentiation, by way of Fisher's exact tests, showed very strong support ($P<0.001$) for the genetic differentiation of *Tetratheca paynterae* and *T. "Die Hardy"*. There was no significant genetic differentiation among subpopulations of *T. paynterae* ($P>0.90$), and between the two populations of *T. "Die Hardy"* ($P>0.90$). However the "exact test" result for the Die Hardy populations are affected by the relatively small sample sizes, and is currently being addressed by the genotyping of more samples. Note though the inclusion of more samples of *T. Die Hardy* will not change the result and conclusion for the *T. paynterae* and *T. "Die Hardy"* comparison.

Contrasting *T. paynterae* and *T. "Die Hardy"*,19 of 45 markers (42.2%) were identified statistically to be significantly differentiated ($P<0.01$). Of these 19 markers, 15 (33.3% of all markers scored) showed highly significant differentiation ($P<0.001$) with fixation of markers and extreme frequency differences (Table 2). Fixed genetic differences reflect a current absence of gene flow between these populations. Reproductive isolation is the strongest test of the species status of two candidate taxa or populations.

Table 2: Marker presence frequency for each of the 15 markers generated by the AFLP primer pair m-cac/e-act that were highly significant (P<0.001) in differentiating between *Tetratheca paynterae* from the Windarling Range and *Tetratheca "Die Hardy"*.

Marker (bp)	***T. paynterae***	***T. "Die Hardy"***
125.0	0.22	1.00
126.3	1.00	0.50
142.3	0.15	1.00
144.2	0.81	0
168.7	1.00	0.30
177.0	0.74	0
183.5	1.00	0
199.7	0.22	1.00
218.0	1.00	0.40
227.0	0.96	0
272.1	0.67	0
275.8	0	1.00
298.7	0	1.00
342.5	0	0.60
442.7	0.30	1.00

Conclusion

Genetic analysis, using AFLP, of 27 samples of *Tetratheca paynterae* and 10 samples of *T. "Die Hardy"* has identified:

1. High levels of genetic variation, and an absence of genetic differentiation, within the Windarling population of *T. paynterae*. This indicates that the Windarling population of *T. paynterae* is effectively a single continuous population, and there is no evidence that the plants to be removed, should mining go ahead, are genetically differentiated from those that would remain, and vice versa.

2. Highly significant genetic differentiation between *T. paynterae* and *T. "Die Hardy"* . This genetic differentiation, and the high proportion of fixed or highly significant frequency differences (33% of all markers scored) strongly supports a formal taxonomic recognition of *T. "Die Hardy"* as a taxon district from *T. paynterae*, and most appropriately at species rank.

3. Genetic differentiation between plants from the two sites from the Die Hardy region, as revealed by the non-overlap of clusters in the ordination of genetic variation. However, samples sizes to date are too small to assign statistical significance to the genetic differentiation between these two sites.

4. To complete these studies, we are currently (i) genotyping more samples from the Die Hardy Range to bring these into line with sample sizes of *T. paynterae*, and (ii) genotyping samples of *T. aphylla* to confirm the placement of taxonomic rank of *T. "Die Hardy"*. These results will be submitted as a supplement to this report when they come to hand.

PART 2: GERMINATION STUDY OF *TETRATHECA PAYNTERAE* FROM THE WINDARLING RANGE.

Introduction

Seed dormancy is a common mechanism in Australian native flora allowing germination of seed when the environmental conditions are most favourable (Adkins and Beeliars, 1997). Seed dormancy mechanisms can be broadly divided into two basic types: seed coat imposed dormancy and embryo imposed dormancy (Bewley and Black, 1994).

Seed coat imposed dormancy occurs via several mechanisms, such as permeability barriers (eg. mucilaginous layer) to water and nutrients, mechanical barriers (ie. hard, tough tissues) to embryo expansion, and germination inhibitors produced in the outer coat (eg. abscisic acid, coumarin, tannins and phenols). Seed coat dormancy may be inactivated by the action of saprophytic fungi, fire, enzymes produced by the embryo and the chemical action of the passage of the seed through the digestive tract of an animal (for permeability and mechanical barriers). Germination inhibitors are leached over time by rainfall or the decay of the seed coat (Adkins and Belliars, 1994).

Embryo imposed dormancy occurs via several mechanisms including gene repression of enzymes required for germination, changes in plant growth regulators, changes in metabolic pathways, and embryo immaturity of the shed seed (Bewley and Black, 1994). There is little evidence of gene repression causing dormancy in Australian native species, however embryo immaturity and the influence of plant growth regulators have been well-documented (Adkins and Beeliars, 1997). In particular the plant growth regulator, gibberellic acid (GA), is synthesised by the embryo in response to environmental conditions and promotes germination in a wide range of Australian native species (Adkins and Beeliars, 1997).

Overcoming seed dormancy is a critical issue in restoration and rehabilitation programs of native Australian flora to achieve more successful germination of target species from seed. The dormancy mechanisms were investigated in *Tetratheca paynterae* seeds from the soil seed bank to determine the appropriate treatments required to break dormancy and thereby optimise germination success from seed. The soil stored seed bank at Windarling was characterised, which included an assessment of the viability of these seeds.

Methods

Soil samples taken from crevices on the W3 Windarling site were sifted for seeds for use in the germination trials. These crevices were specifically targeted for their likelihood of containing seed of *T. paynterae*. A cut test was performed on 50 seeds in which the seeds were cut in half with a blade and scored as viable if the endosperm was white and fleshy, and if an embryo was present.

Eight treatments were conducted on the seeds from the soil seed bank, in which there were four replicates of 25 seeds for each trial. The treatments were as follows:

1. Control (no treatment)
2. Nicking
3. Gibberellic Acid
4. Aerosol smoke
5. Gibberellic Acid and Nicking
6. Aerosol smoke and Nicking
7. Aerosol smoke and Gibberellic Acid
8. Aerosol smoke, Nicking and Gibberellic Acid

Nicking was conducted by gently removing a small slice of the seed coat with a blade under a dissecting microscope. The seeds were then soaked in gibberellic acid (1000ppm) overnight for the appropriate treatments (Treatments 5 and 8), concurrently with unnicked seeds (Treatment 3). Seeds, not to be treated with gibberellic acid, were soaked in water overnight (Treatments 1, 2,4, and 6).

Twenty five seeds were sprinkled into each punnet containing a soil mix (see appendix 4) with four replicates for each treatment (ie. A total of 100 seeds per treatment). The seeds were covered with 1-2cm of potting mixture, and exposed to aerosol smoke for 1 hour where appropriate (Treatments 2, 5, 6, and 8). The punnets were placed in a greenhouse at ambient temperature and watered sparingly over a period of 9 weeks. The number of germinants per punnet was recorded every two to four days.

Results

There were a total of 1354 *T.paynterae* seeds found within 30,000cm^3 of soil collected from crevices below large plants on the W3 Windarling site (45,133 seeds/1m^3). A cut test on 50 of these seeds indicated that 36 seeds (72%) were viable, and 14 seeds (28%) were not viable. The viability of the soil seed bank is critical in interpreting the success of the germination trials.

Germination of *T.paynterae* began 32 days from the time the seeds were sowed, except in the control treatment in which there was no germination observed until 36 days (Figures 6, 6a). New germination of seed continued until approximately 55 days, after which few new germinants were seen (Fig. 6). Significant germination was observed in all treatments, including the control, with germination rates after 65 days post-sowing ranging from 20% (control) to 50% (gibberelic acid and nicking) of all seeds sown. Assuming that the percentage of all seeds that were viable was approximately 72%, then these germination percentages translate to a range of 28% (control) to 69% (gibberelic acid and nicking) of all viable seeds germinated. Various combinations of treatments (eg gibberelic acid and smoking) showed a higher germination success than individual treatments (eg. gibberellic acid only) and the control (Figures 6 and 7).

Figure 6a: Seedlings of *T. paynterae* germinated at Kings Park from seed collected from Windarling

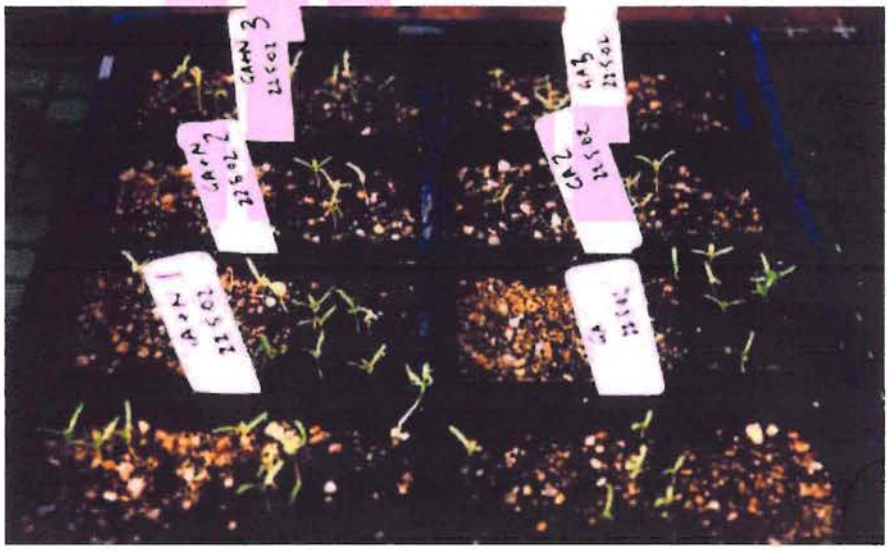


Figure 6: The effect of various treatments on the mean germination rate (calculated as the mean of 4 replicates of 25 seeds) of *Tetratheca paynterae.*

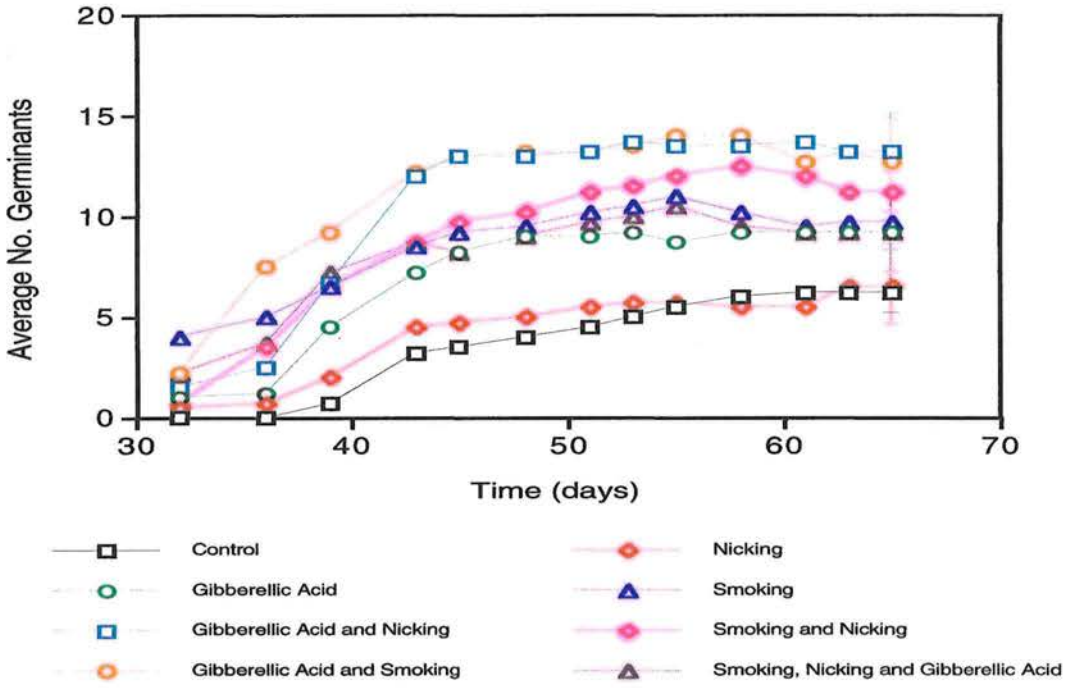
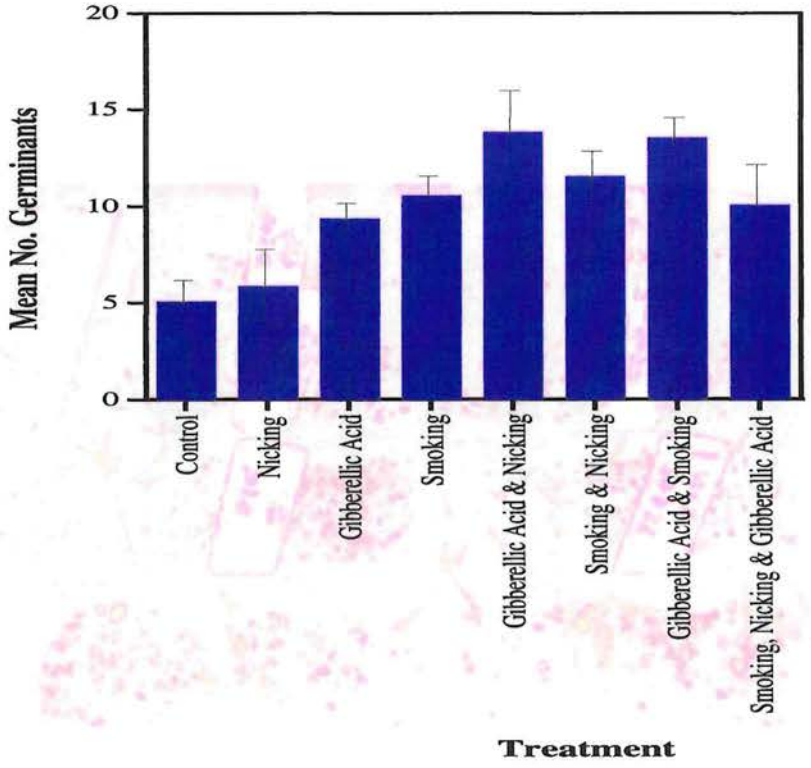


Figure 7: The effect of various treatments on the germination of *Tetratheca paynterae* (calculated as the mean of 4 replicates of 25 seeds) 65 days after the seeds were sowed.



Conclusion

The analysis of soil-stored seed of *T. paynterae* from the W3 Windarling site indicated (i) a substantial soil-stored seed bank in targeted sites, with 1354 seeds collected from 30000cm^3 of soil, (ii) of these seed, approximately 72% were found to be viable, (iii) significant germination of seeds between 32 and 55 days post-sowing, (iv) germination percentages varied from a low of 20% for control to 50% for gibberelic acid with nicking treatments, and combinations of treatments produced greater germination than single treatments or control. However, more detailed research is required to optimise the success and benefits of germination treatments.

PART 3: PROPAGATION STUDY OF *TETRATHECA PAYNTERAE* FROM THE WINDARLING RANGE.

Introduction

Propagation of plants from stem cuttings is a versatile method that readily works with a wide range of plant species and can be used to multiply plants rapidly. It is, however, a vegetative method of propagation and therefore the cuttings are clones of the plant from which the cutting was obtained. Generally the stem tips are used in propagation because this is the site of rapid cell division and growth (Stewart, 1999). Stem cuttings may be propagated from softwood, semi-hardwood, and hardwood species, with hardwood cuttings generally taking longer to strike.

There are a number of treatments that can improve the success of propagation of stem cuttings, including the application of auxins and vitamins (ie. thiamine and riboflavin) to promote root formation. In addition the application of nitrogen and raising the temperature of the propagation medium has a stimulatory effect on root formation in some species (Stewart, 1999). Stem cuttings of a small number of genotypes of *Tetratheca paynterae* and *T. "Die Hardy"* were treated with various concentrations and exposure times of clonex and Thrive® to determine the optimal treatment for propagation.

Methods

Fresh plant material was collected from three plants of *T. paynterae* and one plant of *the T. "Die Hardy"* from W3 Windarling and Die Hardy populations respectively, wrapped in wet paper and stored at 4°C. The material was washed thoroughly with distilled water prior to cutting the tips of the stem in 10cm sections. These sections were then placed in Thrive® for the various lengths of time (0min, 15min, 30min, 45min). Excess Thrive® was rinsed from the cuttings with distilled water and the base of the cuttings were dipped in clonex (either 3000ppm or 8000ppm) up to a depth of approximately 1cm. The cuttings were then planted in punnets (25 per punnet) containing soil mix (2 parts nursery sand (fine sand): 1 part perlite: 1 part peat moss) and placed on a heat bed of 25°C in the greenhouse. The cuttings were watered regularly by mist spray and kept moist at all times. Cuttings were monitored frequently to assess for root and shoot growth.

Results

Significant levels of shoot and root growth from cuttings have been observed for all treatments to date (Figures 8-11). As these cuttings are still producing new shoots and roots, it can not yet be determined at this early stage which treatment is optimal for propagation from cuttings. In particular, the *T. "Die Hardy"* clone only commenced shooting after 73 days. However, the variability observed across clones and treatments suggests that the success of propagation of *T.paynterae* and *T. "Die Hardy"* from cuttings depends more on the condition of the starting material rather than the actual treatments (Figures 12 – 15).

Figure 8a: Shoot growth from a cutting of *T. paynterae* collected from Windarling

Figure 8: The effect of various treatments on the propagation of *Tetratheca paynterae* (CLONE 1) from stem cuttings.

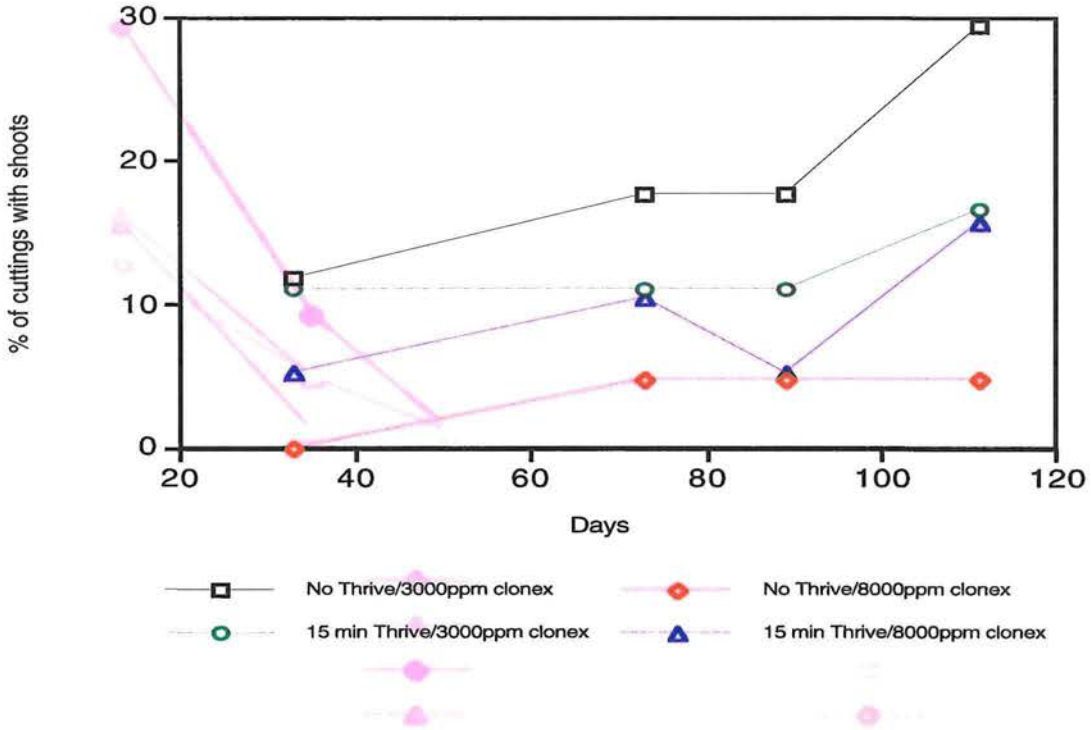


Figure 9: The effect of various treatments on propagation of *Tetratheca paynterae* (CLONE 2) from stem cuttings.

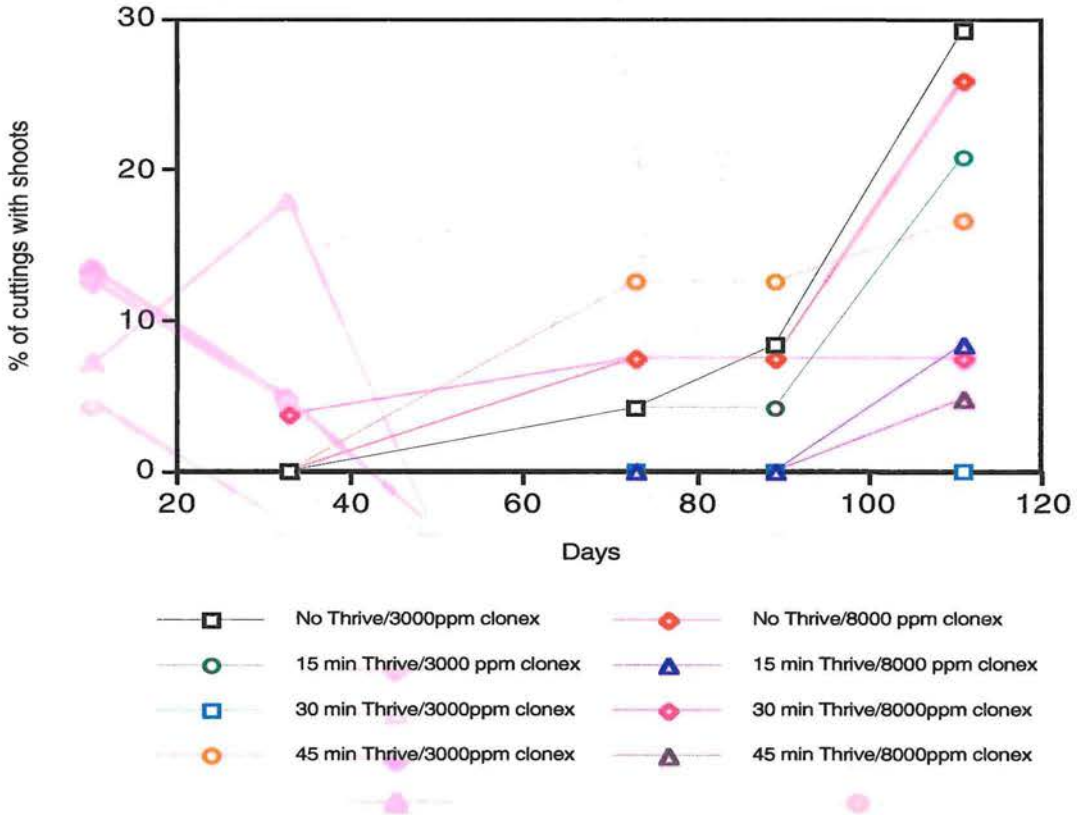


Figure 10: The effect of various treatments on the propagation of *Tetratheca paynterae* (CLONE 3) from stem cuttings.

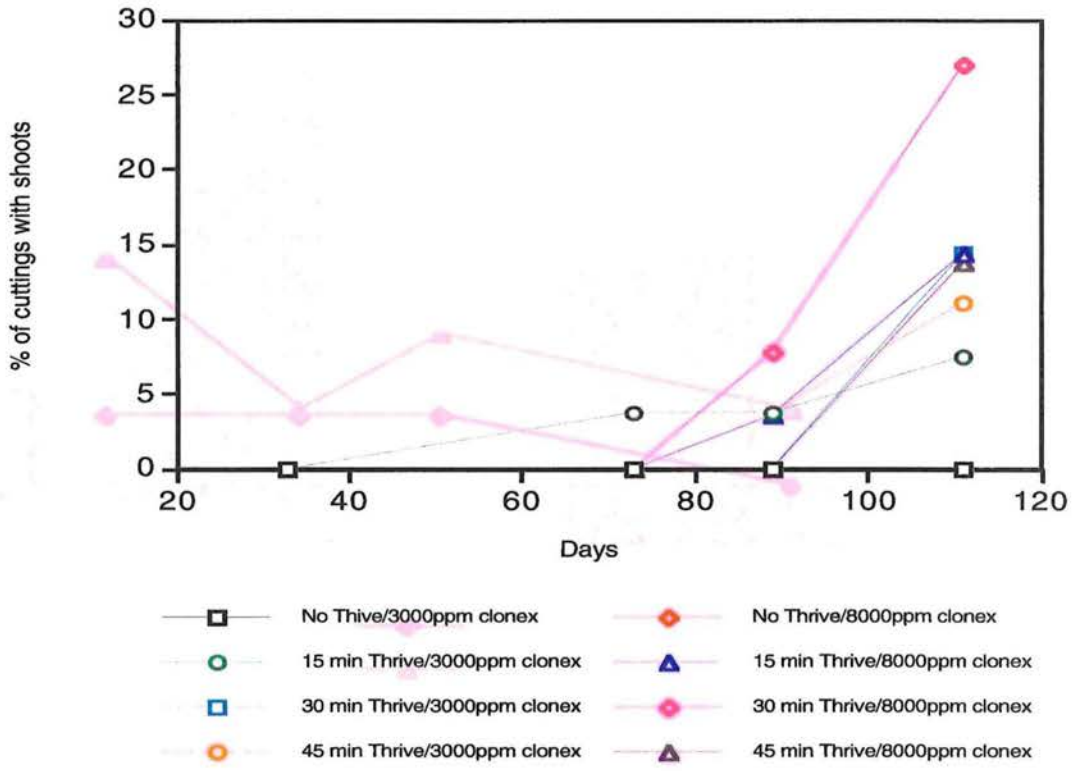


Figure 11: The effect of various treatments on the propagation of *Tetratheca "Die Hardy"* from stem cuttings

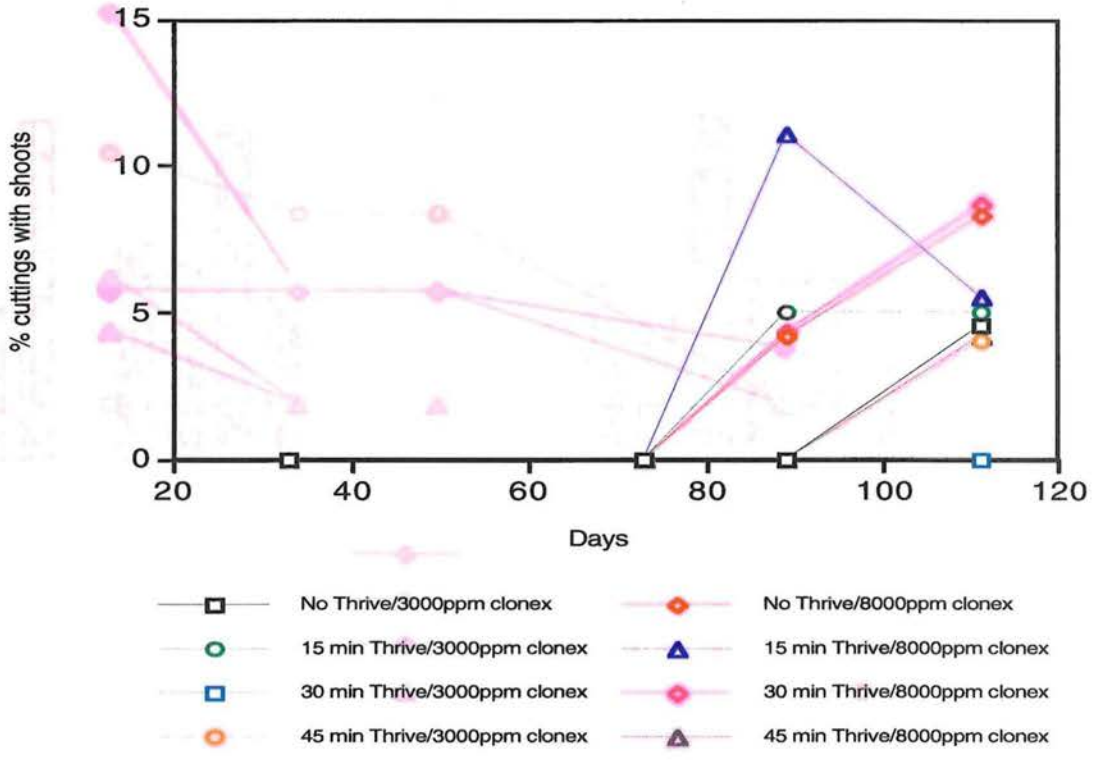


Figure 12: The effect of various treatments on the propagation of *Tetratheca paynterae* (CLONE 1) from stem cuttings after 111 days.

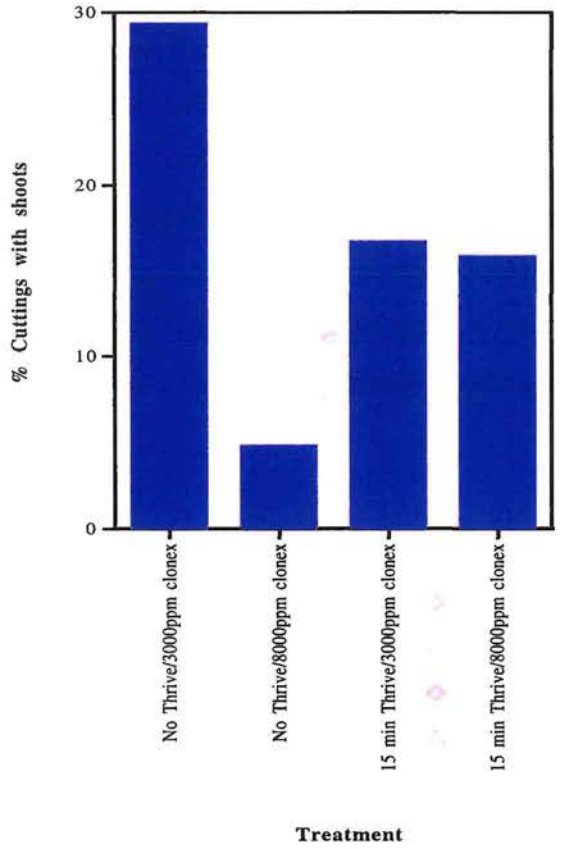


Figure 13: The effect of various treatments on the propagation of *Tetratheca paynterae* (CLONE 2) from stem cuttings after 11 days.

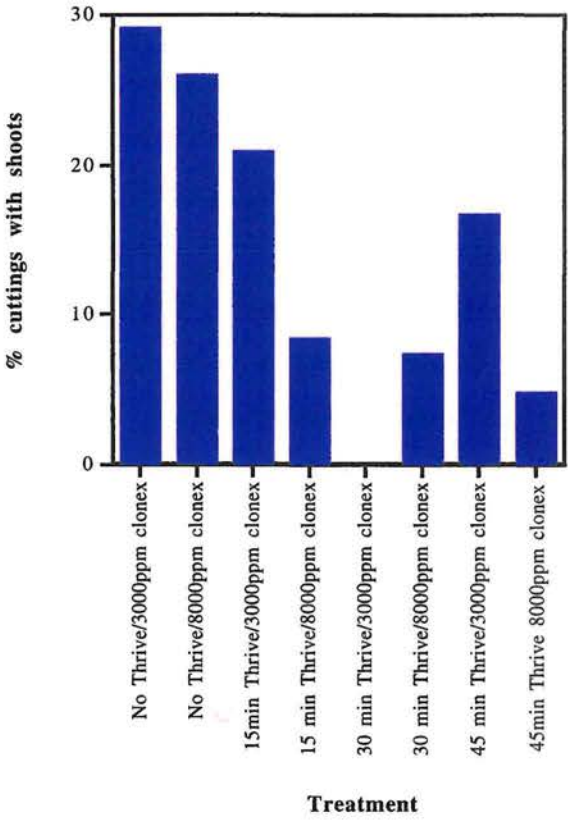


Figure 14: The effect of various treatments on the propagation of *Tetratheca paynterae* (CLONE 3) from stem cuttings after 11 days.

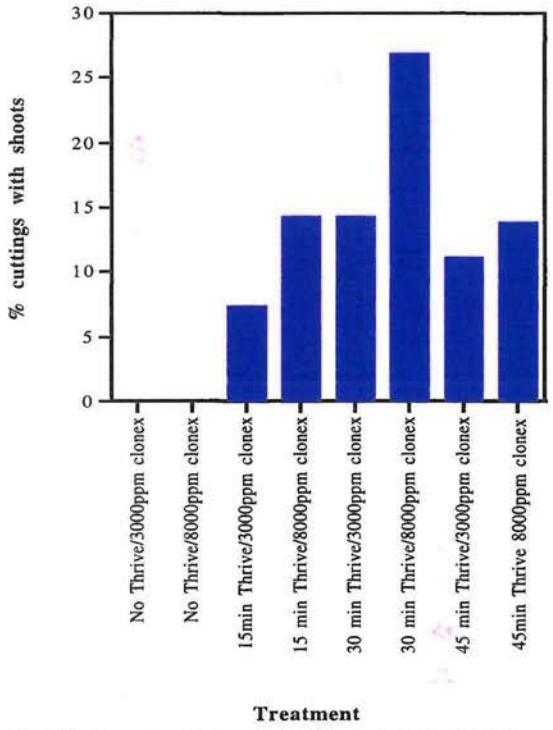
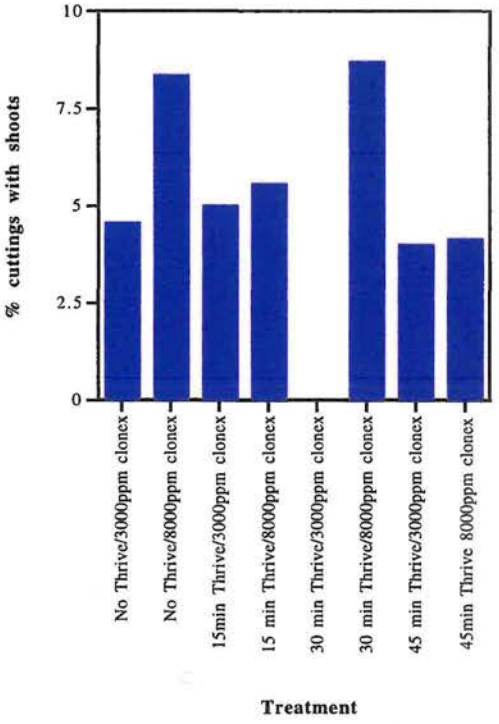


Figure 15: The effect of various treatments on the propagation of *Tetratheca "Die Hard)* from stem cuttings after 111 days.



Conclusion

As this experiment is still running, it cannot yet be determined which treatment is optimal for propagation of *T. paynterae* and *T. "Die Hardy"* from cuttings. However, results to date show a significant level of shoot and root growth from all genotypes over a range of treatments. Furthermore, the variability across genotypes and treatments suggests that the good condition of source material for propagation appears to be the single most important variable for successful propagation from cuttings of *T.paynterae* and *T. "Die Hardy"*. However, more detailed research is required to optimise the success and benefits of propagation treatments.

General Discussion

Using the powerful DNA fingerprinting technique AFLP, genetic analysis of *Tetratheca paynterae* and *T. "Die Hardy"* has clearly shown (i) no significant genetic differentiation within the Windarling Range population of *T. paynterae*; (ii) some genetic differentiation between two populations of *T. "Die Hardy"* from the Die Hardy range, and (iii) very marked genetic differentiation between *T. paynterae* and *T. "Die Hardy"*.

The genetic differentiation found between *T. paynterae* and *T. "Die Hardy"* strongly supports a formal taxonomic recognition of *T. "Die Hardy"*. Of all AFLP markers scored, 42% were significantly differentiated between these two taxa, suggesting that species is the appropriate rank. A very high percentage (33%) of all markers displayed either fixed frequency differences (present in all plants of one population and absent in all plants of the other population) or highly significant frequency differences, which indicates an absence of genetic exchange between these two taxa. This is a powerful test for the recognition of two taxa at species rank. These findings support those of Butcher *et al.* 2002 in which morphometric analyses and chloroplast sequencing clearly distinguished between *T. paynterae* and *T. "Die Hardy"*. Current AFLP analyses of *T.aphylla* will provide a reference to further assess the taxonomic rank of *T. "Die Hardy"*.

In contrast to the *T. paynterae - T. "Die Hardy"* comparison, more or less contiguous sub-populations of *T. paynterae* within the Windarling Range (up to 2.5km apart) were not genetically differentiated, and hence can be regarded as a single panmictic population. That is, these results suggest that there are no internal barriers to gene flow nor microhabitat selection for different genotypes across the Windarling site. Consequently, we found no evidence to suggest any genetic differentiation between the 89% of plants that would be directly affected by proposed mining activities, and the remaining 11% of plants at the western edge of the W3 site.

In contrast, we found some evidence for genetic differentiation between the two *T. "Die Hardy"* populations sampled, which are approximately 3.5km apart. These two *T. "Die Hardy"* populations occur on different peaks and are separated by a large valley, which may be acting as a barrier to gene flow. Depending on the mating system of these plants, the history of isolation and the possibility of historical purging of deleterious alleles through population bottlenecks, isolation and small population size may be leading to inbreeding and loss of genetic variation. If inbreeding is associated with inbreeding depression, these populations are theoretically placed at a higher risk of extinction from genetic decline (Frankham, 1995). Further detailed studies into mating systems, pollen and seed dispersal, pollinators, and inbreeding depression are required to assess the evolutionary consequences and appropriate management strategies of historically small and isolated populations such as these.

Despite the relatively small size and isolation of *T. paynterae* on Windarling range, it still retains relatively high levels of genetic variation (67% of all markers polymorphic) compared to other studies using AFLP (e.g. Winfield *et al* 1998; Russell *et al.* 1999; Krauss 2000; Krauss *et al* 2002). This relatively high level of genetic variation would require careful management if mining occurs on the proposed W3 deposit because a severe reduction in population size (and hence genetic variability) makes it more vulnerable to extinction from demographic and genetic stochasticity. Any proposal for management of *T. paynterae* involving *ex situ* conservation and/or translocation must incorporate and adequately sample this genetic variation. Further, an understanding of the processes that create and maintain this genetic variation is critical.

The results to date of propagation and soil-stored seed bank studies have been extremely positive for any future proposals requiring material for translocations, mine site revegetation and/or *ex situ* conservation of *T. paynterae*. We found (i) large numbers of seed in the soil-stored seed bank from specifically targeted locations, (ii) a high (72%) viability of these seeds, and (iii) significant levels of seed germination for all treatments (including control), with various combinations of treatments producing the best results. In addition, significant levels of root and shoot growth from cuttings were achieved, and

it was noted that the quality of the source material appeared to be the single most important factor in the results obtained. However, more detailed research is required to optimise the success and benefits of propagation and germination for the effective management and conservation of *T. paynterae*.

Furthermore, detailed ecological and physiological studies would be critical in developing an appropriate management strategy for the *in situ* conservation of the DRF *Tetratheca paynterae*. Whilst we have demonstrated some success in the germination of seeds and propagation from cuttings, *in situ* conservation through translocations and/or post-mining re-establishment in revegetation programmes poses significant challenges requiring detailed research to ensure success.

References

Adkins, S. W., and Bellairs, S. M. (1997) Seed dormancy mechanisms in Australian native species. In: *Second Australian native seed biology for revegetation workshop* (Eds. S. M. Bellairs and J.M. Osbourne) pp. 81-91.

Alford, J.J. (1995) Two new species of *Tetratheca* (Tremandraceae), from the Coolgardie and Austin Botanical Districts, Western Australia. *Nuytsia* 10 (2): 143 – 149.

Bewley, J. D., and Black, M. (1994) Seeds. Physiology of development and germination. Second edition. Plenum Press; New York. pp. 445

Butcher, R. (2001) Identification of *Tetratheca* material from the Die Hardy Range. *Unpublished report prepared for Mattiske Consulting Pty Ltd on behalf of Portman Iron Ore Limited, Perth.*

Butcher, R., Byrne, M., and Coates, D. (2002) A study of molecular and morphological variation within and between *Tetratheca* (Tremandraceae) from Windarling and Die Hardy Ranges; Coolgardie District, WA. Prepared for Portman Pty Ltd, Perth. pp.63

Ecologia Environment (2002) Koolyanobbing Iron Ore Expansion Project. Public Environmental Review. Prepared for Portman Pty Ltd, Perth. pp. 242.

Frankham, R. (1995) Inbreeding and extinction: a threshold effect. *Conservation Biology* **9**, 797-799.

Krauss S.L. (2000) Patterns of mating in Persoonia mollis (Proteaceae) revealed by an analysis of paternity using AFLP: implications for conservation. *Australian Journal of Botany* 48:349-356.

Krauss, S.L., B. Dixon, K.W. Dixon (2002) Rapid genetic decline in a translocated population of the endangered plant *Grevillea scapigera. Conservation Biology* 16:1-9.

Miller, M. P. (1997) Tools for population genetic analysis (TFPGA) 1.3: A windows program for the analysis of allozyme and molecular population genetic data.

Mueller, U. G., and Wolfenbarger, L. L. (1999) AFLP genotyping and fingerprinting. *Trends in Ecology and Evolution* **14**, 389 – 394.

Peakall. R., and Smouse, P. (2001) GenAlEx V5: Genetic Analysis in Excel. Population genetic software for teaching and research. Australian National University, Canberra, Australia.

Raymond, M. L., and F. Rousset. (1995) An exact test for population differentiation. *Evolution.* **49**, 1280 – 1283.

Russell, J. R., Weber, J. C., Booth, A., Powell, W., Sotelo-Montes, C., and Dawson, I. K. (1999) Genetic variation of *Calycophyllum spruceanum* in the Peruvian Amazon Basin, revealed by amplified fragment length polymorphism (AFLP) analysis. *Molecular Ecology* **8**, 199-204.

StatSoft, Inc. (1995) STATISTICA for windows. StatSoft, Inc.

Stewart, A. (1999) Let's propagate! ABC Books, Perth. pp. 49 – 67.

Sunnucks, P. (2000) Efficient genetic markers for population biology. *Trends in Ecology and Evolution* **15**, 199 – 203.

Vekemans, X. (2002) AFLP-SURV 1.0 A program for genetic diversity analysis with AFLP (and RAPD) population data.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407 – 4414.

Winfield, M. O., Arnold, G. M., Cooper, F., Le Ray, M., White, J., Karp, A., and Edwards, K. J. (1998) A study of genetic diversity in *Populus nigra* subsp. *betufolia* in the Upper Severn area of the UK using AFLP markers. *Molecular ecology* 7, 3-10.

Appendix 1: DNA extraction using a modified QIAGEN DNeasy protocol

1. Grind plant tissue (~600mg) in liquid nitrogen using a mortar and pestle
2. Add 2ml of Buffer AP1 and 6µl RNAse A
3. Incubate the mixture for 10 minutes at 65 degrees. Mix during incubation by inverting tubes
4. Add 250µl of Buffer AP2 to the lysate mixture and incubate for 5 minutes on ice. (Proteins and Polysaccharides are precipitated in this step)
5. Using the lilac spin column sitting in a 2ml collection tube add the lysate and centrifuge for 2 minutes at maximum speed.
6. Transfer flow-through elute to a new tube without disturbing the cell debri pellet
7. Repeat Step 5 and Step 6 until all the lysate has been centrifuged through the lilac spin column
8. Add 1.5 volumes of Buffer AP3/E to the cleared lysate and mix by pipetting
9. Apply 650µl of the mixture from Step7 to the Dneasy min spin column sitting in a 2ml collection tube. Centrifuge at 1 min at 8000rpm and discard flow-through
10. Repeat Step 9 with remaining sample (several times may be necessary). Discard flow-through and collection tube.
11. Place Dneasy column in a new 2ml collection tube, add 500µl Buffer AW to the Dneasy column and centrifuge for 1 minute at 8000rpm. Discard flow-through and reuse collection tube in Step 12.
12. Add 500µl Buffer AW to the Dneasy column and centrifuge for 2 minutes at maximum speed to dry the membrane.
13. Transfer the Dneasy column to a 1.5ml or a 2ml tube and pipette 40µl of preheated (65 degrees) Buffer AE directly onto the Dneasy membrane. Incubate for 5 minutes at room temperature and then centrifuge for 1 minute at 8000rpm to elute.
14. Repeat Step 13 once more

Appendix 2: Amplified Fragment Length Polymorphism (AFLP)

Amplified fragment length Polymorphism (AFLP) is a recently developed molecular technique. It combines the strengths of both RFLP and RAPD. Random Fragment Length Polymorphism (RFLP) involves the use of restriction enzymes to cut DNA at specific points in the genome, electrophoresis of the resultant fragments and visualisation using specific probes to hybridize with the fragments. The drawbacks of this technique is that it is expensive, time consuming and the information that can be obtained is limited (Mueller and Wolfenbarger, 1999).

With the development of the polymerase chain reaction (PCR), allowing the amplification of DNA, many new molecular techniques such as Randomly Amplified Fragment Length Polymorphism (RAPD) were developed. This technique involves the development of primers which amplify random DNA segments of the genome (Sunnucks, 2000). However a number of problems are encountered with this technique including low reproducibility, and the generation of artifacts (Sunnucks, 2000)

Amplified Fragment Length Polymorphism (AFLP) is a more cost-effective technique. In addition it can screen genomic DNA rapidly, requires no prior genetic knowledge of the organism, can be readily used over a wide range of species or taxa, and is highly reproducible. This technique involves several steps. Firstly the genomic DNA is cut with two restriction enzymes. One of the restriction enzyme cuts frequently (eg. EcoR1) and the other infrequently (eg. Mse1) to generate a series of DNA fragments. Adaptors are ligated to the ends of the fragments to serve as primer binding sites in the subsequent PCR. In the selective PCR a subset of fragments are selected for amplification via fluorescently labelled primers (Vos *et al.*, 1995). The PCR products are electrophoresed on a denaturing polyacrylamide gel and the fluorescent fragments visualised via a laser and transmitted to a computer where it is stored for future reference. The AFLP

fingerprints are then scored according to the presence or absence state of restriction fragments.

AFLP Protocol

(i) Restriction digestion of genomic DNA

For each sample, approximately 200ng of DNA was digested with 1.25 units of EcoRI/MseI restriction enzyme in a reaction volume of 12.5µl, and incubated at 37°C for 2 hours. Samples were then transferred to a 70°C waterbath for 15 minutes, before briefly cooling on ice.

(ii) Ligation of adapters

12µl of adapter ligation solution and 0.5 units of DNA ligase were added to the digested DNA from *(i)*, incubated at 20°C for 2 hours, then diluted 1:10 with 0.1M TE buffer.

(iii) Preselective PCR

1.25µl of the diluted ligation mix was combined with 10µl pre-amplification primer solution, 1.25µl 10X PCR buffer for AFLP, and 0.6 units Taq DNA polymerase in a PCR plate and PCR was performed for 20 cycles of 94°C for 30 sec, 56°C for 2 min, and 72°C for 2min. Subsequently, the pre-amplification mix was diluted 1:50 with 0.1M TE buffer.

(iv) Selective PCR

For each sample 2.5µl of the diluted pre-selective PCR product was added to 7.5ng EcoRI-primer, 15ng MseI-primer, 3.95µl Ultrapure water, 1.0µl 10X PCR buffer, and 0.25 units Taq DNA polymerase. A touch-down PCR reaction commenced with 10 mins at 90°C, followed by 1 cycle of 94°C for 1 sec, 65°C for 2 min, 72°C 2 mins. In subsequent cycles, the annealing temperature was reduced in 1°C steps to 55°, followed by 23 cycles at 56°C. AFLP kits, unlabelled primers, and Taq polymerase were purchased from Invitrogen.

Appendix 3: AFLP genotypes of 27 individuals of *Tetratheca paynterae* from the Windarling Range and 10 individuals of *Tetratheca "Die Hardy"* generated using the m-cac/e-act AFLP primer pair.

	Fragment size (bp)																				
	99.1	104.4	107	110.1	111.5	114.1	115.8	117.9	121.7	122.6	125	126.3	127.7	130.2	131.2	142.3	144.2	152.7	159.4	168.7	177	183.5
T6S	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	1	1	0	1
T7S	1	1	0	0	1	1	0	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1
T8S	1	0	0	0	0	1	1	1	0	1	0	1	0	0	1	0	1	1	1	1	0	1
T17S	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1
T22S	1	1	0	0	0	1	0	1	0	1	0	1	1	0	1	0	1	1	0	1	1	1
T25S	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1	1
T27S	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	1
T28S	1	1	0	0	1	1	0	1	1	0	0	1	0	0	1	0	0	1	0	1	1	1
T32S	1	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1
T33S	1	0	1	0	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1
T34S	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	1
T35S	1	1	0	0	0	1	0	1	1	0	1	1	0	0	1	0	0	1	1	1	1	1
T36S	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	1	0	1
T38S	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1
T40S	1	1	1	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1
T41S	1	0	0	0	1	1	0	1	1	0	1	1	0	0	1	0	1	1	1	1	1	1
T42S	1	0	1	0	1	1	0	1	1	0	0	1	0	0	1	0	1	1	0	1	1	1
T43S	1	0	0	0	0	1	1	1	0	1	1	1	0	0	1	0	0	1	1	1	1	1
T46S	1	0	0	0	1	1	0	1	0	1	1	1	0	0	1	0	1	1	1	1	1	1

T47S	1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	1	1	1	0	1
T49S	1	0	0	1	1	1	0	1	0	0	0	1	0	0	1	0	1	1	1	1	1	1
T52S	1	0	0	0	0	1	0	1	0	0	0	1	0	0	1	1	1	1	1	1	0	1
T53S	1	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1	1

	Fragment size (bp)																				
	99.1	104.4	107	110.1	111.5	114.1	115.8	117.9	121.7	122.6	125	126.3	127.7	130.2	131.2	142.3	144.2	152.7	159.4	168.7	177	183.5
T54S	1	0	0	1	1	1	0	1	0	0	0	1	0	0	1	0	1	1	0	1	1	1
T55S	1	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0	1	1	1
T56S	1	1	1	0	0	1	0	1	1	0	0	1	0	0	1	0	1	1	0	1	0	1
T58S	1	0	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	1	1	1	1	1
TDH19	1	0	1	0	0	1	0	1	0	0	1	1	0	1	0	1	0	1	1	0	0	0
TDH21	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0	0
TDH22	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	0	1	1	1	0	0
TDH23	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	0	1	1	1	0	0
DH4	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	1	0	1	1	0	0	0
DH5	1	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	0
DH6	1	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	0
DH8	1	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1	1	0	0
DH12	1	0	0	0	0	1	0	1	0	0	1	0	0	0	1	1	0	1	1	0	0	0
DH13	1	0	0	0	0	1	0	1	0	0	1	1	0	0	1	1	0	1	1	0	0	0

	Fragment Size (bp)																						
	192.2	197.6	199.7	201.7	210.9	215.2	218	227	236.3	239.2	248.8	260.2	272.1	275.8	279.5	298.7	319.6	320.9	331	342.5	366.4	420.2	442.7
T6S	1	1	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	1
T7S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	0	1	0	1	1	0
T8S	0	0	1	1	0	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	1	1	0
T17S	1	0	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1
T22S	1	0	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0
T25S	1	0	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	1	1	0
T27S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	0	0	1	0	1	1	0
T28S	1	0	0	1	1	1	1	1	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0
T32S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	0	1	0	1	1	1
T33S	0	0	1	1	0	1	1	1	1	1	0	1	0	0	1	0	1	1	1	0	1	1	0
T34S	0	0	1	1	0	1	1	1	1	1	0	1	0	0	1	0	1	1	0	0	1	1	0
T35S	0	0	0	1	1	1	1	0	1	1	0	1	1	0	0	0	1	1	0	0	1	1	0
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T38S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	0	1	0	0	1	1	1
T40S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	0
T41S	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	0
T42S	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0	1	0	0	1	1	1
T43S	1	1	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1	0	0	1	1	0
T46S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	1

T47S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1	0	0	1	1	0
T49S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	1	1	1
T52S	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	0
T53S	1	1	0	1	0	1	1	1	1	1	0	1	1	0	0	0	0	0	1	0	1	1	1

	Fragment Size (bp)																						
	192.2	197.6	199.7	201.7	210.9	215.2	218	227	236.3	239.2	248.8	260.2	272.1	275.8	279.5	298.7	319.6	320.9	331	342.5	366.4	420.2	442.7
T54S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1	1	0	1	1	0
T55S	1	0	0	1	0	1	1	1	1	1	0	1	1	0	0	0	0	1	0	0	1	1	0
T56S	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	1	1	0
T58S	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	0	0	1	0	0	1	1	0
TDH19	0	0	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1
TDH21	0	0	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1	1	1	1
TDH22	0	0	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1
TDH23	0	0	1	1	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1
DH4	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1
DH5	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1
DH6	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1
DH8	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1
DH12	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1
DH13	1	0	1	1	0	1	0	0	1	1	0	1	0	1	1	1	0	0	0	1	1	1	1

Appendix 4: Composition of the Soil Mix used for germination of *Tetratheca paynterae* from seed.

Soil Mix

2 parts composted jarrah sawdust: 1 part nursery sand: 0.5 part coarse river sand

Fertilisers (per cubic metre)

3kg Osmocote

1kg Lime

0.8kg Dolomite

0.5kg Ferrous Sulphate