Government of Western Australia

DEPARTMENT OF Conservation AND LAND MANAGEMENT

Conserving the nature of WA

Genetic Structure in the Priority One Species Genus sp. Yalgoo (J. M. Ward s.n. 11/7/1999)

David J. Coates, Margaret Byrne, Bronwyn MacDonald,

Maggie Hankinson and Joanna Ward

Science Division

Department of Conservation and Land Management

INTRODUCTION

Genus sp. Yalgoo (J.M. Ward s.n. 11/7/1999) represents a new genus of the family Rhamnaceae and is currently listed by the Department of Conservation and Land Management (CALM) as "Priority One - Poorly Known" (Atkins, 2003). It was originally identified from two sites near the Newmont Golden Grove mine (south-east of Yalgoo), one being Gossan Hill, a gold deposit proposed for mining by Newmont Australia and Gindalbie Gold NL.

Field survey to the end of 2004 had located four populations, but subsequent survey (Outback Ecology, 2004) identified ten sites, referred to here as populations, covering a range of approximately 33 km. Populations are restricted to shallow brown sandy clay loams over ironstone of breakaways and rocky outcrops (Outback Ecology, 2004). They have a patchy distribution and some, such as Gossan Hill and the more southerly populations, are significantly geographically isolated from their nearest neighbour. Although all populations, based on morphological studies, were considered to be the same species (Genus sp. Yalgoo) the disjunct nature of these populations suggested that they may be genetically distinct and thus represent separate conservation units or evolutionarily significant units (see Coates, 2000), or possibly separate taxa. This pattern is common in the Western Australia flora and is typical of many rare and threatened species particularly in the south-west of the State (Hopper, 1992).

The aim of this investigation was to use a population genetic approach to assess the level and pattern of genetic divergence between populations and determine whether they represented separate conservation units or taxa within the more broadly defined Genus sp. Yalgoo. In particular, given the proposed mining of Gossan Hill, this investigation was aimed at determining the relationship between the population of Genus sp. Yalgoo on this site and the rest of the populations (see Figure 1) as they are geographically separated from the Gossan Hill site by 14.8km (Population 4) to 32.5km (Populations 8 and 10).

MATERIALS AND METHODS

Sampling

Five populations across the range of the species were sampled. These populations cover each of the geographically discrete populations or population clusters as indicated on the species distribution map (Fig. 1) provided by Newmont Australia Ltd and Gindalbie Gold NL and are recognised as follows:

- Population 5: the Gossan Hill site
- Population 1: representing a meta-population system covering populations 1, 2, 3, 4, 9
- Population 6
- Population 7
- Population 8: representing a meta-population system covering populations 8 and 10.

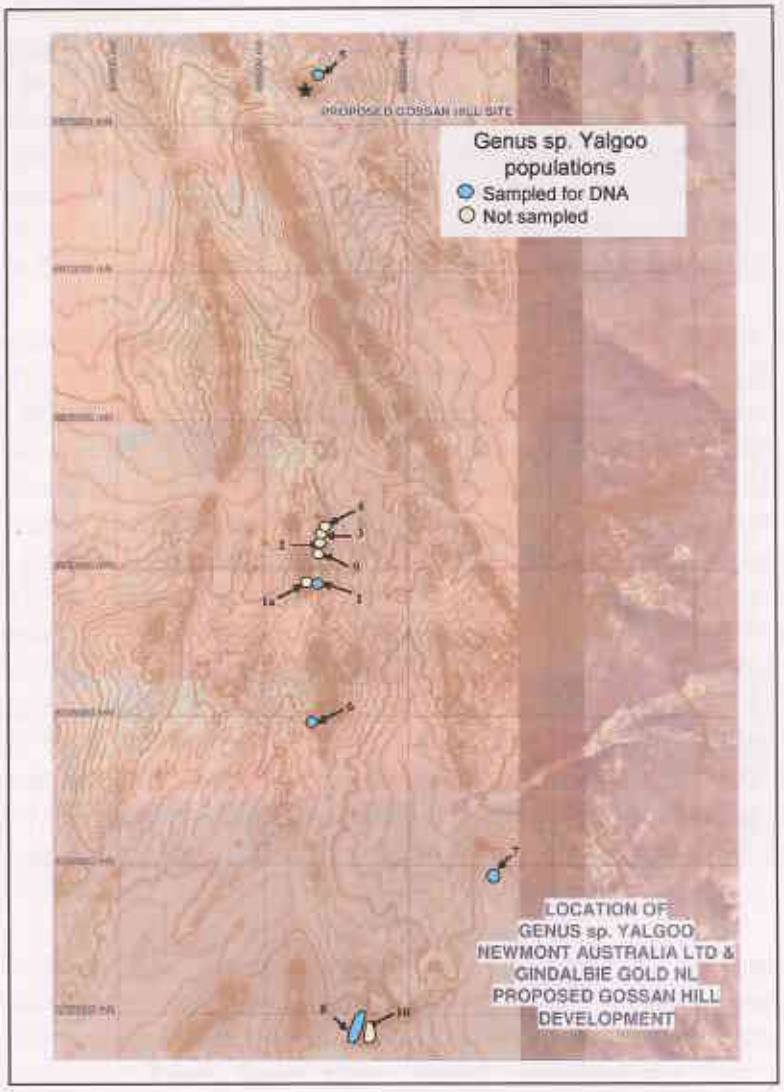

Figure 1. Distribution of Genus sp. Yalgoo

Leaf material was collected from each of 20 plants from each population and transferred within a day of collection to a -80C° freezer for initial storage before DNA extraction.

DNA Extraction

DNA was isolated from 100 mg of leaf material using a Qiagen DNeasy® Plant Mini Prep kit following the manufacturer's protocol. The DNA concentration of each sample was determined using a Hoeffer fluorometer and was then diluted to a working concentration of 50 ng/µL for each individual under study.

DNA Fingerprinting- AFLP procedure

Following extraction, a DNA fingerprint was generated for each sample using the multi-locus AFLP DNA fingerprinting technique (Vos *et al.*, 1995). This technique involves cutting the DNA into fragments with restriction enzymes then ligating the adapters to the fragments. Amplification of specific sets of DNA fragments from the

whole DNA fragment pool can be made using various combinations of primers. For the AFLP assays, 250 ng DNA was digested with 2.5 units of *Eco*RI/*Mse*I restriction enzyme in a reaction volume of 20 µL and incubated at 37°C for 2 hrs, followed by 15 min at 70°C, before briefly cooling on ice. For the ligation of adapters, one unit of DNA ligase and 24 µL of adapter ligation solution were added to the digested DNA sample which was incubated at room temperature overnight. Following ligation the samples were diluted 1:10 with TE buffer. The success of the digestion/ligation reactions were tested by running 1.5 µL of reaction solutions on 8% TAE gels for 1.5 hrs at 80 V, staining with ethidium bromide and examining under UV light.

Pre-selective amplification was performed for each sample by Polymerase Chain Reaction (PCR) using the following reaction protocol: 2.5 µL of the diluted ligation mix was combined with 20µL pre-amplification primer solution, 2.5µL 10 X PCR buffer and 0.5 unit of *Taq* DNA polymerase to make up a 25µL reaction volume, and PCR was performed for 28 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s in a Hybaid Touchdown Thermal Cycler. Subsequently, the pre-amplification mixture was diluted 1:50 with sterile dH_20. Selective amplification by PCR uses primers that match the known adapter sequence plus three selective nucleotides, to reduce the complexity of the profile. For each of the three primer pairs used (E-ACT/M-CTG; E-AGC/M-CAT and E-ACC/M-CTC) the following was added to 2.5 µL of each 1:50 diluted pre-selective DNA sample: 0.75µL *Eco*RI-primer, 2.25 µL *Mse*I-primer, 1 µL 10 X PCR buffer, 3.1 µL sterile dH_20 and 0.5 units of *Taq* DNA polymerase. A touchdown PCR reaction commenced with one cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s, with the annealing temperature reducing in 0.7°C steps over 12 subsequent cycles to 56.6°C, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s.

Following PCR, the fluorescently labelled amplified fragments were analysed by electrophoresis using the Amersham Megabase 96 capillary sequencer at James Cook University. Initially, 48 different primer combinations were screened against two individuals. From these, three primer combinations that produced profiles with clearly scorable fragments of similar intensity were selected for assay on DNA samples from all individuals. The fingerprints generated from the three selected primers were analysed by Amersham Fragment Genotyper software and the AFLP profiles were screened for fragment intensity, as revealed by peak height (Fig. 2). Fragments with peak height greater than 200 were scored for presence (1) or absence (0) of fragments in each individual.

Table 1. Primer combinations used in PCR reactions to generate fingerprints for Genus sp. Yalgoo.

Primer combination	*Eco*R1 primer	*Mse*I primer
E-ACT/M-CTG	gactgcgtaccaattcagc	gacgatgagtcctgagtaacat
E-AGC/M-CAT	gactgcgtaccaattcacc	gacgatgagtcctgagtaactc
E-ACC/M-CTC	gactgcgtaccaattcact	gacgatgagtcctgagtaactg

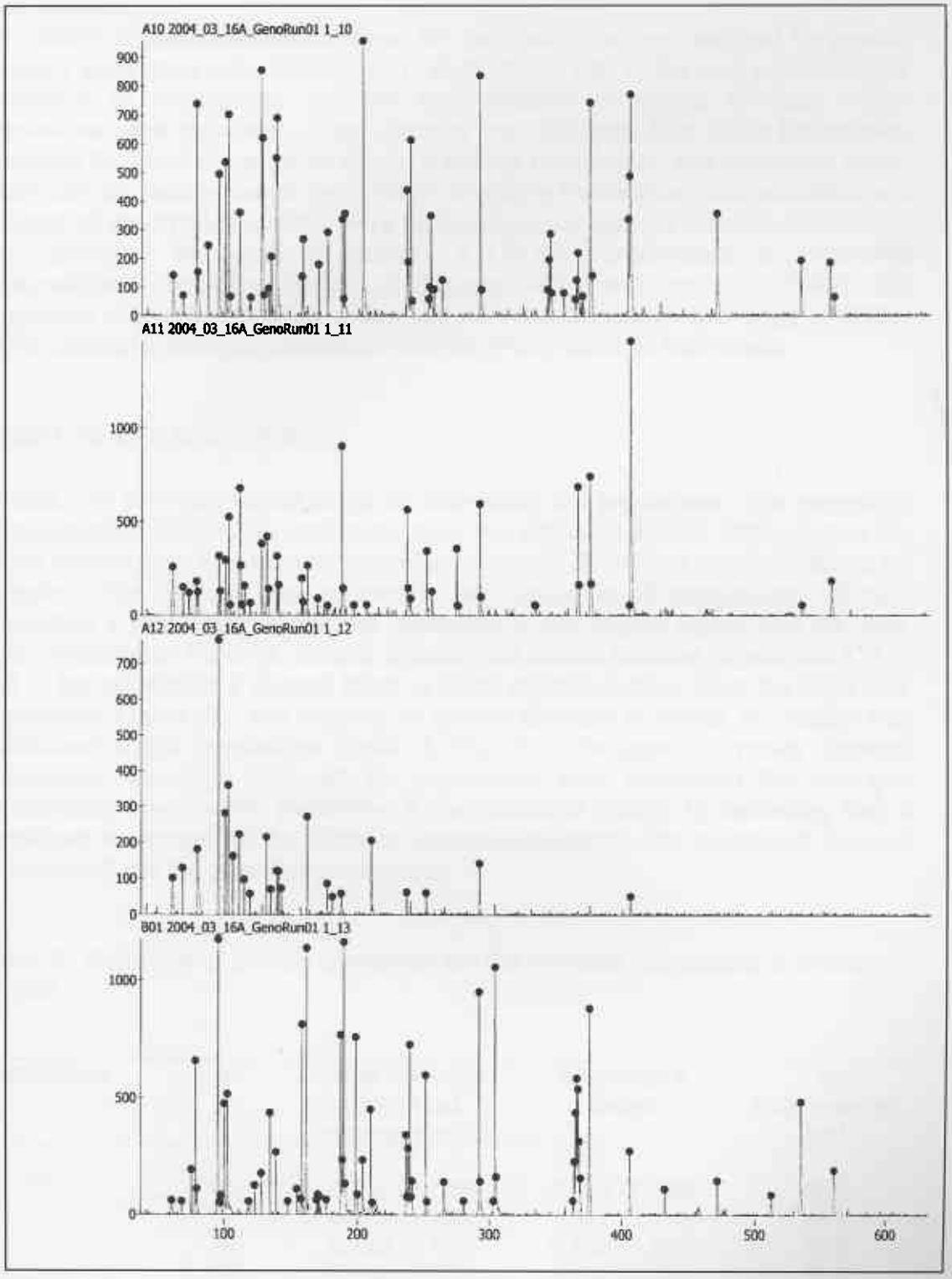

Figure 2. Representative AFLP fingerprints generated by the primer pair E-ACT/M-CTG for four individual plants of Genus sp. Yalgoo from population 1. Fragment size in base pairs is given on the X axis and level of fluorescence is given on the Y axis. The presence/absence of peaks of similar size indicate a genetic polymorphism.

Data analysis

The matrix of presence/absence scores for each individual was analysed for genetic diversity parameters using POPGENE (Yeh & Boyle, 1997). For each population the proportion of polymorphic loci and two measures of genetic diversity within populations were calculated. Gene diversity was calculated from allele frequencies, assuming the populations are in Hardy-Weinberg Equilibrium, and Shannon's Index, which did not make assumptions of Hardy-Weinberg Equilibrium, was calculated as a measure of genotypic diversity. Nei's unbiased genetic distance between populations was calculated and used to construct a UPGMA dendrogram of population relationships. GenAlEx (Peakall & Smouse, 2001) was used to calculate the proportion of genetic variation within and between the populations using AMOVA and to conduct a principal component analysis (PCA) based on individuals.

RESULTS AND DISCUSSION

In total, 219 loci were scored across all individuals and populations. The proportion of polymorphic loci within each population was similar at around 50%. Across the whole species most loci were polymorphic, with only 2% of loci monomorphic in all samples. The level of genetic diversity was similar in all populations, although population 1 was slightly lower and population 8 was slightly higher than the other three populations (Table 2). Genetic distance was similar between populations 1, 5, 6 and 7, but population 8 showed twice as much genetic distance from the other four populations (Table 3). The majority of genetic diversity in Genus sp. Yalgoo was maintained within populations (Table 4, Fig. 3). The genetic diversity between populations was high when all the populations were considered but this was substantially lower when population 8 was excluded (Table 4), indicating that a significant component of the diversity between populations was maintained between population 8 and the other four populations.

Table 2. Estimates of genetic diversity in the five sampled populations of Genus sp. Yalgoo

Population	n	Gene diversity (Expected heterozygosity)	Shannon's Index	% Polymorphic loci
1	16	0.117 (0.160)	0.188 (0.235)	46.6
5	18	0.122 (0.156)	0.197 (0.232)	49.8
6	18	0.120 (0.156)	0.195 (0.229)	51.6
7	19	0.122 (0.153)	0.197 (0.229)	49.8
8	18	0.127 (0.164)	0.203 (0.241)	49.8
Species	**89**	**0.150 (0.146)**	**0.255 (0.202)**	**98.0**

Table 3. Differentiation between the five populations of Genus sp. Yalgoo based on Nei's unbiased genetic distance.

Populations	**1**	**5**	**6**	**7**
1				
5	0.030			
6	0.018	0.023		
7	0.024	0.025	0.012	
8	0.077	0.080	0.061	0.064

Table 4. Results of analysis of molecular variance (AMOVA) for calculation of Phi PT (F_{ST}; estimator of divergence among populations) based on all populations and then with population 8 removed.

	Source	df	SS	MS	Estimated variance	Estimate of Phi PT	Prob
All populations	Among populations	4	274.45	68.61	3.06		
	Within populations	82	1186.22	14.12	14.12	0.178	0.01
Population 8 removed	Among populations	3	110.86	36.95	1.30		
	Within populations	67	935.06	13.96	13.96	0.085	0.01

The similarity of populations 1, 5, 6 and 7, and the differentiation of population 8 is visually demonstrated in the PCA (Fig. 4) and UPGMA (Fig. 5). In both these analyses the Gossan Hill population (population 5) clustered closely with populations 1, 6 and 7 although there was some separation in coordinate 2 of the PCA. The degree of separation between population 5 and populations 1, 6 and 7 is what would be expected given the level of geographical separation and assuming an isolation by distance model for genetic differentiation between populations of the same taxon. In contrast, population 8 was well differentiated from all other populations and occupies separate ordinate space from the other four populations. The key question here is whether population 8 represents a separate conservation unit or evolutionarily significant unit, or is possibly a separate taxon.

Without reference to levels of differentiation between other, related, taxa it is difficult to determine whether the differentiation seen in Genus sp. Yalgoo represents taxonomic difference or within taxon population divergence due to restricted gene flow associated with the geographical separation of the populations. Comparative studies on a number of other rare and geographically restricted taxa in south-west Western Australia and adjacent areas suggest that the level of differentiation observed in Genus sp. Yalgoo is insufficient for the recognition of different taxonomic units.

For example, in species such as *Lambertia orbifolia* and *Stylidium coroniforme*, significant genetic differentiation is associated with more subtle morphological difference between populations (see Coates 2001) and levels of divergence based on estimates of F_{ST} are considerably higher than the 'all populations' estimate for Genus sp. Yalgoo (Table 4). However, comparisons between such estimates need to be treated cautiously as different molecular markers have been used (i.e. allozymes versus AFLPs), but the magnitude of the differences (Genus sp. Yalgoo, 0.178; *Lambertia orbifolia*, 0.441; *Stylidium coroniforme*, 0.250) suggests that the level of differentiation among populations of Genus sp. Yalgoo is unlikely to be associated with any taxonomic differences.

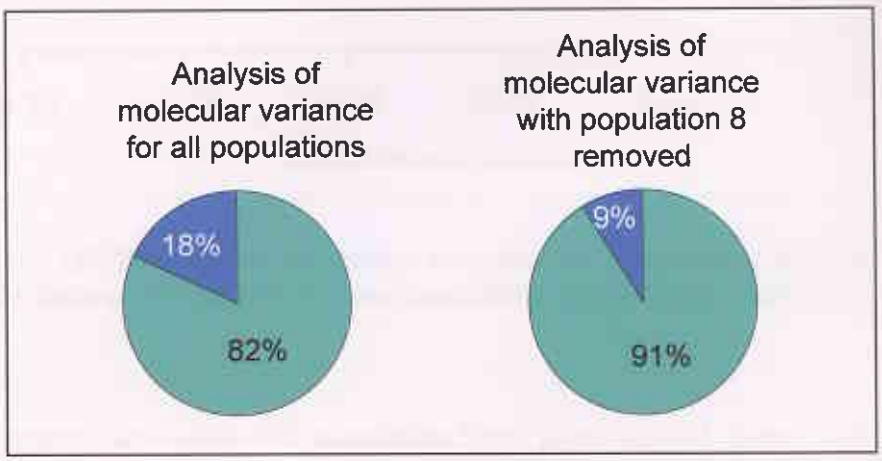

Figure 3. Analysis of molecular variance (AMOVA) for all populations and with population 8 removed, showing the percentage of genetic variation within and among populations.

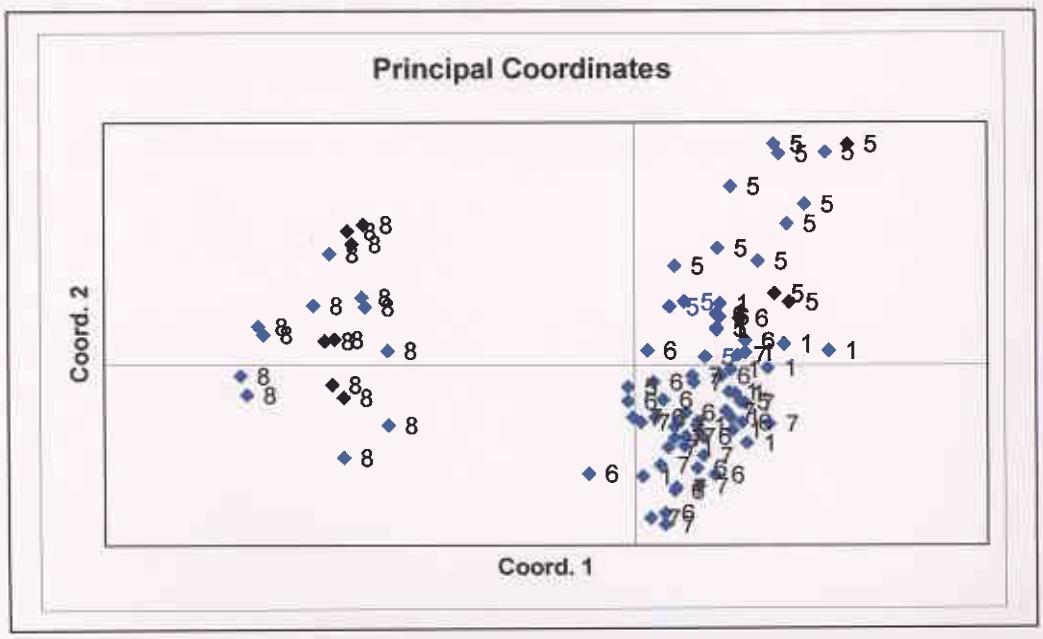

Figure 4. Principal component analysis (PCA) of genetic variation within and between the five populations (1, 5, 6, 7 and 8) of Genus sp. Yalgoo sampled. The numbered points represent individuals from each of the five populations.

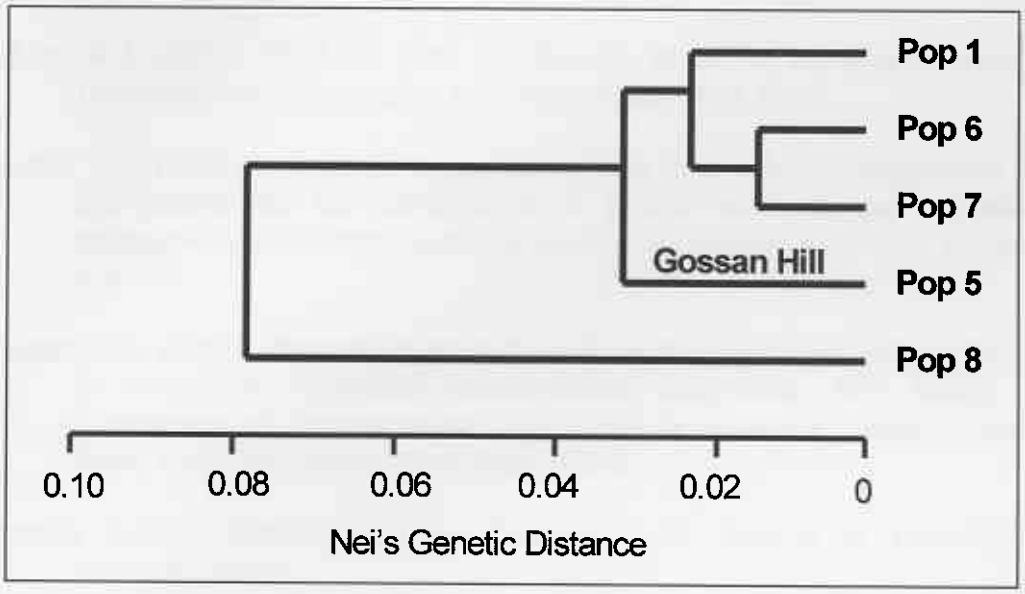

Figure 5. UPGMA tree of population relationships constructed from Nei's unbiased genetic distance for each of the five populations of Genus sp. Yalgoo sampled.

In summary, the Gossan Hill population 5 and populations 1, 6 and 7 are all the same taxon with only minor inter-population genetic differentiation evident. Population 8 shows significantly greater genetic divergence from all others but it seems unlikely that this differentiation is indicative of any taxonomic difference. Further information based on comparative studies with other closely related taxa would be needed to confirm whether this level of divergence is greater than would be expected for inter-population genetic variation within a species such as Genus sp. Yalgoo.

REFERENCES

Atkins, K.J. (2003). Declared Rare and Priority Flora list for Western Australia. Department of Conservation and Land Management, Perth.

Coates, D.J. (2000). Defining conservation units in a rich and fragmented flora: implications for the management of genetic resources and evolutionary processes in south-west Australian plants. *Australian Journal of Botany* **48:** 329-339.

Hopper, S.D. (1992). Patterns of plant diversity at the population and species levels in south-west Australian mediterranean ecosystems. R.J. Hobbs (Ed.) *Biodiversity of Mediterranean Ecosystems in Australia.* Surrey Beatty & Sons., Chipping Norton, New South Wales.

Outback Ecology (2004). Genus sp. 'Yalgoo' (J. M. Ward S. N. 11/7/1999) PN Summary Report.

Yeh, F.C. and Boyle, T.J.B. (1997). POPGENE. Version 1.20. http://www.ualberta.ca/~fyeh/

Peakall, R. and Smouse, P.E. (2001). GenA1Ex V5: Genetic Analysis in Excel. Population genetic software for teaching and research. Australian National University, Canberra, Australia.

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