School of Environmental Biology

Variation and population genetic structure in the Western Australian endemic genus *Geleznowia* Turcz. (Rutaceae)

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Declaration

I declare that all work presented in this thesis is that of myself alone unless otherwise acknowledged. The contents of this thesis have not been submitted previously, in whole or in part, in respect of any other academic award.

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Abstract

The endemic wildflower, *Geleznowia verrucosa* Turcz. (Rutaceae), is widely distributed as small disjunct populations throughout the sandplains of Western Australia (21° 50'S, 116° 12'E to 31° 12'S, 117° 02'E). Although the genus is supposedly monospecific, three morphological forms can be recognised in the field:

(1) a small form (0.5-1 m) with small, often solitary flowers and small leaves;
(2) a large form (1-2 m) with larger, more abundant flowers and larger leaves; and,
(3) an intermediate form exhibiting mixed morphological characters.

The patterns of morphological, reproductive and genetic variation within and between populations of these three forms were investigated. Uni- and multivariate analyses of morphological traits found the large and intermediate forms to be closely allied, and distinguishable from the small form. Responses to controlled pollination experiments indicated that the small form favours selfing but maintains some level of outcrossing, while the large form exhibits a mixed mating system. The intermediate form displays a stronger self-pollination mechanism than the small form.

Patterns of genetic variation were analysed using both allozymes and randomly amplified polymorphic DNA (RAPDs). The allozyme analysis concurred with genetically depauperate expectations of endemic taxa (*A*, 1.4; *P*, 29.6%; H_o, 0.055; H_e, 0.097). Genetic diversity and patterns of allelic distribution, however, differed within the three forms. Lower levels of genetic diversity were found in the small form (H_T, 0.192) compared with the large form (H_T, 0.254), although both forms apportioned this diversity within populations (H_S, 0.122 and 0.164, respectively) rather than between (D_{ST}, 0.070 and 0.090, respectively). In contrast, populations of the intermediate form are highly divergent (G_{ST}, 54%), with genetic diversity apportioned between populations (D_{ST}, 0.121) rather than within (H_S,

0.105). Whereas the morphometric analyses had indicated closer affinity between the large and intermediate forms, both allozyme and RAPD analyses suggested that the intermediate form is closer to the small form than to the large form.

There is circumstantial evidence to suggest that the intermediate form has arisen following hybridisation between the small and large forms. Its hybrid origin is supported by the high level of genetic diversity between the intermediate form populations, as well as its strong autogamous tendencies and mixed morphological characteristics. In a putative zone of hybridisation between the small and large forms, asymmetric introgression was observed, indicating gene exchange between the two forms can occur when they come into contact.

It is speculated that three major events have shaped the evolution of *Geleznowia verrucosa* and contributed to hybridisation between the small and large forms. Firstly, the small form is derived from the large form; associated with this speciation was a shift in reproductive strategy from outcrossing to selfing when the small form migrated into a harsher and more unpredictable environment. Secondly, recurrent Tertiary and Quaternary climatic perturbations have facilitated range expansion and contraction of these forms, generating opportunities for spatially and temporally distributed hybridisation events. Finally, more recent evolution has been driven by population disjunction, limited gene flow, and bottleneck and/or associated founder effects.

From this study it is apparent that the genus *Geleznowia* consists of at least two taxa and a series of hybrid derivatives. On this basis, formal systematic revision of the genus is now warranted. Systematic clarification will also assist with the conservation and management of this valuable natural resource, which is currently under threat.

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CHAPTER 1

General Introduction

One of the most significant challenges for systematists is to determine whether observed variation in any measurable character, reflects a degree of evolutionary divergence sufficient to warrant systematic separation. Organisms are usually comprised of a series of populations distributed throughout a given geographic region (Richardson *et al.*, 1986). Within each population, selection and isolation can lead to the accumulation of genetic differences which, with time, may be expressed as attributes clearly defining them as new species (Nei, 1975; Thorpe, 1976; Loveless and Hamrick, 1984; Levin, 1993). Systematics investigates these natural levels of variation to characterise and classify groups of organisms, and to clarify their evolutionary relationships. In so doing, systematics contributes to both the formal and informal nomenclature of organisms, as well as providing important insights into the evolutionary, historical and biogeographic factors operating within species (Thorpe, 1976; Whiffen, 1978).

Population divergence is driven by many interacting forces. Of primary importance are selection, gene flow, isolation, migration, genetic drift, effective population size and mutation; ecological factors, particularly those influencing reproduction and dispersal, may further contribute to differentiation (Hamrick *et al.*, 1979; Linhart *et al.*, 1981; Hamrick, 1982; Loveless and Hamrick, 1984; Cole and Biesboer, 1992; Carthew, 1993). Chromosomal variation, particularly changes in ploidy level, can also be a significant factor in population divergence and speciation (Grant, 1981). Selection and genetic drift promote population divergence, while gene flow tends to maintain uniformity. Restricted gene flow is often invoked as a major factor contributing to divergence between plant populations (Levin and Kerster, 1974; Slatkin and Maruyama, 1975; Hamrick, 1982; Loveless and Hamrick, 1984; Slatkin, 1985). Many factors influence gene flow. For

example, the shape, density and distribution of populations can restrict pollen movement by influencing pollinator activity, while both biotic and abiotic factors can affect long distance pollen and seed dispersal (Levin and Kerster, 1974; Slatkin and Maruyama, 1975; Hamrick *et al.*, 1979; Hamrick, 1982; Loveless and Hamrick, 1984; Slatkin, 1985).

Breeding systems are acknowledged to be a crucial factor influencing evolutionary patterns in plants. Breeding systems determine genotype frequencies in subsequent generations, as well as influencing population parameters such as neighbourhood size, gene flow and selection (Levin and Kerster, 1974; Jain, 1975; Grant, 1981; Hamrick, 1982, 1989; Loveless and Hamrick, 1984). Predominantly selfed species often exhibit lower genetic diversity than their predominantly outcrossed congeners (Hamrick and Godt, 1989). Self pollinated species also tend to apportion genetic diversity between populations rather than within, thereby promoting population differentiation. In contrast, the alleles of outcrossers tend to be more widely distributed, reducing population subdivision (Levin, 1978; Brown, 1979; Hamrick *et al.*, 1979; Gottlieb, 1981; Loveless and Hamrick, 1984; Hamrick and Godt, 1989; Cole and Biesboer, 1992).

Population size and distribution can also contribute to population differentiation. Small, localised and isolated populations are more susceptible to drift and fixation (Loveless and Hamrick, 1984). Locally distributed populations, such as those of endemic taxa, are frequently small and fragmented, exposing them to many of the factors promoting divergence. Further, these populations may be subject to inbreeding and founder effects (Hamrick *et al.*, 1979; Coello *et al.*, 1993; Baskauf *et al.*, 1994; Purdy *et al.*, 1994; Watson *et al.*, 1994). Not surprisingly, many endemic taxa are genetically depauperate, although genetic diversity is partitioned in a similar manner to that of more widespread species (Hamrick and Godt, 1989; Baskauf *et al.*, 1994). Yet not all localised and endemic taxa exhibit low levels of genetic diversity. Several studies have revealed endemic taxa with high levels of variation (Hiebert and Hamrick,

1983; Gottlieb *et al.*, 1985; Karron, 1987; Karron *et al.*, 1988; Murawski and Bawa, 1994; Lewis and Crawford, 1995).

Identifying systematic divergence

The phenotypic expression of genetic divergence has traditionally been employed by plant systematists to delineate taxa. Such readily observable morphological characters remain the primary means of identification in Angiosperm systematics (Heywood, 1967; Davis and Heywood, 1991). Successful classification requires morphological characters which are not highly variable or susceptible to environmental modification, and which show consistency in expression (Davis and Heywood, 1991). The floral characters of Angiosperms appear to be relatively stable and provide many useful diagnostic features (Metcalf, 1983; Stace, 1989).

Although morphological and anatomical characters can highlight similarities and contrast differences between many species (Thorpe, 1976), phenotypic variation and plasticity can be problematic (Palmer *et al.*, 1988; Castiglione *et al.*, 1993; Liu and Furnier, 1993). In species with large distributions, geographic patterns can also occur (Thorpe, 1976). A further consideration is the difficulty of character choice, which is often reliant on the systematist's own experience (Heywood, 1967; Davis and Heywood, 1991). In some groups, clonality, hybridisation and introgression can complicate systematic decisions (Liu and Furnier, 1993).

The role of hybridisation and introgression in plant evolution and speciation is complex. Hybridisation can increase intraspecific genetic diversity, facilitate the origin and transfer of genetic adaptations, create new ecotypes and species, and reinforce or break down reproductive barriers (Rieseberg, 1997). With the increased use of molecular markers to assess genetic divergence, the extent of hybridisation within many plant groups is only now becoming apparent (Rieseberg, 1995).

Where morphological variation between taxa is subtle, or difficult to

characterise, quantitative statistical methods can be useful (Heywood, 1967; Liston, 1970; Sneath and Sokal, 1973). In numerical systematics, statistical methods are used to group organisms according to character states (Sneath and Sokal, 1973). Although univariate analysis can suggest patterns or trends within and between groups, multivariate analyses can often impart more information by investigating relationships between a suite of characters (Heywood, 1967; Liston, 1970; Sneath and Sokal, 1973; Thorpe, 1976). These characters are used to construct a matrix of resemblances which is then analysed to reveal and summarise relationships between groups (Sneath and Sokal, 1973). Numerous statistical algorithms are now available for matrix construction and analysis. A major difficulty for many systematists is to determine which of these statistical procedures is appropriate for the investigation at hand.

Molecular markers and systematics

Although traditional and numerical systematic techniques have successfully classified many taxa, some of the more cryptic groups remain difficult to clarify. In such taxa, cytological, embryological, biochemical and molecular investigations may provide insights not readily apparent using traditional characters (Heywood, 1967; Stebbins, 1971; Crawford, 1983; Harbourne and Turner, 1984; Hedberg, 1988; Stace, 1989; Brown, 1990; Clegg and Durbin, 1990). For many systematic investigations, molecular markers provide a means of assessing genetic divergence between taxa, while levels and patterns of genetic variation can be valuable for evaluating both the mode(s) of speciation, as well as the underlying causes of morphological variation (Watson *et al.*, 1994). A wide range of molecular markers are available for population genetic investigations and systematic clarification.

Isozymes (Markert and Moller, 1959) are protein markers which constitute multiple molecular forms of the same enzyme. When these molecular forms are coded by different alleles at the same locus, the term allozyme is used (Gottlieb, 1981). Both terms are, however, often used interchangeably.

Changes to the amino acid sequence of genomic DNA often alters the charge, and sometimes the conformation, of an enzyme, affecting its electrophoretic mobility (Shields *et al.*, 1983; Weeden and Wendel, 1989). These differences in mobility are assumed to reflect changes to the DNA coding sequences, providing a useful set of molecular markers with which to examine genetic differentiation and population structure (Crawford, 1989; Brown, 1990; Murphy *et al.*, 1996). By comparing levels of observed heterozygosity with those expected under the Hardy-Weinberg equilibrium, some of the evolutionary mechanisms which contribute to divergence can be elucidated (Murphy *et al.*, 1996).

More recently, polymorphisms revealed by DNA markers have become increasingly popular for assessing genetic variation and systematic relationships (Weising *et al.*, 1995). As with allozymes, these techniques survey the genotype rather than the phenotype. They are also generally applicable to any type of DNA, which can be extracted from relatively small amounts of tissue; additionally, the DNA sequence is identical regardless of age, tissue type analysed or environmental effects, providing many characters for analysis (Kazan *et al.*, 1993; Torres *et al*, 1993).

In plants, molecular DNA markers occur in the chloroplast (cpDNA), mitochondrial (mtDNA) and nuclear genomes. The chloroplast genome is large, complex and highly conserved (Clegg, 1990; Dowling *et al.*, 1996). Investigations using cpDNA markers have revealed a range of within and between population variation, but the utility of cpDNA markers at this level may depend on the species' under investigation (Byrne and Moran, 1994; Dowling *et al.*, 1996). Slow evolutionary rates within the cpDNA genome make it a useful marker to assess interspecific and higher level taxonomic relationships (Palmer, 1987), and several recent investigations have focused on relationships within Asteraceae (Baldwin *et al.*, 1990; Michaels *et al.*, 1993), Agavaceae (Bogler and Simpson, 1995), Leguminosae, (Doyle *et al.*, 1997), Crassulaceae (van Ham and Hart, 1998), Saxifragaceae (Soltis *et al.*, 1996; Soltis and Soltis, 1997) and between

several monocot families (Duvall *et al.*, 1993). In contrast, the utility of plant mtDNA for population studies is limited by the possible incorporation of foreign DNA sequences, frequent rearrangements and differences in genome size between taxa (Clegg, 1990).

Many nuclear genes can now be accessed to act as genetic markers (Dowling *et al.*, 1996). Several recent investigations of plant molecular diversity have specifically focused on the nuclear encoded ribosomal RNA (rDNA) gene family (Clegg, 1990; Byrne, 1996). Variation detected within and between populations using rDNA markers also varies between species (Byrne, 1996), and many studies have centred on phylogenetic relationships (Doyle *et al.*, 1990; Baum *et al.*, 1994; Bogler and Simpson, 1996; Moller and Cronk, 1997).

Irrespective of the type of DNA utilised, the markers are commonly generated by either cutting the DNA at specific sites or amplifying sections of the DNA between primer sites to produce fragments of varying size (Tao *et al.*, 1993; Bachmann, 1994). The Restriction Fragment Length Polymorphism (RFLPs) technique utilises restriction enzymes to cut DNA at specific sites, revealing the chance loss or gain of restriction sites in a particular gene or region of the DNA (Hoelzel and Dover, 1991; Bachmann, 1994; Dowling *et al.*, 1996). Visualisation requires the use of target DNA pieces being hybridised to the DNA that has been digested with restriction enzymes.

In contrast, Polymerase Chain Reaction (PCR) amplifies short DNA segments between specific sites, revealing sequence changes within the PCR priming sites (Bachmann, 1994; Dowling *et al.*, 1996). Randomly Amplified Polymorphic DNA (RAPDs) is a PCR technique which amplifies DNA using single short oligonucleotide primers of an arbitrary sequence; this method generates large numbers of small DNA sequences of varying lengths (Williams *et al.*, 1993). Microsatellites are another PCR technique which uses specifically designed primers to detect short tandem repeated

sequences (Bachmann, 1994; Byrne, 1996; Palumbi, 1996). Requiring little DNA, being highly polymorphic and codominant, microsatellites can be a particularly useful molecular marker (Hillis *et al.*, 1996)

The choice of an appropriate marker is often tempered by both the question under investigation, and the resources available to the investigator. Population subdivision, geographic variation and heterozygosity can be detected using allozymes, RFLPs, and microsatellites; these techniques, and RAPDs, can also distinguish between clones. Allozymes and RFLPs are useful for systematic and hybridisation investigations, while allozymes and microsatellites are good mating-system markers (Weising *et al.*, 1995; Dowling *et al.*, 1996; Hillis *et al.*, 1996). RAPDs may also be useful for systematic investigations where species are closely related, and homology between bands has been established (Rieseberg, 1996).

Allozyme electrophoresis is relatively inexpensive, easy and rapid to perform (Brown and Weir, 1983; Brown *et al.*, 1989; Hamrick, 1989; Weeden and Wendel, 1989; Brown, 1990). Although RFLPs provided many more polymorphic loci for analysis, they are expensive, time consuming, can be technically demanding and require large amounts of relatively pure DNA (Chalmers *et al.*, 1992; Grattapaglia *et al.*, 1992; Skroch *et al.*, 1992; Kazan *et al.*, 1993). Microsatellites are similarly limited by the time and expense required to develop suitable primers. In comparison, RAPDs are relatively easier and faster to assay, and are preferable for investigating large numbers of individuals at a few loci (Welsh *et al*, 1991; Grattapaglia *et al.*, 1992; Tingey *et al.*, 1992; Kazan *et al.*, 1993; Tao *et al.*, 1993). Unlike RFLPs and microsatellites, RAPDs do not require a knowledge of the genome, use small amounts of DNA, and many primers are now commercially available (Williams *et al.*, 1990; Williams *et al.*, 1991; Bachmann, 1994).

Systematic investigations using morphological and molecular markers have contributed greatly to our understanding of evolutionary relationships

and the processes contributing to population and systematic divergence in recent years. Direct comparisons of these methodologies can, however, be problematic and congruence elusive (Hillis, 1987; Patterson *et al.*, 1993). Differences in sample size and analytic technique can contribute to incongruence, while homology and homoplasy also present difficulties (Hillis, 1987; Patterson *et al.*, 1993). Perhaps of greater significance for direct comparisons of such markers, is the sensitivity of the characters themselves to selection. In comparing morphological and molecular data, a common assumption is that morphological characters should reflect molecular evolution, or that both morphological and molecular characters have evolved in parallel. These characters can, however, have different environmental sensitivities and may evolve independently (Harrison, 1986; Michaud *et al.*, 1995). In these circumstances, incongruence between analyses is to be expected.

Meaningful interpretation of morphological and molecular variation hinges on an understanding of the reproductive biology and mating system of an organism. It is therefore important to have some understanding of the breeding system, population genetic structure, patterns of gene flow, and the reproductive output of populations (Brown and Allard, 1970; Jain, 1975; Grant, 1981; Ellis and Sedgley, 1992).

Diversity within the Western Australian flora

In some regions of the world, complex evolutionary processes have resulted in remarkably high species diversity and richness in vascular plants. The southwest region of Western Australia is one such area that is characterised by high levels of speciation and endemism. In this corner of the continent, some 75% of the 8000 known plant species do not occur outside the region (Hopper, 1979; 1992).

Many factors have contributed to the high biological diversity of this region. Strong affinities with the South African flora and diverse patterns of population genetic structure, breeding systems and life histories,

emphasise the antiquity of the floral element (Hopper, 1992). Punctuating this extensive evolution have been geological events, sea level fluctuations and a climatic shift, all of which have contributed to a heterogeneous soil mosaic in a fragmented landscape (Hopper, 1979; 1992). Population isolation brought about by such events has further facilitated speciation (James and Hopper, 1981). At the same time, no major extinction episodes have occurred, leaving many relictual species.

More recent evolution within the southwest region has been driven by climatic change, leading to the recognition of three rainfall-vegetation zones: the high-rainfall zone (800-1400 mm annual rainfall); the transitional-rainfall zone (300-800 mm); and, the arid-zone (less than 300 mm) (Hopper, 1979) (Figure 1.1). Major differences in the concentration of genera occur within each zone (Hopper, 1992). The transitional-rainfall zone, in particular, contains more localised endemics, infraspecific variants, cryptic species complexes and natural hybrids than any other (Hopper, 1979). Additionally, few species are distributed across the whole zone, with most confined to smaller ranges in allopatric replacement series with their congeners (Hopper, 1992).

The occurrence of monotypic taxa within such a speciated landscape is intriguing. Such taxa possibly represent refugial relicts (Hopper, 1979) and their investigation may present valuable insights into the processes of speciation within the zone. Since few monotypic taxa exist, empirical evidence regarding their genetic diversity, systematic divergence and speciation processes is difficult to obtain. Such taxa may be created through either extinction of closely related species, or a rapid speciation event which brings about morphological divergence from closely related taxa. For ancient taxa, such as those within the Western Australian flora, it may be impossible to determine which of these events is responsible for any given monotypic species.

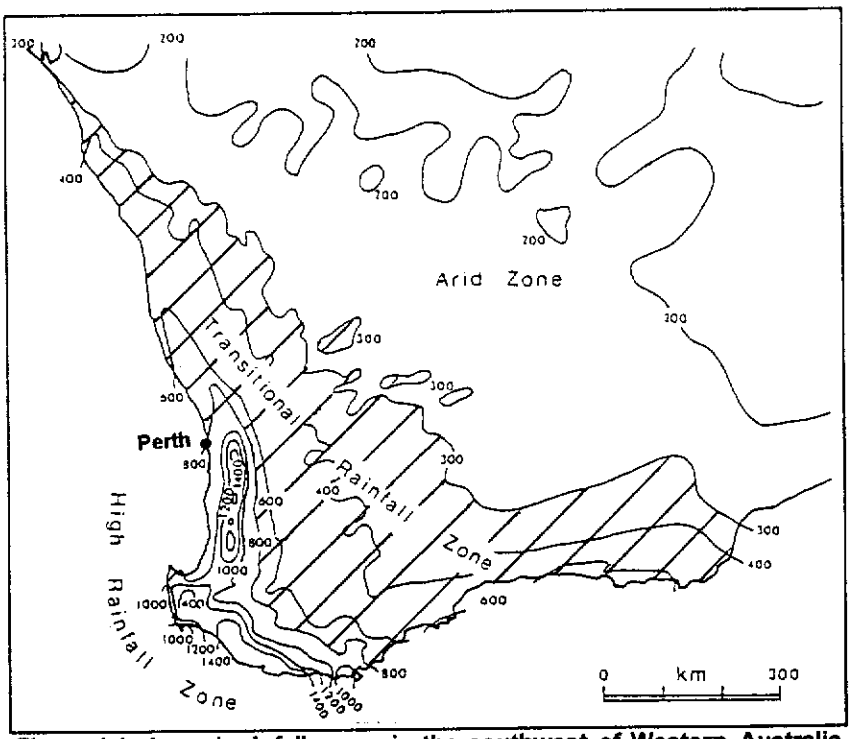


Figure 1.1: Annual rainfall zones in the southwest of Western Australia (from Hopper, 1979).

Geleznowia verrucosa

Geleznowia verrucosa Turcz. (Rutaceae) is, at present, a monotypic species of the predominantly Australia tribe Boronieae (Smith-White, 1959). This endemic species is widely distributed within the transitional-rainfall zone, extending from Cape Range in the north of Western Australia, to Dowerin in the southeast (Elliot and Jones, 1986; Hnatiuk, 1990; Keighery and Gibson, 1993; Broadhurst, 1995) (Figure 1.2).

G. verrucosa is a small woody shrub (less than 2m) exhibiting terminal clusters of bright yellow flowers. Each flower is comprised of five small, thin petals surrounded by five sepals and further enclosed by larger bracts (Figure 1.3). These attractive floral qualities and longevity following harvest, have resulted in many wild populations of *G. verrucosa* being commercially exploited (Paynter and Dixon, 1990).

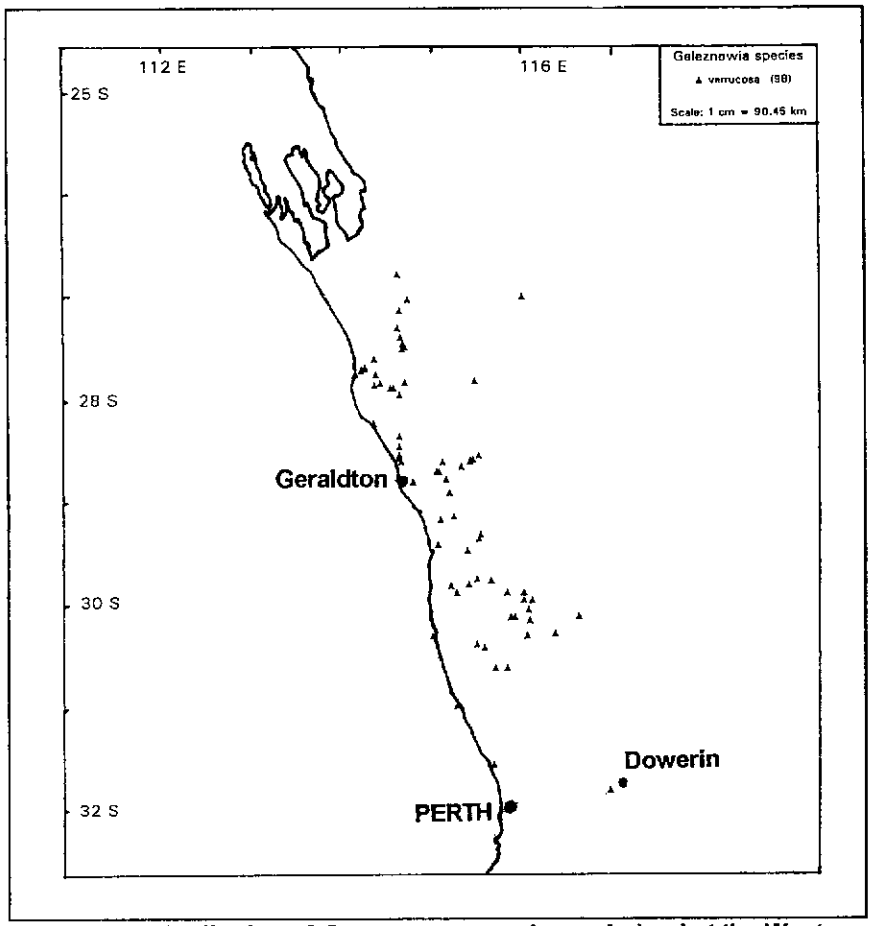


Figure 1.2: Distribution of *G. verrucosa* specimens lodged at the Western Australian Herbarium.

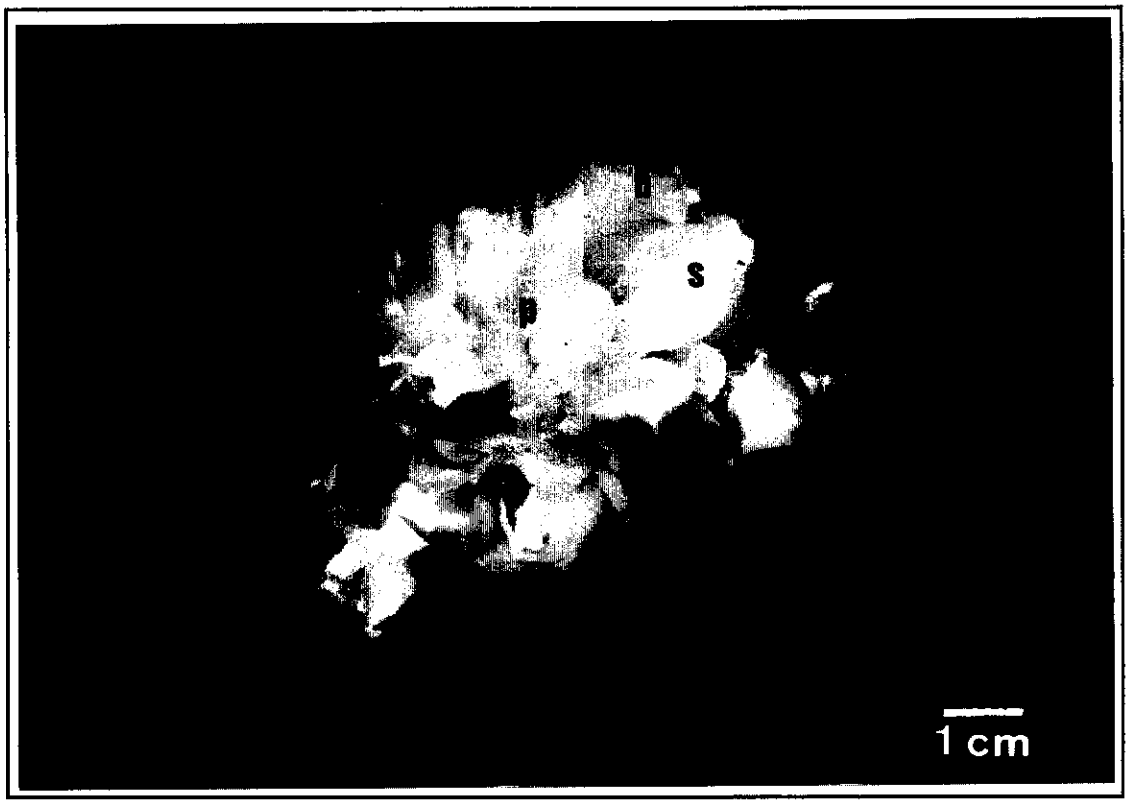


Figure 1.3: Photograph of *G. verrucosa* flowers (courtesy of S. Roche). p, petals (closed); s, sepals; b, bracts.

Little is known about the reproductive strategy, ecology or demographics of the genus. Populations occur in a variety of vegetation types ranging from low heath to low forest (see Muir's 1977 classification), although most are associated with thickets and heath of mid-dense canopy cover (30-70%). Populations are generally small, consisting of 15-30 plants, and disjunct, with some rare populations of over 400 individuals occurring. The most uniting feature of all populations is their restriction to sandy soils (Broadhurst, 1995).

Fecundity in *G. verrucosa* is reported to be high (D. Growns, pers. comm.), with each flower capable of setting up to five seeds. Seed germination was somewhat difficult and erratic until recent investigations highlighted smoke treatment to be a significant germination factor (Paynter and Dixon, 1991; Dixon *et al.*, 1995). Further, *G. verrucosa* is now thought to be a disturbance opportunist with rapid turnover in the soil seed store (pers. obs; Roche, 1998). High fecundity may be a strategy to ensure reproductive continuity under these circumstances.

The genus was originally named after the Russian botanist Nikolai Ivanovich Zheleznov (1816-1877) (Baines, 1981). Bentham and Mueller (1863) described first the genus as three species: *G. verrucosa*, *G. macrocarpa* and *G. calycina* (Figure 1.4), with systematic separation based on sepal and carpel shape, and sepal size in comparison to petals. A revision in 1980, however, failed to find sufficient morphological differences to justify separation (Paul Wilson, pers. comm.), and a monospecific status was adopted (Baines, 1981; Armstrong, 1983; Elliot and Jones, 1986; Elliot, 1990).

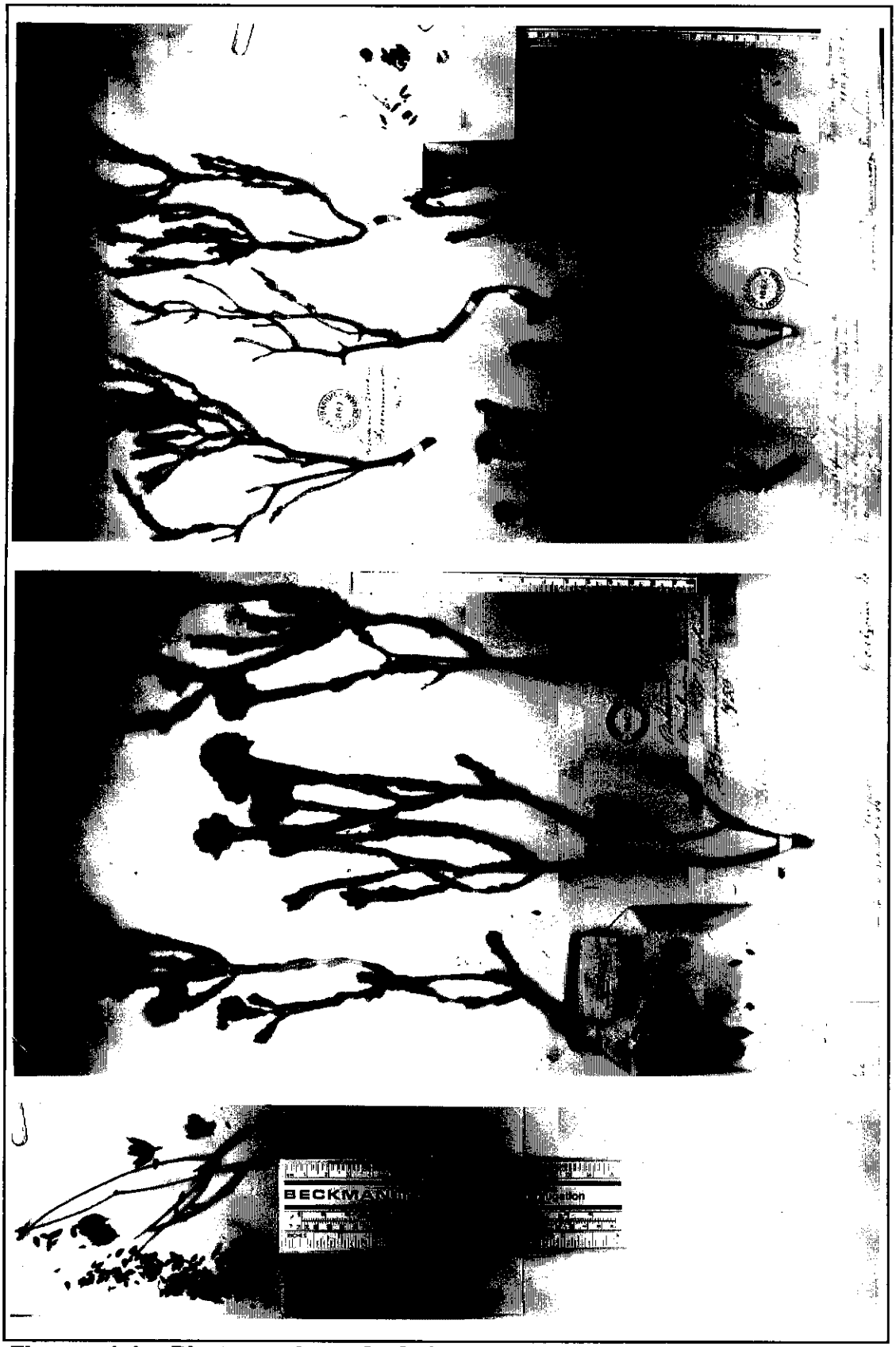


Figure 1.4: Photographs of *Geleznowia* type specimens. Top, *G. verrucosa*; middle, *G. calycina*; bottom, *G. macrocarpa* (courtesy of D. Foreman, Kew Gardens London).

Field observations clearly indicate that significant morphological diversity exists within the genus (Figure 1.5). Some populations comprise a taller (1-2 m) form with abundant, large flowers and large leaves, while other populations consist of shorter plants (0.5-1 m) with smaller, often solitary flowers and smaller leaves. Between these forms are a number of variable populations exhibiting intermediate characteristics. Although the different forms rarely co-occur, those that do appear to maintain their morphological integrity.

Aims of this investigation

The objectives of this research are to investigate the patterns of morphological and genetic variation within *Geleznowia verrucosa*, to determine the reproductive biology and population genetic structure and, to resolve the systematic uncertainty surrounding the genus.

Field sites

Seventeen populations (20 sites) representing various forms were located from within the known range of the species (Table 1.1, Figure 1.6). At each population, with the exception of Pleshkes and Hutt River, twenty plants were randomly selected, tagged and their locations mapped. Material was collected from each of these plants as required. The unusually large Hutt River population was divided into two sites of twenty plants at opposing ends of the population. The Pleshkes population was also large and both the small and large forms co-occurred. This population was divided into three sites comprising both forms and a transect running across the integration zone between them. Ten plants were selected from each form and twenty plants from across the integration zone. A fire in late 1994 destroyed all Spalding Park plants, limiting the collection of material from that population to that used in the morphological analysis only. Specimens from all populations were lodged at the Western Australian Herbarium (PERTH).

a

c

d

f

h

b

e

g

i

Figure 1.5: Photographs of the three *G. verrucosa* forms. a. Indarra Reserve (small); b. Ajana West 1 (intermediate); c. Arinya (small); d. Hutt River (intermediate); e. Geraldton (large); f. Pleshkes Small (small); g. Pleshkes Large (large); h. Red Bluff (large); i. Meanarra Hill (large).

Table 1.1: Location of sampled *G. verrucosa* populations. n_{est}, estimated population size; *n*, no. of plants sampled.

Form	Population	n_{est}	*n*	Lat. (°S)	Long. (° E)
Small	Arinya	50	20	31° 19' 86	116° 58' 61
	Coorow 1	20	20	29° 54' 14	116° 00' 08
	Coorow 2	20	20	29° 59' 05	115° 56' 17
	Indarra Reserve	20	20	28° 41' 67	115° 19' 80
	Maiseys	30	20	31° 24' 38	117° 00' 77
	Pleshkes Small	60	10	28° 01' 41	114° 17' 85
Intermediate	Ajana West 1	30	20	27° 50' 51	114° 28' 21
	Ajana West 2	25	20	27° 51' 88	114° 29' 92
	Hawks Head	30	20	27° 48' 76	114° 28' 11
	Hutt River 1	200	20	28° 05' 59	114° 28' 30
	Hutt River 2	200	20	28° 05' 47	114° 28' 04
	Nanns	25	20	28° 00' 52	114° 29' 36
	West Binnu	20	20	28° 01' 19	114° 28' 16
Large	Geraldton	24	20	28° 35' 42	114° 37' 95
	Meanarra Hill 1	25	20	27° 41' 66	114° 13' 01
	Meanarra Hill 2	25	20	27° 41' 61	114° 12' 80
	Pleshkes Large	400	10	28° 01' 16	114° 17' 77
	Red Bluff	30	20	27° 44' 79	114° 09' 04
Transect	Pleshkes Hybrid	400	20	28° 01' 35	114° 17' 82

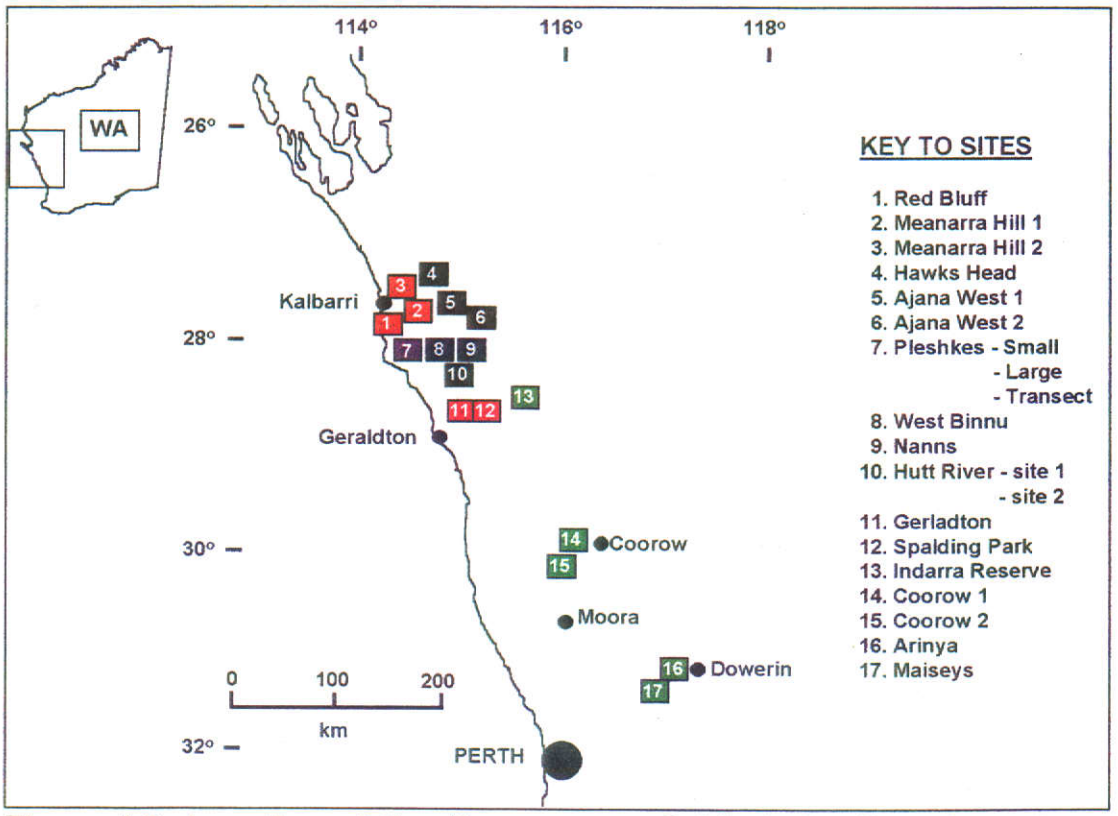


Figure 1.6: Location of the *G. verrucosa* field study sites. Small form; intermediate form; large form; mixed forms.

Organisation of this thesis

The experimental work of this investigation encompassed several complementary approaches. As with any systematic study, morphological attributes were considered foremost. In Chapter 2, observed morphological variation in *G. verrucosa* was investigated to determine whether this could be translated to recognisable taxa. This chapter also examined chromosomal variation in the different forms to determine whether morphological variation could be attributed to structural differences and/or ploidy levels.

Since breeding systems in plants profoundly influence genetic variability, Chapter 3 examined both the reproductive biology and mating system of the genus. This was further expanded to compare and contrast variation between the forms and their ability to interbreed. An understanding of the reproductive biology assisted with interpretation of levels of observed genetic variation and population genetic structure investigated using allozyme markers in Chapter 4.

To corroborate allozyme evidence, relationships between a subset of populations from the three forms were explored using RAPD markers in Chapter 5. The co-occurrence of the small and large forms at the Pleshkes population provided an opportunity for a direct analysis of morphological and genetic patterns across the zone of hybridisation in Chapter 6.

In Chapter 7, the major findings of this research are synthesised and summarised.

CHAPTER 2

Morphological variation and chromosome number

Introduction

Systematics, one of the oldest fields of biological science, provides a means by which organisms can be readily distinguished into groups (Stace, 1989). The classification of organisms not only allows accurate recognition, but also provides for collective assumptions about these groups to be made. Since scientific investigations are usually conducted on a subset of organisms, their correct systematic assignment allows the outcome of such endeavours to be extrapolated to the group as a whole. Although the characters most useful for determining systematic relatedness vary upon the group under examination, those which are visible and readily recognisable are commonly favoured.

Morphological variation

Many groups of organisms exhibit some degree of variability. In spatially distributed species, clinal, mosaic and discontinuous patterns of geographic variation can often be explained by ecological influences, rather than by the processes of speciation (Bradshaw, 1965; Heywood, 1967; Thorpe, 1976; Richardson *et al.*, 1986). *G. verrucosa* is widely distributed throughout the transitional-rainfall zone of the southwest of Western Australia. Although the degree of observed morphological variation may reflect patterns of ecological variability across the large species' range, its occurrence in a region known for species richness, endemism, population disjunction and cryptic speciation (Hopper, 1979; 1992) suggests this variation may also have a systematic basis.

In situations where morphological variation is subtle or cryptic, quantitative statistical methods may be more useful for resolving systematic differences (Heywood, 1967; Liston, 1970; Sneath and Sokal, 1973).

Morphometric analyses have successfully clarified relationships in several enigmatic Australian genera, in particular, the large and somewhat problematic genus *Eucalyptus* (Williams and Ladiges, 1985; Cook and Ladiges, 1991; Chappill and Ladiges, 1992; Pryor *et al.*, 1995), and the unusual *Epacris tasmanica* complex (Crowden and Menadue, 1990).

Multivariate analyses use a suite of characters to either classify individuals into groups, or, to examine their relationships by ordination which may, or may not, lead to the recognition of groups (Conn, 1984). Common classification procedures include cluster and discriminant analyses. Cluster analysis identifies similar entities according to predetermined selection criteria, generating clusters of high internal homogeneity and high external heterogeneity (Hair *et al.*, 1992). A hierarchical or tree-like structure is then constructed using either agglomerative or divisive methods (James and McCulloch, 1990; Hair *et al.*, 1992). Discriminant analysis (discriminant function analysis) not only seeks to place objects into groups, but also attempts to predict group membership (Tabachnick and Fidell, 1989; Hair *et al.*, 1992). Linear combinations of the independent (predictor) variables are constructed by maximising the between-group variance relative to within-group variance (Hair *et al.*, 1992; Norusis, 1994). Calculated discriminant functions provide an indication of the separation between groups (Tabachnick and Fidell, 1989).

Principle component analysis and multidimensional scaling are frequently applied ordination procedures. Principal component analysis attempts to reduce the dimensions of a single group of data by producing a smaller number of abstract variables using a matrix of computed eigenvalues and eigenvectors (Sneath and Sokal, 1973; Tabachnick and Fidell, 1989; James and McCulloch, 1990). Multidimensional scaling employs interobject distances to estimate nonlinear, monotonic relationships, displaying them geometrically (James and McCulloch, 1990; Norusis, 1994). While both methods produce similar results, multidimensional scaling can consider asymmetric dissimilarity matrices, contend with tied

or missing data, and provide a balance between large intercluster distances and the fine differences between members within a cluster (Sneath and Sokal, 1973).

Chromosome number and polyploidy

Another component of variation within a species, often critical for understanding systematic and evolutionary patterns, is chromosome variation. Polyploidy, in particular, has been shown to be a major factor in the evolution of many plant groups. The propensity of higher plants towards polyploidy is reflected by its occurrence in almost every Angiosperm family (Roose and Gottlieb, 1976; Grant, 1981; Gottlieb, 1982; Weeden, 1983; Soltis and Rieseberg, 1986).

Although previously considered to be maladaptive, polyploidy may confer significant adaptive advantages on organisms (Soltis and Rieseberg, 1986; Crawford, 1989). Biochemical diversity associated with polyploidy may provide a buffer for environmental change as well as facilitating the evolution of new and adaptive characters, and colonisation into new niches (Roose and Gottlieb, 1976; Levin, 1983; Soltis and Rieseberg, 1986; Crawford, 1989; Weeden and Wendel, 1989). As a strong barrier to gene exchange, polyploidy can also bring about rapid speciation, creating new species within a few generations (Smith-White, 1954; Love and Love, 1974; Levin, 1983).

Prolonged isolation, a high degree of endemism and the distinctive character of the Australian flora, provide a unique evolutionary insight into speciation within the region (Smith-White, 1959). The fertility of polyploids, wide geographical ranges and low frequency of multivalents indicate these are a well established component of the flora (Smith-White, 1954). As a family, Rutaceae exhibits substantial chromosome variation with polyploidy being an important evolutionary development (Smith-White, 1954; Stace *et al.*, 1993). In the tribe Boronieae, systematic relationships and evolution are reflected by chromosomal variation (Smith-White, 1954). Within the largest

genus of the tribe, *Boronia*, extensive dysploidy and polyploidy occur (James, 1981). The subtribe Eriostemoninae, to which *G. verrucosa* belongs, exhibits chromosomal variation of n = 13, 14, 16, 17, 19, 28 and 32 (Smith-White, 1954; Stace *et al.*, 1993). *G. verrucosa* has a basic chromosome complement of n = 14 (Smith-White, 1954).

Aims of morphometric and chromosomal analyses

Traditionally, systematic investigations and revisions have relied upon material lodged in herbarium collections. Although useful, the assumption is that these collections are representative of the natural variation present within taxa. Classical systematic methods can also be invasive and, occasionally, impractical due to the intrinsic value of some specimens. In some instances, the age and condition of specimens can distort characters. Consequently, this investigation focused on natural variation observed in the field.

This investigation aimed to analyse the morphological variation within and between populations of *G. verrucosa* using morphometric techniques and to examine whether the patterns obtained agreed with morphological variation observed in the field. It also sought to determine whether the observed morphological variation in *G. verrucosa* could be attributed to polyploidy.

Materials and methods

Morphological variation

Background and sampling strategy

Fresh floral and vegetative material was collected from the tagged plants within each study site (Chapter 1, Table 1.1 and Figure 1.6); these were sealed in plastic bags, stored at 4°C and transported to the laboratory for measurement. Poor flowering of some populations and a fire in late 1994 at Spalding Park, reduced the data sets of some populations to less than 20 plants. Although ideally all collections would have been made in 1994,

unpredictable seasonal variation in flowering, the large geographic distances between populations, and the discovery of new populations as research progressed, meant that collections were also made in 1995.

Character assessment and measurement

Quantitative characters were employed in this investigation for several reasons. Firstly, no distinct morphological differences between PERTH specimens had previously been noted (Paul Wilson, pers. comm.). In contrast, the size of leaves, flowers and plants appeared to be important diagnostic characters in the field. It was also apparent after examining PERTH specimens that a bias towards the intermediate and large forms existed, with the small form represented by a single specimen. Secondly, this assessment was intended to complement molecular techniques and not as a comprehensive taxonomic analysis *per se*. Thirdly, qualitative characters, such as flower and leaf colour, and flower abundance, could not be consistently scored and were consequently ignored. Only adult characters were used as seedling propagation was both difficult and unreliable.

The length, width and area of six mature leaves, bracts, sepals and petals from each of the selected plants within each population were measured using a digital analyser (DIAS II, Delta-T Devices, Cambridge, England). To provide a better idea of shape, width and area measurements were discarded and length:width ratios calculated (Cook and Ladiges, 1991). The number of abaxial glands on each leaf were counted at 20x magnification under a binocular dissecting microscope and converted to the number of glands/mm^2. To make the ratios more linear, $\log_{10}$ transformations were performed (James and McCulloch, 1990). The thickness of a further six leaves was determined using a pair of electronic digital calipers (Max-Cal) (± 0.03 mm).

Univariate analyses were undertaken on the ten measured or calculated characters while multivariate analyses were conducted using the same

characters plus three additional derived characters intended to reflect size relationships between bracts, sepals and petals (Table 2.1).

Table 2.1: Quantitative characters used for morphometric analyses.

Character (unit)	Code
1. Leaf length (mm)	**LL**
2. Log_{10} Leaf length:width ratio (mm^2)	**Log_{10} LL:W**
3. Leaf thickness (mm)	**LT**
4. Log_{10} Number of leaf glands per mm^2	**Log_{10} GL/mm^2**
5. Bract length (mm)	**BL**
6. Log_{10} Bract length:width ratio (mm^2)	**Log_{10} BL:W**
7. Sepal length (mm)	**SL**
8. Log_{10} Sepal length:width ratio (mm^2)	**Log_{10} SL:W**
9. Petal length (mm)	**PL**
10. Log_{10} Petal length:width ratio (mm^2)	**Log_{10} PL:W**
Additional multivariate characters	
11. Log_{10} Bract:Sepal length ratio	**Log_{10} BL:SL**
12. Log_{10} Bract:Petal length ratio	**Log_{10} BL:PL**
13. Log_{10} Sepal:Petal length ratio	**Log_{10} SL:PL**

Univariate analyses

To establish the degree of natural variation within populations, individual characters for each plant were analysed by one-way Analysis of Variance (ANOVA) and means compared using Tukey's Compromise. This test allowed for unequal treatment numbers and overcame Type I errors associated with Tukey's Honestly Significant Difference test and Type II errors of the Student-Newman-Keuls (Abacus Concepts, Inc., Berkeley, CA, 1989). To determine whether the different forms could be distinguished, character means for each population were also analysed and compared in the same manner. All analyses were conducted using SuperANOVA (Abacus Concepts, Inc., Berkeley, CA, 1989).

Multivariate analyses

Multivariate analyses explored the data at two levels. Character means for each plant were used to search for consistent groupings in discriminant

and principle component analyses, while character means for each population were used to assess associations between populations in cluster analysis and nonmetric multidimensional scaling. To overcome the unequal contribution of some characters and the uneven range of values, all characters were standardised by zscores prior to analyses. Computations were undertaken using SPSS Version 6.1 (SPSS Inc., Chicago, IL).

(a). Discriminant analysis

Discriminant analysis sought to place individual plants into groups and assess the reliability of group membership (Tabachnick and Fidell, 1989; Hair *et al.*, 1992). Plants were coded according to form: small = 1, intermediate = 2 and large = 3. Plants in the transect at Pleshkes were coded by the form they most resembled with suspected hybrids classed as the intermediate form. Discriminant scores for each plant were calculated from the discriminant coefficients and classified according to Bayes' rule using the standard SPSS algorithms. A combined-groups scatterplot of the first two discriminant function variables for each plant was produced to visualise associations between individuals, populations and forms.

(b). Principle component analysis

A coefficient correlation matrix of plant character means was calculated and principal component factors with eigenvalues greater than one were extracted. To visualise associations between individuals, populations and forms, a scatterplot of the first two factors for each plant was generated.

(c). Cluster analysis

An agglomerative hierarchical classification of the twenty populations was undertaken using the Euclidean distance measure of similarity and the unweighted pair-group method using arithmetic means (UPGMA) clustering algorithm. This algorithm averages all distances between pairs of objects in different clusters, accurately reflecting real distances between individuals whilst avoiding extremes associated with single or complete

linkage clustering (Sneath and Sokal, 1973). Distances were rescaled to between 0 and 25 according to SPSS specifications.

(d). Nonmetric multidimensional scaling

Euclidean distances between populations were calculated and a two-dimensional Euclidean model produced. Kruskal's stress measure and the squared correlation coefficient were calculated to determine how well the ordination fitted the original dissimilarities.

Cytology

Sampling Strategy

Seed was collected from a random sample of five plants per population in 1994 and 1995, and germinated after smoke treatment at Kings Park and Botanic Gardens Perth, Western Australia. Despite the beneficial effects of smoke treatment on germination in this species (Dixon *et al.*, 1995), germination was low, limiting the number of root tips available for examination. Root tips were harvested in the morning, approx. 3-5 days after germination, pre-treated in 0.1% colchicine for 2-3 h, fixed in ethanol:acetic acid (3:1 v/v) for 24 h, rinsed and stored at 4°C in 70% ethanol (v/v).

Protocol

Prior to staining, root tips were hydrolysed in 1M HCl at 60°C for 5 min, rinsed in distilled water and stained with Schiff's Reagent (Sigma, S-5133) for 30-60 min. The stained portion of each tip was then removed, macerated on a slide, counterstained with 0.5% of aceto-carmine (Gurr, C.I. 75470), squashed and viewed at 1000x magnification (Olympus Vanox) using both normal and phase contrast microscopy. Where possible, chromosomes in five cells per root tip were counted for each plant.

Results

Morphological variation

Univariate analyses

Tukey's Compromise comparisons of characters within populations showed individual patterns of variation depending upon both the population and character being assessed (data not included). For example, while some characters within a population showed no variation, others were highly variable. Additionally, a character which showed no variation in one population, was highly variable in another.

Comparisons of character means between populations also failed to statistically establish clear morphological trends (Table 2.2). Although the small form exhibited significantly shorter and smaller shaped leaves, bracts and sepals, many characters did not differentiate significantly between the forms ($\alpha = 0.05$). In addition, the Pleshkes Small population exhibited several characters such as leaf, bract and sepal characters which were considerably larger than those of other small form populations.

Table 2.2: Tukey's Compromise comparison of character means between populations. Means not shown, same letter indicates means did not differ at $\alpha = 0.05$.

Form	Population	Code	*n*	LL	Log_{10} LL:W	LT	Log_{10} GL/mm^2	BL	Log_{10} BL:W	SL	Log_{10} SL:W	PL	Log_{10} PL:W
Small	Arinya	A	20	4.60^{kl}	1.07^{f}	0.55^{gh}	0.19^{cd}	6.32^{g}	1.26^{k}	7.33^{j}	1.37^{k}	7.24^{h}	1.34^{e}
	Coorow 1	C1	20	5.02^{k}	1.17^{e}	0.66^{bc}	0.07^{fg}	7.53^{g}	1.50^{j}	8.34^{i}	1.54^{ij}	7.64^{gh}	1.38^{de}
	Coorow 2	C2	20	4.89^{k}	1.16^{e}	0.73^{a}	0.06^{g}	6.47^{g}	1.36^{j}	8.30^{i}	1.50^{j}	7.79^{efgh}	1.38^{de}
	Indarra Reserve	IR	20	4.13^{l}	1.04^{f}	0.58^{efgh}	0.25^{abc}	7.11^{g}	1.38^{j}	9.10^{i}	1.58^{i}	7.62^{gh}	1.38^{e}
	Maiseys	M	20	5.25^{k}	1.16^{e}	0.65^{bcd}	0.18^{cd}	9.16^{f}	1.65^{h}	10.39^{h}	1.76^{h}	8.05^{efg}	1.44^{cd}
	Pleshkes Small	PS	10	6.30^{ij}	1.45^{cd}	0.59^{defg}	0.17^{cde}	12.43^{d}	1.94^{fg}	13.46^{abcde}	1.95^{bcde}	8.29^{cdef}	1.46^{bc}
Intermediate	Ajana West 1	AW1	20	7.29^{fg}	1.61^{b}	0.68^{ab}	0.06^{g}	14.75^{ab}	2.16^{ab}	13.71^{abcd}	1.99^{b}	8.74^{abcd}	1.53^{ab}
	Ajana West 2	AW2	20	7.51^{efg}	1.61^{b}	0.64^{bcde}	0.13^{def}	15.29^{a}	2.19^{a}	14.40^{a}	2.10^{a}	9.33^{a}	1.57^{a}
	Hawks Head	HH	20	6.18^{j}	1.44^{d}	0.69^{ab}	0.20^{cd}	12.33^{d}	2.01^{def}	14.27^{a}	1.97^{bcd}	9.00^{ab}	1.52^{ab}
	Hutt River 1	HR1	20	6.52^{hij}	1.44^{d}	0.62^{cdef}	0.28^{ab}	14.23^{abc}	2.08^{bcd}	12.76^{defg}	1.93^{bcdef}	8.23^{defg}	1.50^{bc}
	Hutt River 2	HR2	20	6.79^{ghij}	1.46^{cd}	0.60^{cdefg}	0.29^{a}	14.88^{ab}	2.12^{abc}	13.00^{cdef}	1.96^{bcd}	8.19^{defg}	1.47^{bc}
	Nanns	N	20	7.06^{fgh}	1.52^{c}	0.61^{cdefg}	0.19^{cd}	13.60^{bcd}	2.02^{def}	13.52^{abcde}	1.98^{bc}	8.95^{ab}	1.53^{ab}
	West Binnu	WB	16	6.97^{ghi}	1.49^{cd}	0.58^{efgh}	0.23^{abc}	11.05^{e}	1.91^{g}	12.97^{cdef}	1.94^{bcdef}	9.39^{bcde}	1.49^{bc}
Large	Geraldton	GP	20	7.75^{def}	1.60^{b}	0.49^{i}	0.05^{gh}	13.10^{cd}	2.05^{cde}	12.15^{fg}	1.91^{cdefg}	8.10^{efg}	1.48^{bc}
	Meanarra Hill 1	MH1	20	9.25^{b}	1.74^{a}	0.55^{gh}	0.23^{abc}	12.31^{d}	1.94^{fg}	12.39^{fg}	1.87^{fg}	8.77^{abcd}	1.53^{ab}
	Meanarra Hill 2	MH2	20	10.03^{a}	1.79^{a}	0.52^{hi}	0.21^{bcd}	13.47^{bcd}	1.97^{efg}	13.11^{bcdef}	1.88^{efg}	8.87^{abc}	1.53^{ab}
	Pleshkes Large	PL	10	8.96^{bc}	1.73^{b}	0.61^{cdef}	0.15^{h}	14.07^{abc}	2.12^{abc}	14.06^{ab}	1.98^{bc}	8.37^{bcde}	1.48^{bc}
	Red Bluff	RB	20	10.04^{a}	1.80^{a}	0.58^{efgh}	0.13^{def}	12.51^{d}	1.94^{fg}	12.52^{efg}	1.84^{g}	8.87^{abc}	1.48^{bc}
	Spalding Park	SP	16	8.03^{de}	1.66^{b}	0.58^{efgh}	0.05^{gh}	14.33^{abc}	2.12^{abc}	11.78^{g}	1.90^{defg}	7.68^{fgh}	1.44^{cd}
Transect	Pleshkes Hybrid	PH	20	8.35^{cd}	1.65^{b}	0.57^{fgh}	0.11^{efg}	15.08^{a}	2.13^{abc}	13.90^{abc}	1.96^{bcd}	8.39^{bcde}	1.48^{bc}

LL, Leaf length; Log_{10} LL:W, Log_{10} Leaf length:width ratio; LT, Leaf thickness; Log_{10} GL/mm^2, Log_{10} number of leaf glands per mm^2; BL, Bract length; Log_{10} BL:W, Log_{10} Bract length:width ratio; SL, Sepal length; Log_{10} SL:W, Log_{10} Sepal length:width ratio; PL, Petal length; Log_{10} PL:W, Log_{10} Petal length:width ratio.

Multivariate analyses

(a). Discriminant analysis

A priori classification of the 371 individuals fitted the three forms well with 91.1% of plants correctly classified (Table 2.3). Leaf and bract characters (LL, Log_{10} LL:W, BL, Log_{10} BL:W,) and the bract/sepal/petal ratios (Log_{10} BL:PL, Log_{10} SL:PL and Log_{10} BL:SL) contributed most to the first discriminant function, while the remaining characters contributed to the second discriminant function (Figure 2.1, left). Differences between the *a priori* and predicted classifications for the small form were due to all Pleshkes Small plants and five of the small form Pleshkes Hybrid plants being placed with the intermediate form. Two other small form Pleshkes Hybrid plants were classified as belonging to the large form. Several intermediate form plants were classified as large form plants: Ajana West 1 (1), Hutt River 2 (1), Nanns (1), West Binnu (2). Three intermediate form plants at Pleshkes Hybrid were also placed with the large form. Two large form plants at Pleshkes Hybrid were classed as the intermediate form along with several large form plants at Geraldton (3), Pleshkes Large (2) and Spalding Park (1).

Removing the transect plants at Pleshkes Hybrid and repeating the analysis increased the number of correctly classified individuals to 94.3% (Table 2.3). Leaf characters and the bract ratios (LL, Log_{10} LL:W, Log_{10} BL:PL, and Log_{10} BL:SL) contributed most to the first discriminant function, while the remaining characters contributed to the second discriminant function (Figure 2.1, right). The small form plants at Pleshkes Small were again classified as belonging to the intermediate form. Fewer of the intermediate form plants were classified as the large form (Ajana West 1, 2; Hutt River 2, 1; Nanns, 1; West Binnu, 2), as were large form plants considered to belong to the intermediate form (Geraldton, 2; Pleshkes Large, 1; Spalding Park, 1).

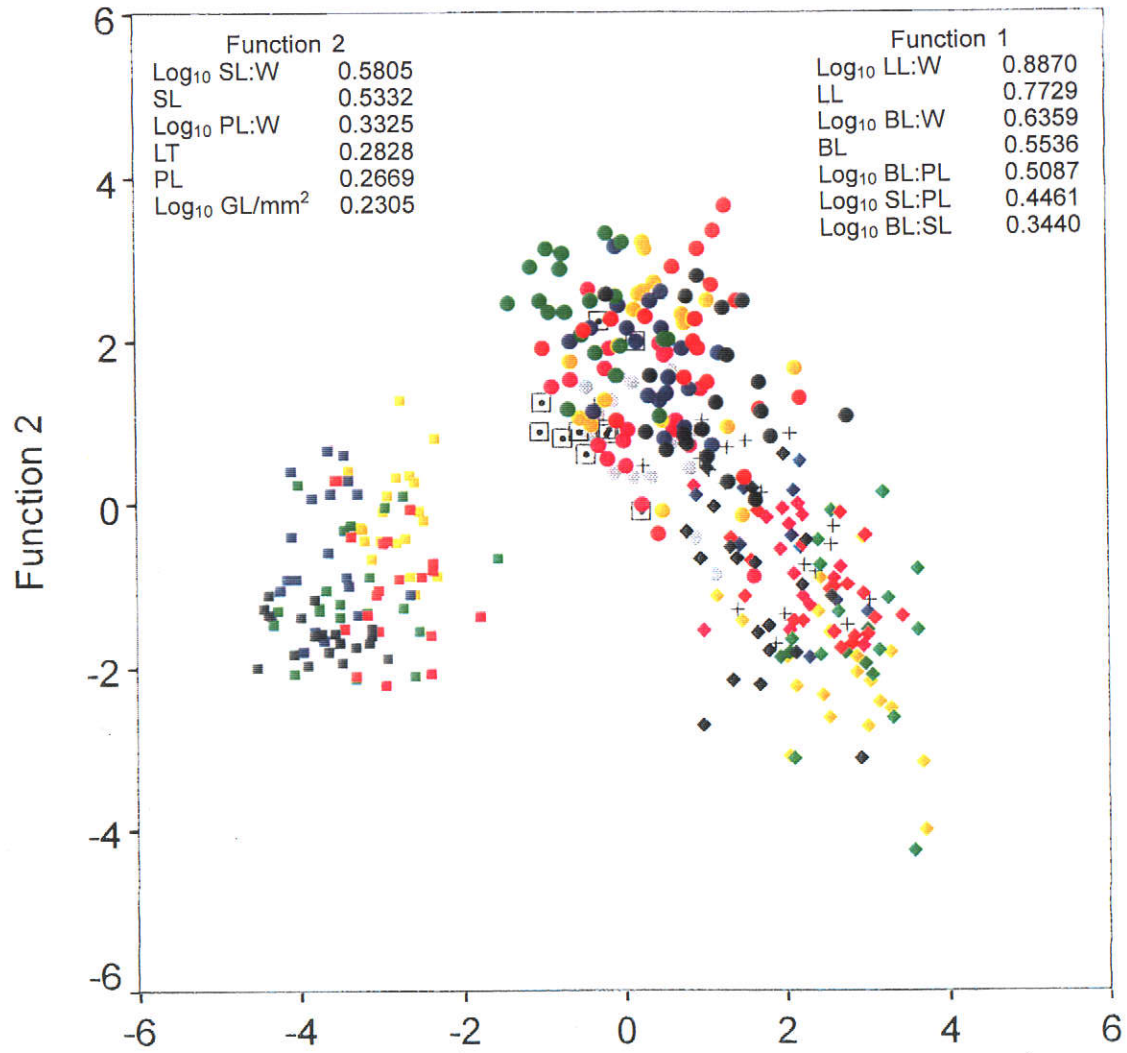
Table 2.3: Percentage of *G. verrucosa* plants correctly predicted following *a priori* classification by form using all plants and with Pleshkes Hybrid plants removed (No PH). Number in parentheses.

A priori classification by form		Predicted classification: *Small*		*Intermediate*		*Large*	
Small	All plants (117)	85.5%	(100)	12.8%	(15)	1.7%	(2)
	No PH (110)	90.9%	(100)	9.1%	(10)	0%	(0)
Intermediate	All plants (141)	0%	(0)	94.3%	(133)	5.7%	(8)
	No PH (135)	0%	(0)	95.6%	(129)	4.4%	(6)
Large	All plants (113)	0%	(0)	7.1%	(8)	92.9%	(105)
	No PH (106)	0%	(0)	3.8%	(4)	96.2%	(102)

The scatterplot revealed that while most of the small form plants grouped together, those at Pleshkes Small were closely allied with plants of the intermediate form (Figure 2.1, left). Pleshkes Hybrid plants were variously placed with both the intermediate and large forms. Although these latter forms were not clearly separated, plants did tend to group with their respective forms. Removing the Pleshkes Hybrid plants from the analysis did not significantly improve separation between the intermediate and large forms or change the placement of Pleshkes Small plants (Figure 2.1, right).

(b). Principle components analysis

Four principle component factors were extracted and accounted for 90.7% of the total variance with bract length (BL), leaf length (LL), leaf thickness (LT) and the bract:petal length (Log_{10} BL:PL) contributing to this variation. The first two factors extracted explained 73.4% of the total variance, the first factor contributing to 60.8% and the second factor contributing to 12.6%. Associations between populations within the three forms were not as clear from scatterplots of the factor loadings as those obtained in discriminant analysis (Figure 2.2, left). Although most of the small form plants were separated from the other forms, plants at the Maiseys population were scattered between the small and intermediate form plants. Removing the Pleshkes Hybrid plants did not alter the total variance, the factors extracted, or further clarify the associations between forms and populations (Figure 2.2, right).



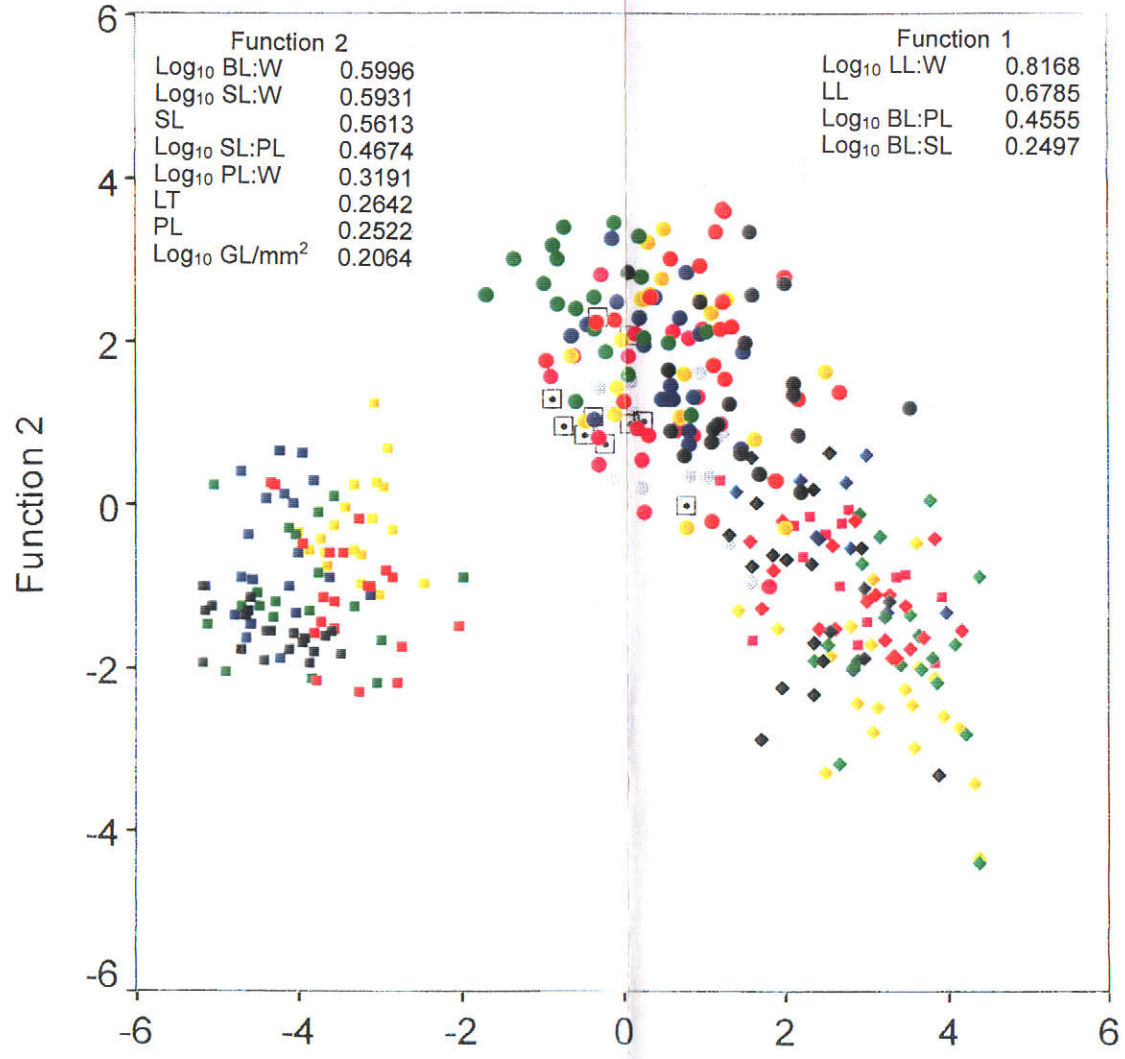


Figure 2.1: Scatterplot of canonical discriminant functions for all *G. verrucosa* individuals (left) and with Pleshkes Hybrid individuals removed (right).

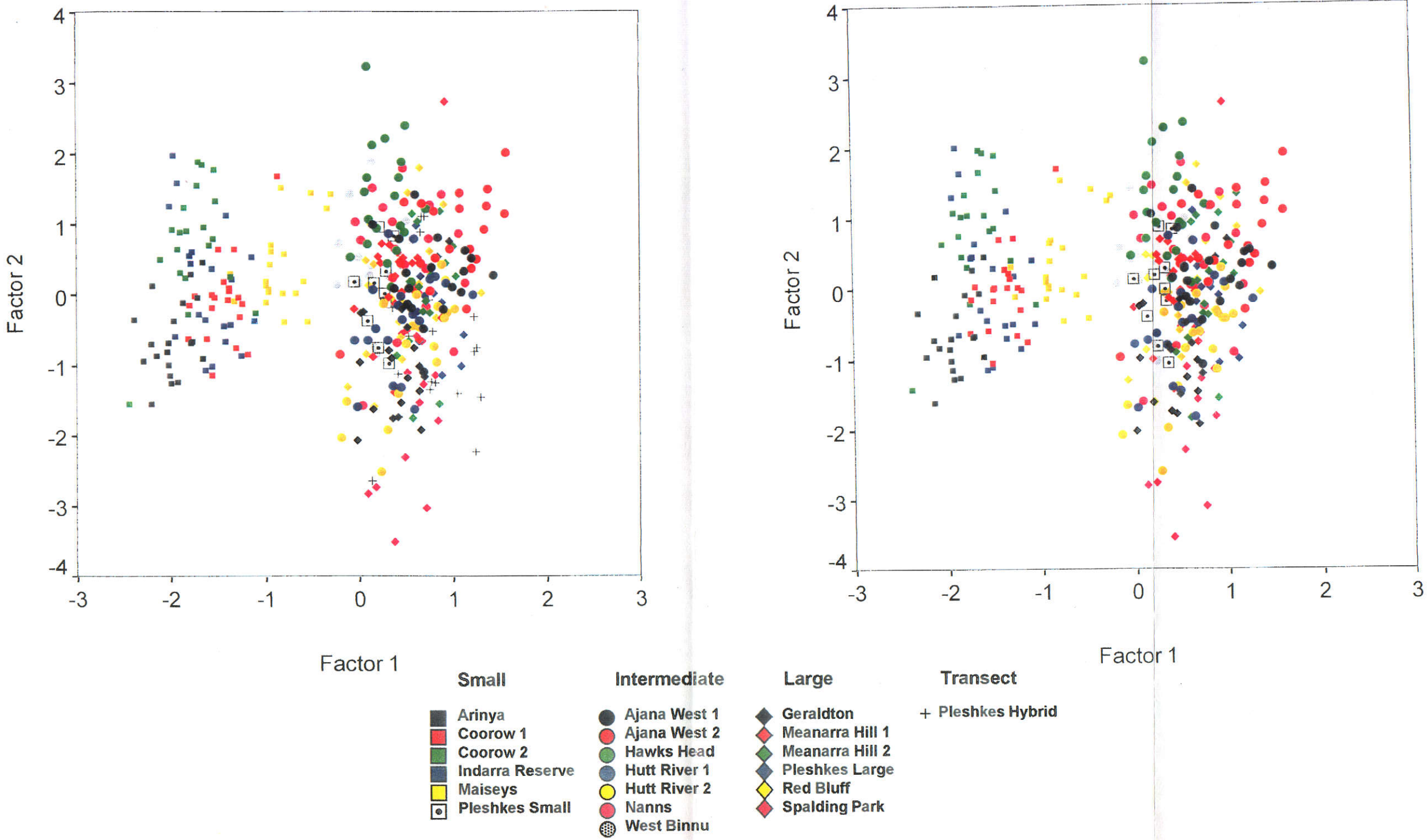


Figure 2.2: Scatterplot of the first two principal component factors for all *G. verrucosa* individuals (left) and with Pleshkes Hybrid individuals removed (right).

(c). Cluster analysis

Cluster analysis generated two distinct clusters (Figure 2.3). Populations belonging to the small form, with the exception of Pleshkes Small, formed one cluster and the remaining populations the other. Within the latter cluster, the intermediate and large form populations were separately grouped. The small form at Pleshkes (Pleshkes Small) was placed within the intermediate form.

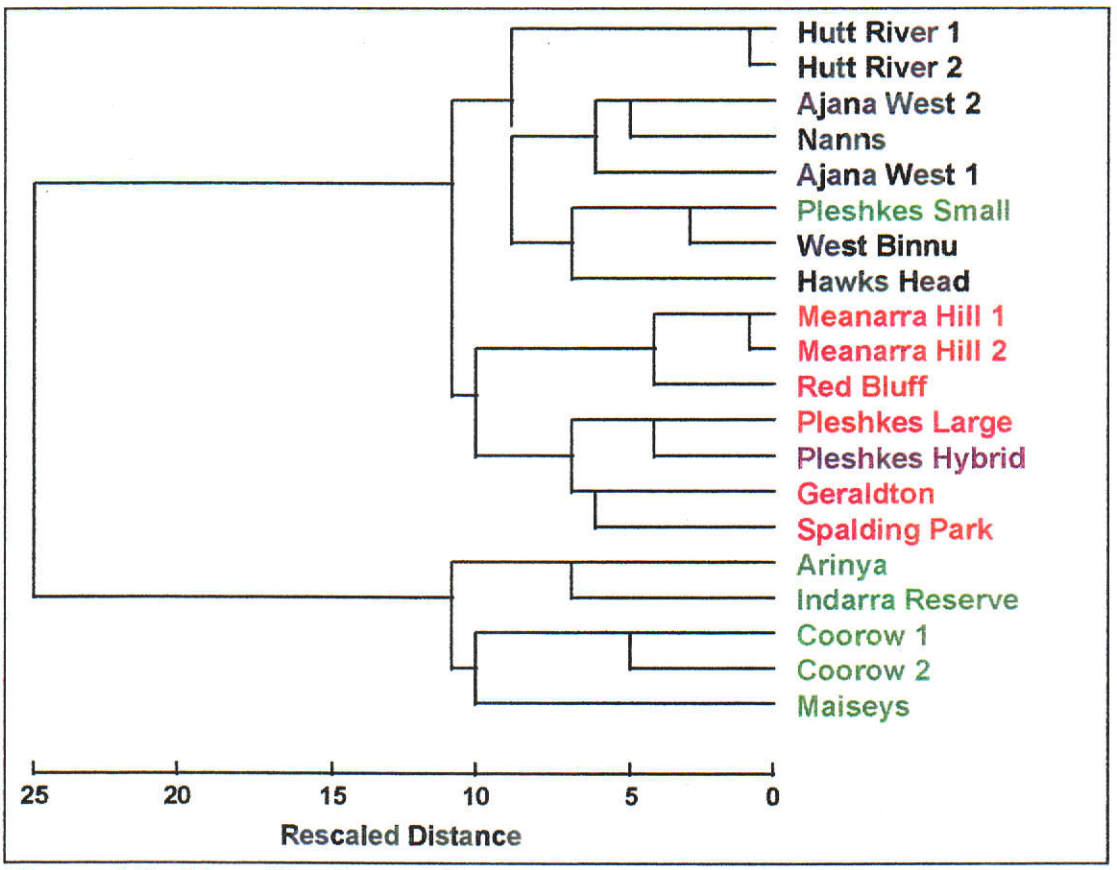


Figure 2.3: Classification of *G. verrucosa* populations based on Euclidean distances and UPGMA algorithm. Small form; **intermediate form;** large form; transect.

(d). Nonmetric multidimensional scaling

Kruskal's stress measure (0.11) and the squared correlation coefficient (r^2 = 0.97) indicate the two dimensional Euclidean model described the data reasonably well. When represented graphically, the ordination highlighted several important features (Figure 2.4). Although the intermediate and large

form populations were not readily separated, the small form populations at Arinya, Coorow and Indarra Reserve were clearly a discrete group. The remaining small form population at Maiseys was placed between this group and the other forms. Pleshkes Small was closely allied with the intermediate form populations. The large form populations at Geraldton and Spalding Park fell some distance away from the main intermediate-large group.

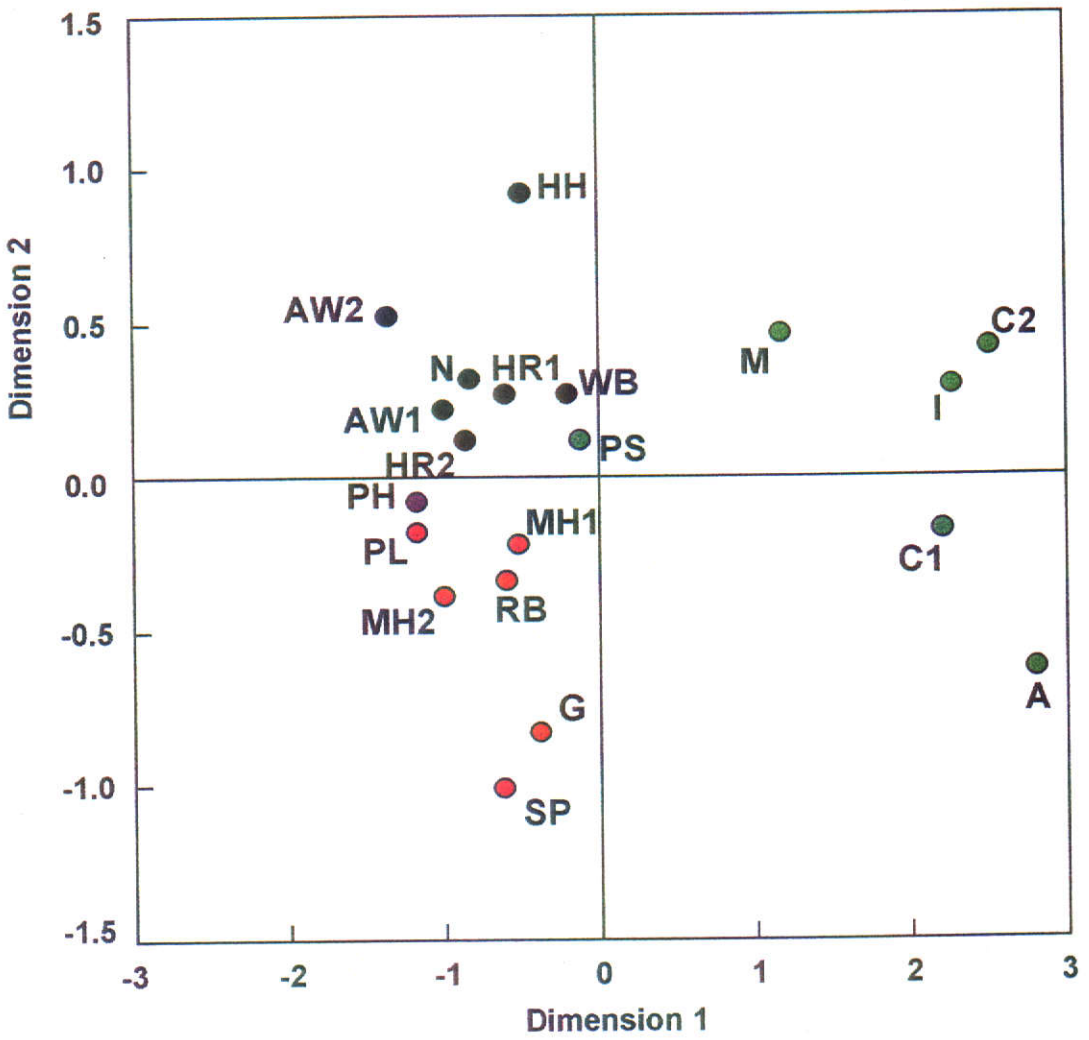


Figure 2.4: Nonmetric multidimensional scaling of *G. verrucosa* populations. Small form; intermediate form; large form; transect. Population codes outlined in Table 2.2.

Cytology

Although chromosomes at various mitotic stages were observed, good chromosome spreads were difficult to obtain. Chromosomes were also small and karyotyping was not possible. No polyploidy was observed and all three forms exhibited a chromosome complement of $2n = 28$ (Figure 2.5 and Appendix 1).

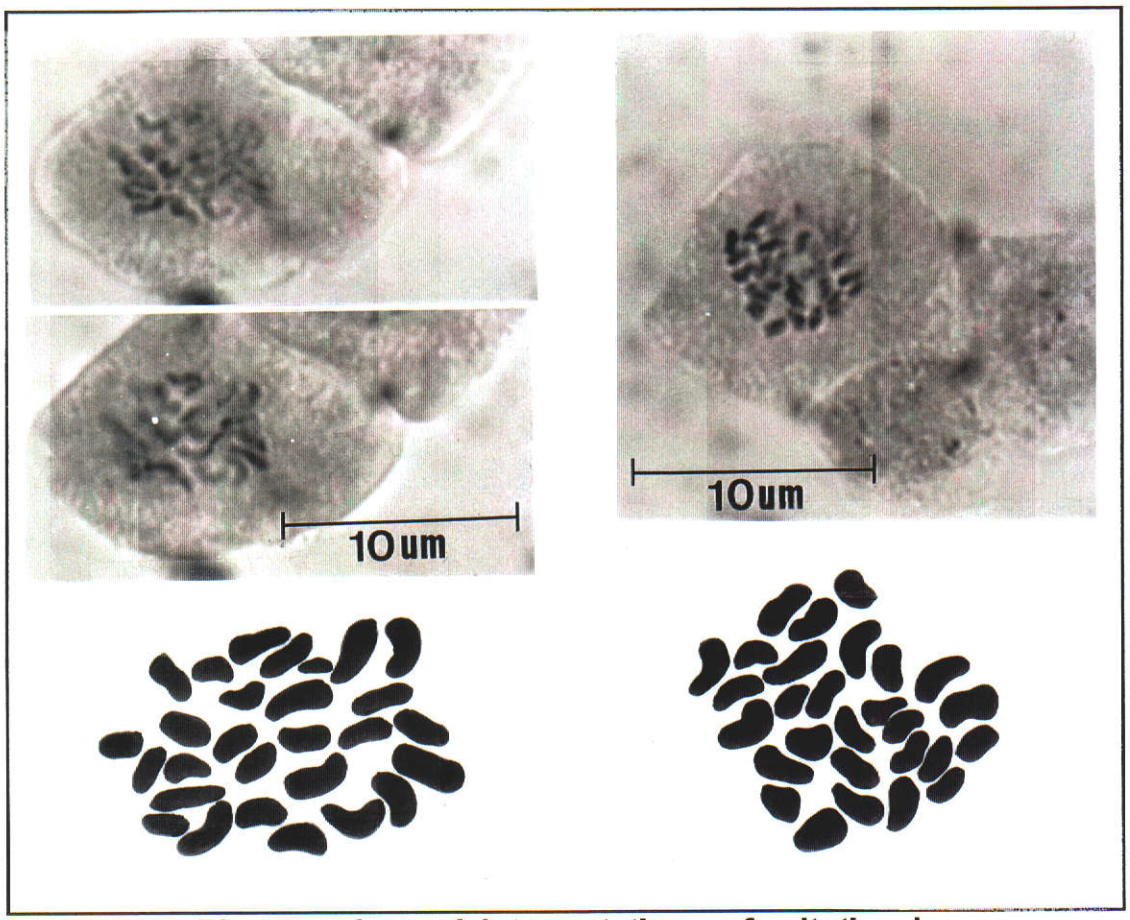


Figure 2.5: Photographs and interpretations of mitotic chromosomes from root tips of two *G. verrucosa* forms. Left, Geraldton (large form); right, Ajana West 1 (intermediate form).

Discussion

Discriminant and cluster analyses and nonmetric multidimensional scaling all indicate that *G. verrucosa* is morphologically diverse and that the small form constitutes a morphologically discrete taxon. The major distinguishing characters are the length and shape of leaves and bracts, and size

relationships between the bracts, sepals and petals. This corresponds well with empirical evidence that the small form is characterised by smaller leaves and flowers. Separation between the intermediate and large forms was, however, less clear. The morphological variation observed could not be attributable to polyploidy. Although polyploidy and variation in chromosome number are common within Rutaceae (Smith-White, 1954; Stace *et al.*, 1993), neither polyploid, nor other chromosomal variants, were observed.

Since morphological variation can often be explained by geographic distribution and associated ecological factors (Bradshaw, 1965; Heywood, 1967; Richardson *et al.*, 1986), the observed morphological diversity present within *G. verrucosa* may reflect such conditions. The small form occurs to the southeast of the species' range, the large form towards the north while the intermediate form is distributed in between (Chapter 1, Figure 1.6). Several factors, however, suggest that an environmental gradient does not satisfactorily account for the morphological variation in this genus.

Although somewhat rare, different forms do co-occur at two sites. Several small form plants, which were not included in this investigation, exist near the intermediate form plants at Ajana West 2. At the Pleshkes population, significant numbers of plants belonging to both the small and large forms occur. Despite their close proximity, however, these co-occurring forms maintain their morphological integrity. This integrity has been further highlighted by a recent planting of all forms in a common experimental plot just outside of Perth. Further, the flowering patterns of the two forms at Ajana West 2 are also distinct, exhibiting little overlap (Chapter 3), suggesting that environment and/or ecological influences do not explain the morphological differences observed.

Plants belonging to the small form at Pleshkes, however, are consistently classified with intermediate form populations. While these plants exhibit

morphological characteristics similar to those of other small form populations, they possess slightly larger flowers. The forms at Pleshkes also exhibit considerable overlap with respect to commencement and length of flowering (Chapter 3). The co-occurrence of the small and large form plants at this site provides an opportunity for hybridisation and introgression to take place. Hybridisation between these forms may include the incorporation of genetic components from the large form into the small, leading to an overall increase in size and changes in flowering patterns. Morphological and genetic variation in this population are further explored in Chapter 6.

The misclassification of several Geraldton plants in discriminant analysis is also interesting and suggests that hybridisation may be an important evolutionary event within *G. verrucosa*. This unusual population is located at the centre of the species' range. The plants at this site exhibit a habit that is similar to the small form plants at Arinya, but display larger and more abundant flowers per inflorescence reminiscent of the large form. Being centrally located, and within an area of overlap between the small and large forms, suggests these 'misclassified' plants may be of hybrid origin.

The major argument against hybridisation in *G. verrucosa* is population disjunction and limited gene flow. However, disjunction may not have always characterised this genus. The present day distribution of flora within the southwest region largely reflects climatic fluctuations and geological events from late Tertiary-Quaternary time to the present day (Hopper, 1979). It is conceivable that hybridisation between populations of *G. verrucosa* has been facilitated by closer proximity and a more intimate distribution in the past. The possibility of hybridisation between the small and large forms does present some difficulties for systematic clarification.

The small form is readily distinguished from the other forms by the size and shape of its flowers and leaves, and fewer flowers per inflorescence. Differentiating between the large and intermediate forms is more

problematic. Although this latter form may represent an ecotype, the possibility that it is of hybrid origin cannot be discounted. By determining possible genetic relationships between the three forms using molecular markers, and the ability of the forms to interbreed, their systematic relationships may be further elucidated and evidence of hybridisation established.

CHAPTER 3

Reproductive biology and mating system

Introduction

Plant reproductive strategies play an important role in gene transmission, population genetic structure, selection response and speciation (Brown and Allard, 1970; Jain, 1975; Grant, 1981; Barrett and Eckert, 1990; Lyons and Antonovics, 1991; Ellis and Sedgley, 1992; Barrett *et al.*, 1996). Many diverse and dynamic plant mating systems have evolved from the necessity to balance reproductive requirements with genetic and ecological demands (Clegg, 1980; Lloyd, 1980; Barrett and Eckert, 1990; Brown, 1990). Underlying this great diversity are five modal breeding systems - habitual (predominant) outbreeding, habitual inbreeding, apomixis, facultative amopomixis and vegetative reproduction (Jain, 1976).

Evolutionary shifts in the breeding strategies of plants are usually associated with a move from obligate outbreeding to predominant self-fertilisation, although reverse shifts are known (Stebbins, 1970; Gottlieb, 1973; Grant, 1975; Jain, 1976). Floral architecture, and its role in pollen transfer, is an important evolutionary influence on plant mating systems (Holsinger, 1996). Shifts in reproductive strategy are often correlated with changes in floral morphology, biology and resource allocation. Selfing populations frequently exhibit smaller petals, stamens and styles, lower nectar and pollen production, and lower pollen:ovule ratios, essentially investing fewer resources in floral attractants while setting more seed (Jain, 1976; Cruden, 1977; Schoen, 1977, 1982; Solbrig and Rollins, 1977; Wyatt, 1984; Barrett, 1988; Ritland and Ritland, 1989). In contrast, predominantly outcrossing species possess larger and more abundant flowers, but set a lower proportion of fruit and seeds (Solbrig and Rollins, 1977; Lyons and Antonovics, 1991).

Some 20% of Angiosperms are self-fertile, although many selfing species maintain some level of genetic recombination through outcrossing (Nei, 1975; Brown, 1990; Cole and Biesboer, 1992). Predominantly selfed species are often genetically less diverse than their outcrossing congeners, apportioning diversity between populations rather than within (Levin, 1978; Brown, 1979; Hamrick *et al.*, 1979; Gottlieb, 1981; Hamrick and Godt, 1989). Selfing is particularly widespread within colonising species, ensuring reproductive success and efficiency, and the fixation of highly advantaged adaptive genotypes (Stebbins, 1957; Schemske and Lande, 1985; Barrett and Eckert, 1990; Hamilton and Mitchell-Olds, 1994; Richards, 1997). Selfing may, however, contribute to inbreeding depression through the homozygous expression of deleterious genes, or the loss of heterozygous advantage (Richards, 1997).

The alleles of outcrossing plants tend to be widely distributed, effectively reducing population subdivision (Loveless and Hamrick, 1984; Cole and Biesboer, 1992). Outcrossing maintains genetic recombination, promotes genetic diversity and creates new and adaptive individuals, some of which may exhibit increased fitness (Clegg and Allard, 1973; Loveless and Hamrick, 1984; Mitton, 1989; Mopper *et al.*, 1991; Strauss and Karban, 1994; Richards, 1997). Many species combine the advantages of both reproductive strategies, exhibiting a mixed mating system (Grant, 1975) while others, and their hybrid derivatives, have replaced sexual reproduction with asexual means (Rutishauser *et al.*, 1969; Stebbins, 1971; Hignight *et al.*, 1991).

Many complex and diverse reproductive strategies exist within the Australian flora. Some of the more intriguing strategies include elimination of selfed progeny through recessive lethals (Banyard and James, 1979; Coates and James, 1979; James, 1979; Coates, 1981), and clonality (Kennington *et al.*, 1996). Although unusual reproduction mechanisms, such as adventitious embryony, apospory and diplospory, exist within

Rutaceae (Grant, 1981; Torres, 1989; Richards, 1997), the reproductive strategy(ies) of most Australian family members is unknown.

Plant reproductive strategies can be determined using both traditional techniques and molecular markers. Levels of natural seed set and that following manipulation, provide measures of fecundity, reproductive isolation, compatibility systems and selfing/outcrossing rates (Dafni, 1992). Pollen and stigma biology and interaction can further elucidate reproductive strategy(ies) (Dafni, 1992). Plant mating systems can be assessed using molecular markers such as allozymes. Although several fertility measures and estimates have been developed, the mixed mating system model is commonly employed (Brown *et al.*, 1989; Barrett and Eckert, 1990; Brown, 1990). This model calculates both random mating and self-fertilisation, based on the joint behaviour of genotypes at one or more marker loci through one mating event (Clegg, 1980; Brown *et al.*, 1985; Brown *et al.*, 1989; Barrett and Eckert, 1990; Brown, 1990). It assumes that mating events are due to random outcrossing (with probability t) or self-fertilisation (with probability $s = 1 - t$), gene frequency distribution between pollen is identical over all maternal plants, the rate of outcrossing is independent of maternal genotype and there is no selection of markers between fertilization and progeny census (Clegg, 1980; Hamrick, 1989).

Aims of this investigation

The reproductive strategy(ies) employed by *G. verrucosa* is unknown. An understanding of the reproductive mechanisms is considered essential for interpreting patterns of genetic and morphological variation. The presence of reproductive isolating mechanisms would also provide evidence of possible divergence between the various forms. This investigation aimed to determine the reproductive strategy(ies) employed by *G. verrucosa*. More specifically, it sought to ascertain whether the three forms share a common reproductive strategy, and whether reproductive barriers exist between the forms. An assessment of the mating system is also undertaken using allozyme markers.

Materials and methods

Sampling strategy

G. verrucosa has a wide distribution with three forms readily distinguishable (Chapter 1). Experiments were conducted on five populations chosen to ensure that the three forms and their distributions were represented in these analyses. The large form was represented by the Meanarra Hill 1 and Geraldton populations, the intermediate form by the Hutt River 2 population, and the small form by the Arinya and Coorow 1 populations. This sampling strategy included the most northerly and southerly located populations (Chapter 1, Figure 1.6). Plants (4-6) were randomly selected for experimental manipulation within each population.

Floral biology

Flowering events were observed both in the field and laboratory. In the field, several flowers were tagged on 3-4 plants in each population and observed from early morning to late afternoon. Events from flower opening to closing were observed, recorded and photographed. Flower samples transported to the laboratory were allowed to open naturally and were similarly treated.

Flowering phenology

Observations of flowering were undertaken at all study sites during 1995 and 1996. Due to the large distances between field sites, it was not always possible to determine the exact date of flowering and where possible, approximate dates were obtained with the assistance of landowners.

Pollen viability

Flowers collected from each population were placed in sealed plastic bags, stored at 4°C and transported to the laboratory where they were allowed to open naturally, and the anthers to dehisce. An initial trial was undertaken to assess the germinability of pollen from a single population. Sucrose solutions of 5, 10, 20, 30, 40 and 50% (w/v) incorporating 50% H_3BO_3 and 50% $Ca(NO_3)_2$ were prepared and droplets placed in small petri dishes (Dafni, 1992). Fresh pollen was added to each drop, inverted and

left overnight. The droplets were stained with methylene blue and examined for germination. No germination was observed in any solution and the experiment was discontinued.

Single anthers from 4-5 flowers of each plant were bulked and divided into two samples for staining. Pollen grains of the first sample were stained with Alexander's stain (Alexander, 1980) and two hundred scored as either red (viable) or green (non-viable). The second sample was stained with fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970), left for 10 min in a humid environment and then viewed by fluorescence microscopy using a violet exciter filter (λ 395-415 nm). Five hundred pollen grains were scored as either bright (viable), dullish (viable) or non-stained (non-viable).

Stigma receptivity

Stigmas (4-6 mm) from flowers at various stages of opening were examined both in the field and laboratory to determine receptivity. Pistils were detached from the ovary and placed in 3% hydrogen peroxide (H_2O_2) (Galen and Kevan, 1980; Dafni, 1992). The size, abundance and distribution of bubbles from the stigma were qualitatively assessed.

Pollen tube growth

To determine whether pollen tube growth was influenced by pollen source, a small number of flowers were hand pollinated with pollen from either within (Xsame) or outside the population with another form (Xother) (Table 3.1). These were collected 24 h after pollination, fixed in formalin:proprionic acid:ethanol (40:50:10 v/v) and stored at 4°C in 70% ethanol (EtOH). Pistils were later autoclaved in sodium sulphate (50 g/L) for 30 min at 121°C, stained with decolourised aniline blue for 24 hours and viewed under fluorescent light using a violet exciter filter (λ 395-415 nm) (Martin, 1958; Dafni, 1992). Styles were examined for pollen germination, pollen tube growth and abnormalities.

Table 3.1: Pollen source for the Xother pollination treatment.

Population	Maternal form	x	Paternal form	Pollen source
Arinya	*Small*	x	*Large*	Meanarra Hill 1
Coorow 1	*Small*	x	*Large*	Geraldton
Hutt River 2	*Intermediate*	x	*Large*	Pleshkes Large
Geraldton	*Large*	x	*Small*	Indarra Reserve
Meanarra Hill 1	*Large*	x	*Intermediate*	Nearby population

Pollination experiments

Since propagation of *G. verrucosa* was difficult and growth of germinants slow, the pollination experiments were conducted in the field during 1995 and 1996. This also provided an opportunity to assess seed set under natural conditions. In 1995, the protocol to remove untreated buds, flowers and fruits to allow easy identification of treated flowers elicited a wounding response, and all flowers abscised, necessitating repetition in 1996.

Large distances between field sites also presented logistical problems when determining the commencement of flowering. An unexpected short flowering season at Hutt River 2 in 1996 meant flowering was well advanced when experiments commenced, limiting the number of flowers available for treatment. At Coorow 1 and Arinya, no other forms occurred in the vicinity and cross pollination with a different form (Xother) was undertaken using pollen collected from the Geraldton population during a previous field trip. To maintain viability for the Arinya and Coorow 1 experiments, pollen was stored in plastic vials under dark, cool conditions and viability assessed by fluorescein diacetate prior to use.

The successful 1996 protocol involved identifying treated flowers within an inflorescence with colour-coded cotton thread tied around individual peduncles. Newly opened flowers with undehisced anthers were manipulated as outlined in Table 3.2. Pollen sources for the Xother pollination treatment are listed in Table 3.1. Hand pollination was undertaken by wiping dehiscing anthers over stigmas until pollen was clearly visible. At Arinya and Coorow 1, the stored pollen was transferred

using a fine hair brush. Potential pollinators were excluded by covering inflorescences with nylon stocking bags and applying a clear sticky substance (Bird-Off®, Rentokil Pty Ltd) around the stem below the tie. To prevent seed from treated and untreated flowers mixing, the fruit of treated flowers were collected before maturity and seed set assessed. Although the fruit were green, seeds (3-4 mm) were well formed and empty carpels clearly distinguishable.

Table 3.2: Pollination treatments undertaken on *G. verrucosa* populations (after Dafni, 1992).

Code	Test	Treatment	Comments
Open	Open-pollination	Untreated, unbagged	To evaluate pollination under natural conditions.
Selfed	Self-pollination	Untreated, bagged	To evaluate levels of self-pollination and the effects of pollinator exclusion.
Xsame	Cross-pollination within populations	Emasculated, pollen from within site	To determine whether barriers to fertilisation exist between plants within a population.
Xother	Cross-pollination between forms	Emasculated, pollen from outside site	To determine whether barriers to fertilisation exist between forms.
Pollen Excl.	Non-sexual	Emasculated, bagged	To evaluate whether non-sexual reproduction occurs, excluding pollination-promoted hormonal activity producing seed set without fertilisation.

One-way Analyses of Variance (ANOVA) were conducted using SuperANOVA (Abacus Concepts, Inc., Berkeley, CA, 1989) to determine whether treatments varied within and between populations. Means were compared using Tukey's Compromise (Chapter 2) performed at $\alpha = 0.05$. To remove any bias created by the large differences between the open-pollinated and all other treatments, the former values were removed, the ANOVA performed again and contrasts between means re-assessed.

To assess the effects of the cross-pollination treatments, an index of self-incompatibility (ISI) (Zapata and Arroyo, 1978; Kenrick, 1986) was calculated for both the Xsame and Xother treatments:

$$ISI = \frac{\text{fruit set from self-pollination}}{\text{fruit set from cross-pollination}}$$

This calculation indicates the compatibility of pollen to set seed. Values of <0.2 indicate self-incompatibility, 0.2-0.9 partial self-compatibility, 1.0 self compatibility and >1.0 preferential self-pollination (Zapata and Arroyo, 1978; Kenrick, 1986).

Pollinators

Day-time and nocturnal opportunistic observations were undertaken during field visits. Type, and activity of invertebrates, were noted.

Mating system

Seeds from 10-15 plants within the five experimental populations were collected from Arinya, Hutt River 2 and Meanarra Hill 1 in 1995, and, due to poor seed set in that year, in 1996 at Coorow 1 and Geraldton. Seeds (10-12) from each plant were germinated for approx. 14 days under sterile conditions and seedlings frozen at -72°C until allozyme analysis was undertaken. Germination was sporadic, despite smoke treatment being considered an effective germination promoter (Dixon *et al.*, 1995), reducing the number of seedlings available for analysis.

Seedlings were ground with 100 mL of modified extraction buffer (Systma and Schaal, 1985), centrifuged for approx. 1.5 min and 12 mL of extract loaded onto pre-soaked cellulose acetate plates (Titan III, Helena Laboratories, Beaumont, TX). The plates were transferred to running tanks maintained at 5°C and electrophoresed at 5 mAmps/gel. All protocols are detailed in Chapter 4.

All progeny were initially screened for the aspartate aminotransferase (*Aat*) and leucine aminopeptidase (*Lap*) enzyme systems since these were variable and readily scorable within and between populations (Chapter 4).

Very low levels of polymorphism were detected between progeny within most of the populations. The investigation was subsequently limited to the Arinya population, representing the small form, and Meanarra Hill 1, representing the large. A further examination of other enzyme systems for these populations also revealed little to no polymorphism, limiting the analysis to the *Lap* system in progeny from the Meanarra Hill 1 population. Progeny arrays (10-12) for each of the 11 plants were scored and a single-locus estimate of the mixed mating model for the *Lap* locus was obtained using the computer program developed by K. Ritland (Ritland and Jain, 1981).

Results

Floral biology

G. verrucosa produces inflorescences of bright yellow flowers clustered at the ends of branches (Chapter 1, Figure 1.3). Several large yellow bracts surround smaller, similar coloured sepals with shorter, thinner and darker yellow petals inside. Colour changes from yellow to red were observed in some populations although it was not determined whether this was a response to aging, environment or pollination. Inflorescences form a complex cluster composed of flowers at various developmental stages, from buds through to fruit. The number of flowers in each inflorescence is population-dependent with the large form having larger and more flowers per inflorescence, while the small form produces smaller and fewer flowers (Figure 3.1).

Observations suggest *G. verrucosa* is a facultative selfer. Mature buds generally open during early morning, although on overcast days, opening may be delayed. Mature buds open within a short space of time and flowers remain open for a single day. The sequence of flowering events begins when the bud bursts open with some force, splaying petals outwards; sepals are already extended outwards at this time (Figure 3.2, Diagrams 1-3). Upon opening, the long style, which is bent over in the closed bud, is

released and straightens. The style and stamens then move away from each other. By mid-morning when the style and anthers are furthermost from each other (Diagram 4), the pollen is released, limiting the possibility of self pollen falling on the stigma. After midday the style and stamens begin to move towards each other and by mid-afternoon, are close together (Diagram 5). The petals begin to close in the late afternoon, enclosing both pistil and stamens (Diagram 6). Occasionally a flower closes but with its style trapped on the outside; these may represent buds which fail to fruit.

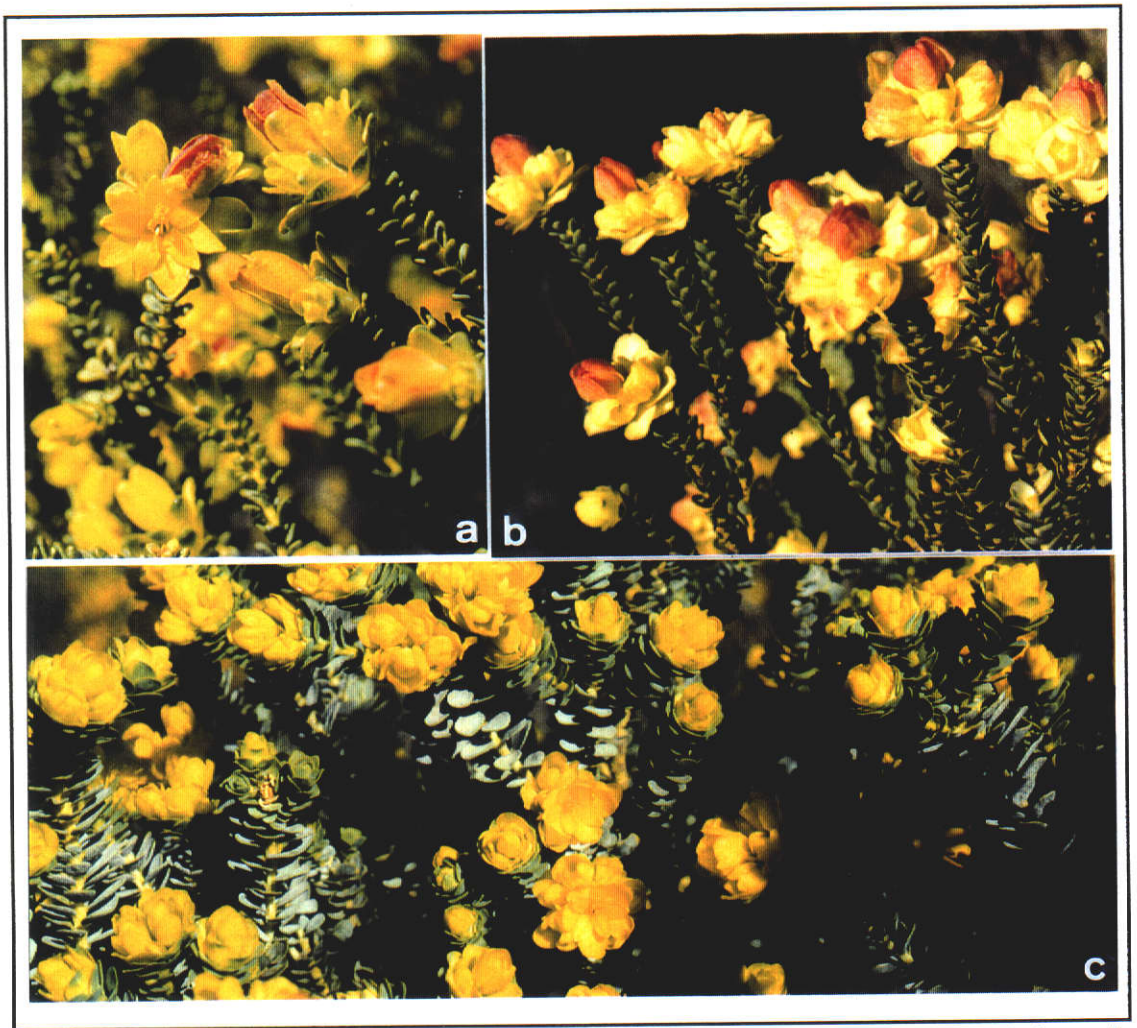


Figure 3.1: Flowers of the three *G. verrucosa* forms. a, Coorow 1 (small form); b, Ajana West 1 (intermediate form); c, Red Bluff (large form).

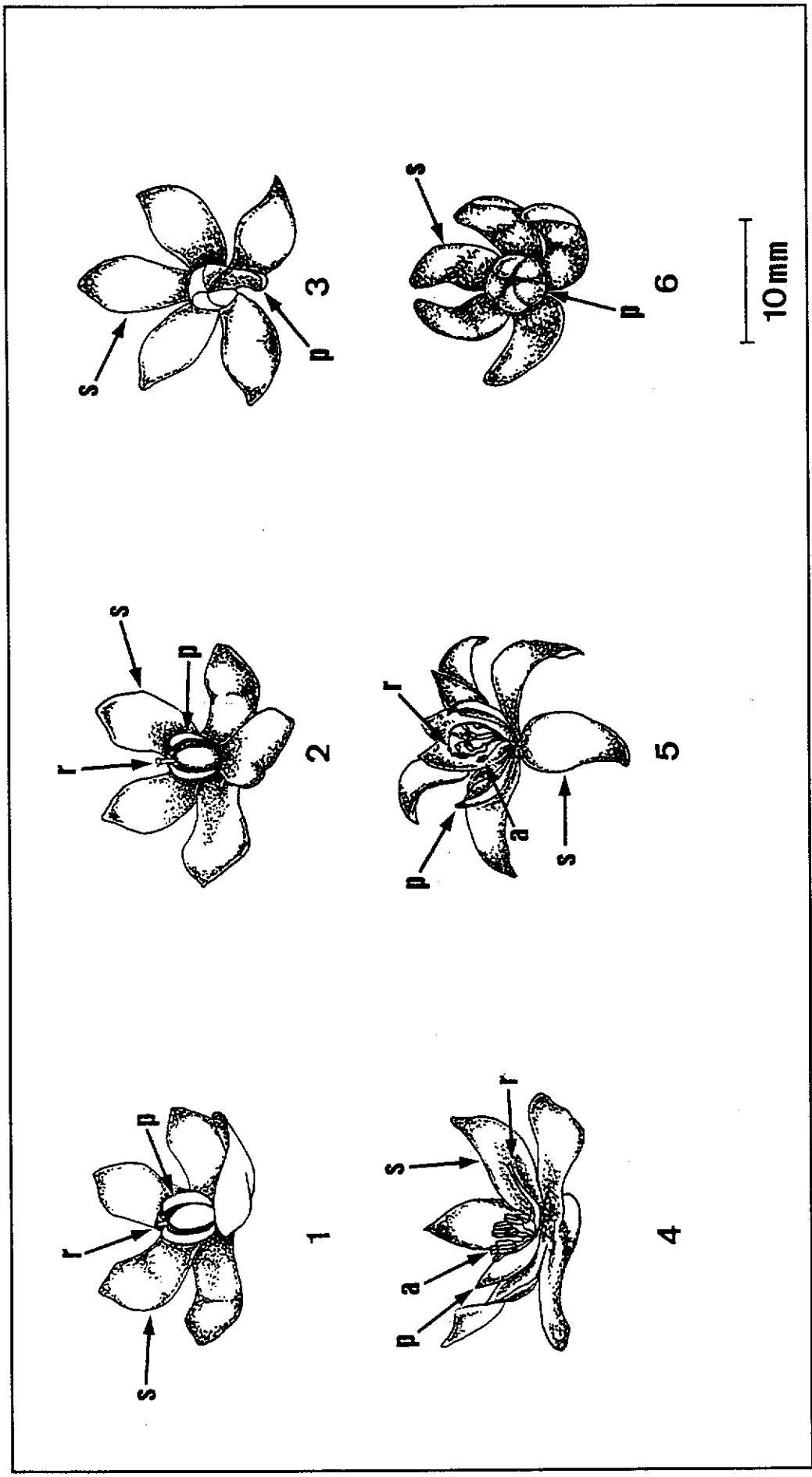


Figure 3.2: Flowering stages in *G. verrucosa* (drawings by E. Ladhams). Explanations outlined in "Floral biology". a, anthers; p, petals; r, style; s, sepals.

Flowering phenology

Although field observations were made on all populations, the five experimental populations were observed most closely. Commencement and length of flowering differed between populations and years (Table 3.3). Flowering commenced as early as May or as late as September and generally extended for 2-4 months depending on the population. In 1996, the flowering season of most populations was considerably reduced.

Table 3.3: Flowering seasons for all *G. verrucosa* populations over two years. Populations used for reproductive experiments in bold. Number of months in parentheses.

		Flowering season	
Form	**Population**	**1995**	**1996**
Small	**Arinya**	**Sept - Oct (2)**	**Aug - Nov (4)**
	Coorow 1	**Aug - Oct (3)**	**Sept - Nov (3)**
	Coorow 2	Aug - Oct (3)	Sept - Nov (3)
	Indarra	July - Oct (4)	Aug - Oct (3)
	Maiseys	Oct - Nov * (2?)	Aug - ? (?)
	Pleshkes Small	May - Aug (4)	May - Aug (4)
Intermediate	Ajana West 1	June - Sept (4)	July - Aug (2)
	Ajana West 2	June - Sept (4)	July - Aug (2)
	Hawks Head	June - Sept (4)	July - Aug (2)
	Hutt River 1	May - Aug (4)	May - July (3)
	Hutt River 2	**May - Aug (4)**	**May - July (3)**
	Nanns	June - Sept (4)	July - Aug (2)
	West Binnu	July - Sept (3)	July - Aug (2)
Large	**Geraldton**	**July - Oct (4)**	**Aug - Oct (3)**
	Meanarra Hill 1	**June - Sept (4)**	**July - Aug (2)**
	Meanarra Hill 2	June - Sept (4)	July - Aug (2)
	Pleshkes Large	June - Sept (4)	July - Sept (3)
	Red Bluff	June - Sept (4)	July - Aug (2)
Transect	Pleshkes Hybrid	June - Sept (4)	July - Sept (3)

* Population located in October 1995.

Pollen viability

A suitable medium for germinating *G. verrucosa* pollen was not found and viability was assessed using Alexander's and fluorescein diacetate stains only. Aborted and non-viable pollen stained with Alexander's was easily distinguished by colour, size and shape. Fluorescein diacetate produced three fluorescence reactions - bright, dullish and translucent. The translucent pollen grains were smaller, misshapen and regarded as

inviable. Although the differential staining between bright and dullish may have reflected the loss of viability, combined scores resulted in values close to those obtained with Alexander's stain. All populations exhibited high pollen viability values for both stains (Figure 3.3).

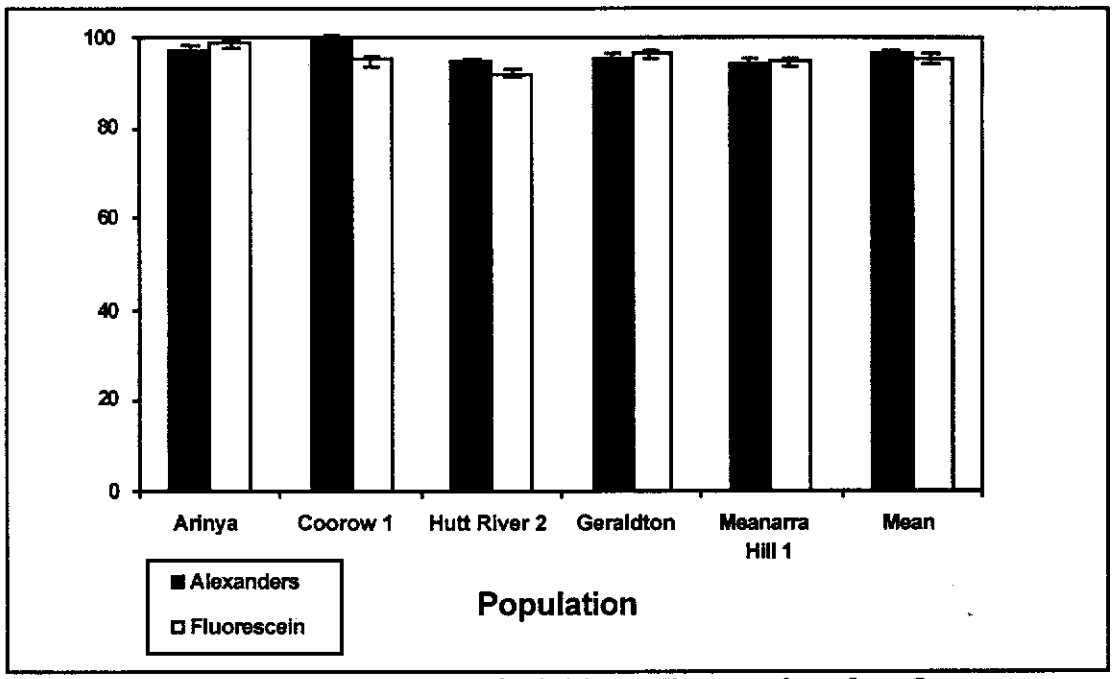


Figure 3.3: Mean percentage of viable pollen grains for *G. verrucosa* populations. Bars indicate standard errors.

Stigma receptivity

Stigma receptivity was weak when buds were green, but increased with maturity; receptivity was maintained well after anthesis, even as flowers were closing (Table 3.4).

Table 3.4: Stigma receptivity for *G. verrucosa* buds and flowers.

Flower stage	Observations
1. Unopened bud, green	• Small bubbles produced slowly from surface
2. Unopened bud, green-yellow	• Small bubbles produced slowly from surface
3. Unopened bud, yellow	• Few, larger bubbles produced from surface
4. Open flower, undehisced anthers	• Larger bubbles produced from surface
5. Open flower, 50% anthers undehisced	• Larger bubbles produced from surface
6. Open flower, all anthers dehiscing	• Larger bubbles produced faster from surface
7. Closing flower, all anthers dehisced	• Larger bubbles produced faster from surface

Pollen tube growth

All pollen tubes grew in the stigmatic tissue towards the ovary whether the flower was pollinated with Xsame or Xother pollen and no abnormal pollen tube growth was observed. Observations were reduced at Meanarra Hill 1 and Arinya, where little to no pollen had adhered to some of the stigmas but at Geraldton pollination appeared successful and good pollen tube growth was observed. While pollen tube growth at Geraldton and Meanarra Hill 1 extended the length of the style, at Arinya growth was not as pronounced. Too few flowers were available at Coorow 1 and Hutt River for this experiment.

Pollination experiments

Total seed set for all pollination treatments ranged from 518 in open-pollinated flowers to 10 in flowers where pollen was excluded (Figure 3.4). Open-pollinated flowers set significantly more seed than any other treatment, while similar levels of seed set were observed in self- and cross-pollinated flowers.

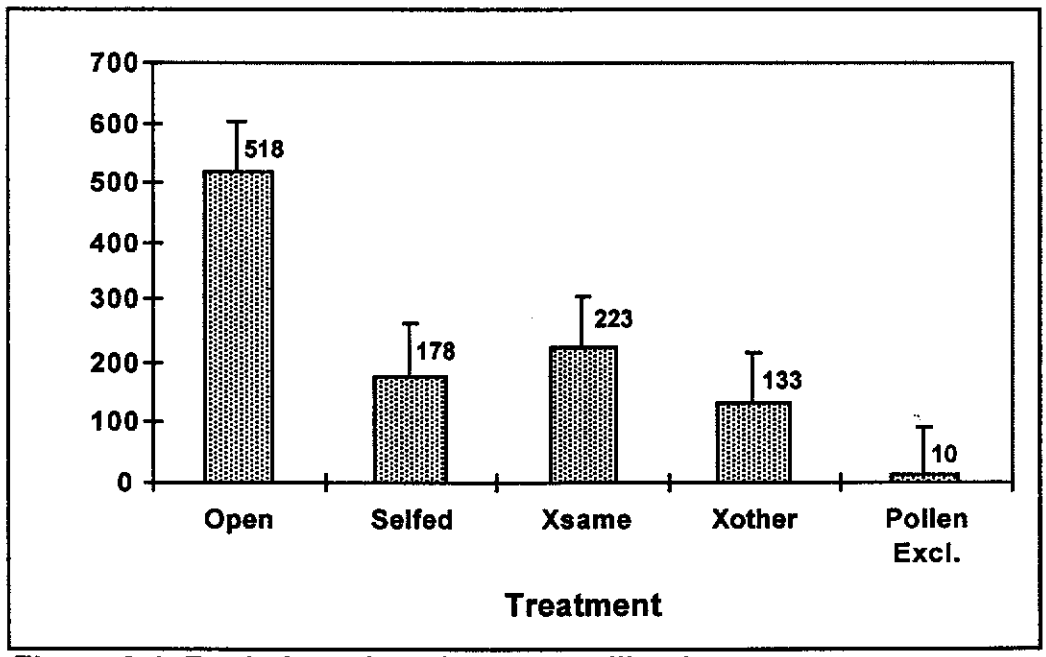


Figure 3.4: Pooled seed set for each pollination treatment. Total seed set shown; bars indicate standard error. Treatments outlined in Table 3.2.

Mean seed set per flower was lower than anticpated. Although *G. verrucosa* flowers can potentially set 5 seeds per fruit, the mean seed set by open-pollinated flowers was highest at 2.5 seeds per flower; this was still only half of the maximum possible (Table 3.5). All other treatments set less than two seeds per flower and ranged from 21-33% of the potential seed set.

Table 3.5: Mean and percentage of potential seed set per flower for all pollination treatments. Treatments outlined in Table 3.2.

	Treatment				
Seed set	**Open**	**Selfed**	**Xsame**	**Xother**	**Pollen Excl.**
Mean	2.50	1.41	1.66	1.09	0.17
% potential	50%	28%	33%	21%	<1%

The ISI for pollination within populations (Xsame) was 0.80, suggesting partial self-compatibility. Pollination using pollen sources from outside populations (Xother) produced an ISI of 1.34, indicating preferential self-pollination (Zapata and Arroyo, 1978; Kenrick, 1986).

Pollination success within and between populations

Pollination treatments exhibited little variation between plants within populations (Table 3.6). No significant differences were observed for any pollination treatment at Arinya and Coorow 1. Some differences occurred in the Selfed, Xsame and Xother pollination treatments at Hutt River 2, the open-pollination treatment at Meanarra Hill 1 and the Open and Xsame treatments at Geraldton. The Arinya and Coorow 1 population set a small amount of seed despite pollen exclusion (Pollen Excl.).

As there was little variation in seed set between plants within populations, data were pooled to establish whether treatments differed within populations, and to look for evidence of underlying trends. Populations responded differently to the treatments (Table 3.7a). All populations except Geraldton, set significantly more open-pollinated seed than for any other treatment. At Geraldton, open-pollinated seed set was comparable to that

of both cross-pollination treatments. No variation occurred between the remaining treatments at Arinya and Meanarra Hill 1, while at Coorow 1, the selfed and Xsame treatments produced more seed than the Xother and pollen exclusion treatments. Self-pollination at Hutt River 2 produced more seed than both cross-pollination treatments; insufficient flowers were available for the pollen exclusion treatment at this population. Removing the open-pollinated values from the analysis altered these patterns at Hutt River 2 only, where treatments were no longer significantly different (Table 3.7b).

Comparisons of treatments between populations highlighted several important differences (Table 3.8). Open-pollination seed set at Geraldton and Meanarra Hill 1 was considerably lower than in the other populations, while significantly more ($\alpha = 0.05$) self-pollinated seed was set at Hutt River 2. The Xsame treatment produced similar levels of seed set between Coorow 1 and Geraldton and between Arinya and Meanarra Hill 1 with intermediate levels set at Hutt River 2. Low seed set occurred following the Xother treatment at Arinya, Coorow 1 and Meanarra Hill 1.

Table 3.6: Tukey's Compromise comparison of mean seed set per flower for each pollination treatment between plants within each population. Same letter indicates no significant difference ($\alpha = 0.05$). Standard error in parentheses. Treatments outlined in Table 3.2. - , insufficient flowers for treatment.

			Treatment									
Form	Population	PI	*n*	Open	*n*	Selfed	*n*	Xsame	*n*	Xother	*n*	Pollen excl.
Small	Arinya	A	7	3.14^{a} (0.67)	5	0.00^{a} (0.00)	7	0.86^{a} (0.71)	5	0.00^{a} (0.00)	2	0.00^{a} (0.00)
		B	4	4.25^{a} (0.25)	3	0.00^{a} (0.00)	-	-	-	-	-	-
		C	7	3.29^{a} (0.36)	4	2.00^{a} (0.91)	7	1.14^{a} (0.63)	7	0.00^{a} (0.00)	2	2.00^{a} (2.00)
		D	4	4.00^{a} (0.41)	4	1.50^{a} (0.96)	4	0.00^{a} (0.00)	4	1.00^{a} (1.00)	1	4.00^{a} (0.00)
		E	5	2.40^{a} (0.51)	5	0.00^{a} (0.00)	4	0.33^{a} (0.21)	4	0.00^{a} (0.00)	1	0.00^{a} (0.00)
		F	6	3.83^{a} (0.31)	4	2.00^{a} (0.91)	5	1.14^{a} (0.63)	5	0.00^{a} (0.00)	4	0.00^{a} (0.00)
	Coorow 1	1	5	4.20^{a} (0.37)	3	3.00^{a} (1.00)	3	4.00^{a} (0.41)	3	0.00^{a} (0.00)	5	0.00^{a} (0.00)
		2	4	4.25^{a} (0.25)	4	3.25^{a} (0.85)	4	2.50^{a} (0.50)	4	0.00^{a} (0.00)	2	0.00^{a} (0.00)
		6	2	3.50^{a} (0.50)	1	0.00^{a} (0.00)	1	3.00^{a} (0.00)	1	0.00^{a} (0.00)	2	0.50^{a} (0.50)
		A	12	3.83^{a} (0.24)	11	1.46^{a} (0.53)	10	1.58^{a} (0.36)	10	0.20^{a} (0.20)	12	0.08^{a} (0.03)
		B	3	4.33^{a} (0.67)	2	0.50^{a} (0.50)	-	0.50^{a} (0.50)	-	-	1	0.00^{a} (0.00)
		X	5	4.20^{a} (0.49)	4	0.75^{a} (0.75)	5	2.50^{a} (0.76)	5	0.00^{a} (0.00)	6	0.00^{a} (0.00)
Intermediate	Hutt River 2	3	6	4.33^{a} (0.33)	4	3.75^{a} (0.63)	4	0.00^{b} (0.00)	4	0.00^{b} (0.00)	-	-
		5	5	4.00^{a} (0.32)	6	4.33^{a} (0.33)	2	0.00^{b} (0.00)	4	0.00^{b} (0.00)	-	-
		8	2	4.50^{a} (0.50)	1	4.00^{a} (0.00)	1	4.00^{a} (0.00)	1	5.00^{a} (0.00)	-	-
		A1	4	5.00^{a} (0.00)	3	1.33^{ab} (1.33)	2	3.00^{ab} (2.00)	4	4.25^{a} (0.48)	-	-
		A2	5	3.60^{a} (0.68)	2	4.50^{a} (0.50)	2	5.00^{a} (0.00)	2	4.00^{a} (0.00)	-	-
		N3	5	4.80^{a} (0.20)	3	0.00^{b} (0.00)	4	0.00^{b} (0.00)	4	0.00^{b} (0.00)	-	-
Large	Geraldton	3	15	1.73^{b} (0.37)	14	1.29^{a} (0.49)	16	2.20^{ab} (0.45)	16	1.69^{a} (0.38)	10	0.00^{a} (0.00)
		5	11	0.82^{b} (0.35)	14	1.29^{a} (0.49)	7	4.00^{a} (0.37)	7	2.71^{a} (0.87)	8	0.00^{a} (0.00)
		6	9	1.22^{b} (0.55)	10	0.90^{a} (0.43)	7	0.78^{b} (0.36)	7	1.57^{a} (0.65)	8	0.00^{a} (0.00)
		10	5	3.60^{a} (0.93)	7	1.00^{a} (0.66)	4	2.17^{ab} (0.48)	4	2.25^{a} (1.11)	-	-
		19	7	4.29^{a} (0.42)	8	0.00^{a} (0.00)	10	2.86^{a} (0.46)	10	2.00^{a} (0.52)	7	0.00^{a} (0.00)
	Meanarra Hill 1	11	9	3.00^{a} (0.47)	8	0.00^{a} (0.66)	12	0.33^{a} (0.33)	12	0.83^{a} (0.29)	6	0.00^{a} (0.00)
		13	6	2.33^{ab} (0.33)	7	0.00^{a} (0.00)	-	0.00^{a} (0.00)	-	-	6	0.00^{a} (0.00)
		15	9	1.11^{b} (0.42)	10	0.40^{a} (0.49)	6	1.14^{a} (0.71)	6	0.17^{a} (0.17)	8	0.00^{a} (0.00)
		A	6	3.00^{a} (0.45)	8	0.00^{a} (0.43)	4	1.00^{a} (0.32)	4	0.00^{a} (0.00)	-	-

Table 3.7a: Tukey's Compromise comparison of mean seed set per flower for pollination treatments within populations. Same letter indicates no significant difference (α = 0.05). Treatments outlined in Table 3.2. -, insufficient flowers. *N*, number.

		Treatment									
Form	Population	*n*	Open	*n*	Selfed	*n*	Xsame	*n*	Xother	*n*	Pollen Excl.
Small	Arinya	33	3.42^{a} (0.21)	25	0.88^{b} (0.29)	30	0.80^{b} (0.26)	25	0.16^{b} (0.16)	10	0.80^{b} (0.53)
	Coorow 1	31	4.03^{a} (0.15)	25	1.68^{b} (0.36)	27	2.19^{b} (0.29)	23	0.09^{c} (0.09)	28	0.07^{c} (0.05)
Intermediate	Hutt River 2	27	4.33^{a} (0.18)	19	3.05^{b} (0.46)	15	1.33^{c} (0.56)	19	1.58^{c} (0.50)	-	-
Large	Geraldton	47	2.00^{a} (0.27)	53	0.98^{b} (0.22)	46	2.37^{a} (0.25)	44	1.96^{a} (0.26)	33	0.00^{c} (0.00)
	Meanarra Hill 1	30	2.30^{a} (0.26)	33	0.12^{b} (0.12)	30	0.53^{b} (0.21)	22	0.50^{b} (0.18)	20	0.00^{b} (0.00)

Table 3.7b: Tukey's Compromise comparison of mean seed set per flower for pollination treatments within populations, open-pollination treatment excluded. Same letter indicates no significant difference (α = 0.05). Treatments outlined in Table 3.2. -, insufficient flowers. *N*, number.

		Treatment							
Form	Population		Selfed	*n*	Xsame	*n*	Xother	*n*	Pollen Excl.
Small	Arinya	25	0.88^{a} (0.29)	30	0.80^{a} (0.26)	25	0.16^{a} (0.16)	10	0.80^{a} (0.53)
	Coorow 1	25	1.68^{a} (0.36)	27	2.19^{a} (0.29)	23	0.09^{b} (0.09)	28	0.07^{b} (0.05)
Intermediate	Hutt River 2	19	3.05^{a} (0.46)	15	1.33^{a} (0.56)	19	1.58^{a} (0.49)	-	-
Large	Geraldton	53	0.98^{b} (0.22)	46	2.37^{a} (0.25)	44	1.96^{a} (0.26)	33	0.00^{c} (0.00)
	Meanarra Hill 1	33	0.12^{a} (0.12)	30	0.53^{a} (0.21)	22	0.50^{a} (0.18)	20	0.00^{a} (0.00)

Table 3.8: Tukey's Compromise comparison of mean seed set per flower for pollination treatments between populations. Same letter indicates no significant difference (α = 0.05). Treatments outlined in Table 3.2. -, insufficient flowers. *N*, number.

		Treatment									
Form	Population	*n*	Open	*n*	Selfed	*n*	Xsame	*n*	Xother	*n*	Pollen Excl.
Small	Arinya	33	3.42^{b} (0.21)	25	0.88^{bc} (0.29)	30	0.80^{b} (0.26)	25	0.16^{b} (0.16)	10	0.80^{a} (0.53)
	Coorow 1	31	4.03^{ab} (0.15)	25	1.68^{b} (0.36)	27	2.19^{a} (0.29)	23	0.09^{b} (0.09)	28	0.07^{b} (0.05)
Intermediate	Hutt River 2	27	4.33^{a} (0.18)	19	3.05^{a} (0.46)	15	1.33^{ab} (0.56)	19	1.58^{a} (0.49)	-	-
Large	Geraldton	47	2.00^{c} (0.27)	53	0.98^{bc} (0.22)	46	2.37^{a} (0.25)	44	1.96^{a} (0.26)	33	0.00^{b} (0.00)
	Meanarra Hill 1	30	2.30^{c} (0.26)	33	0.12^{c} (0.12)	30	0.53^{b} (0.21)	22	0.50^{b} (0.18)	20	0.00^{b} (0.00)

The ISI values varied between populations (Table 3.9). The small form at Arinya and Coorow 1 was partially self-compatible when pollinated with Xsame pollen, but preferentially self-pollinating when pollinated with Xother pollen sourced from the large form. The intermediate form at Hutt River 2 was preferentially self-pollinating regardless of pollen source, while the large form at Geraldton and Meanarra Hill 1 were partially self-compatible irrespective of pollen source.

Table 3.9: Index of self-incompatibility (ISI) for *G. verrucosa* populations. ●, partially self-compatible; ◆, preferential self-pollination.

Treatment	Population	Form maternal	x	paternal	ISI	
Xsame	**Arinya**	*Small*	x	*Small*	0.92	●
	Coorow 1	*Small*	x	*Small*	0.78	●
	Hutt River 2	*Intermediate*	x	*Intermediate*	2.65	◆
	Geraldton	*Large*	x	*Large*	0.52	●
	Meanarra Hill 1	*Large*	x	*Large*	0.25	●
Xother	**Arinya**	*Small*	x	*Large*	5.50	◆
	Coorow 1	*Small*	x	*Large*	21.00	◆
	Hutt River 2	*Intermediate*	x	*Large*	1.77	◆
	Geraldton	*Large*	x	*Small*	0.66	●
	Meanarra Hill 1	*Large*	x	*Intermediate*	0.36	●

Pollination success within and between forms

Although populations responded to the various pollination treatments on an individual basis, some form-specific trends were apparent. To investigate this further, data for the different forms were pooled and compared (Figure 3.5). The large form set considerably less open-pollinated seed than either the small and intermediate forms. Selfing was highest in the intermediate form and lowest in the large form, while all forms set similar levels of seed following Xsame pollination. The Xother treatment elicited similar levels of seed set in the large and intermediate forms but significantly less seed in the small form. The small form also set some seed formed despite pollen exclusion.

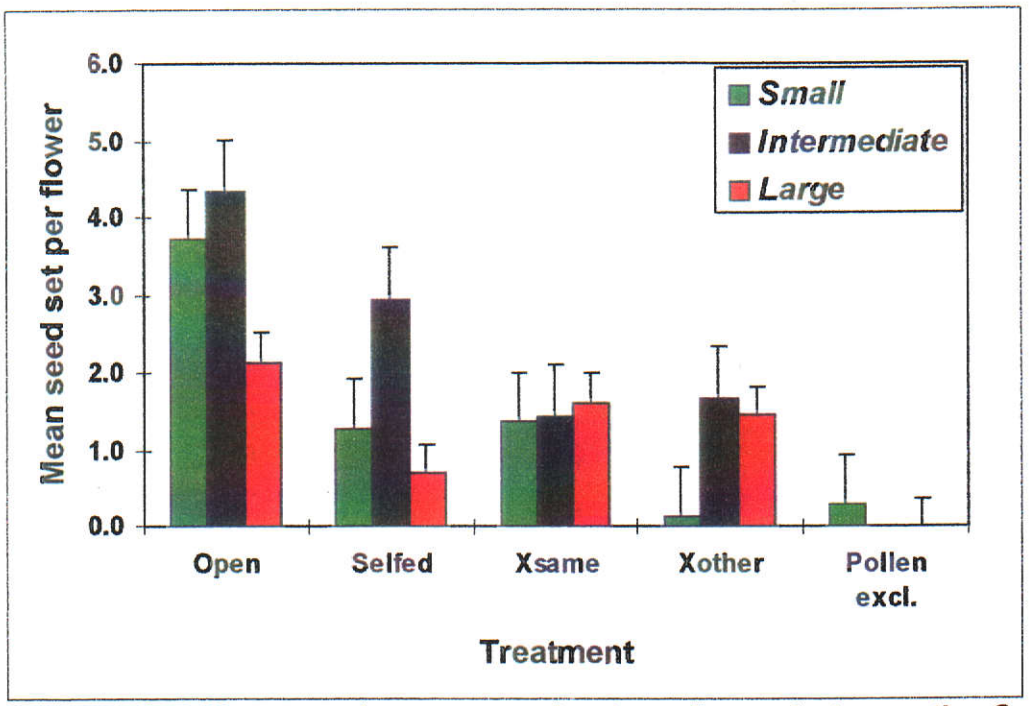


Figure 3.5: Differences in mean seed set per flower between the *G. verrucosa* forms for the pollination treatments. Bars indicate standard errors. Treatments outlined in Table 3.2.

Pollinators

No significant diurnal or nocturnal pollinators were observed visiting *G. verrucosa* plants during flowering. A small moth (Lepidoptera) was occasionally observed and the presence of larvae in flower buds suggested that these act as a nursery. Ants (Hymenoptera) were observed removing pollen on two plants at the Geraldton population. Although the insects did not appear to come into contact with the style, it is possible that some accidental pollination does occur. Small beetles (Coleoptera) were also observed during flowering. The small numbers of observed insects suggests none were significant pollinators of *G. verrucosa*.

Mating system

The lack of polymorphism detected in progeny from plants at the Arinya population and the low levels observed at Meanarra Hill 1 limited the mating system analysis. Allelic segregation was apparent within progeny at Meanarra Hill 1 (Figure 3.6). The estimate of outcrossing for this population was 0.418 (s.e. 0.087), indicating a mixed mating system with relatively

high levels of inbreeding within the population.

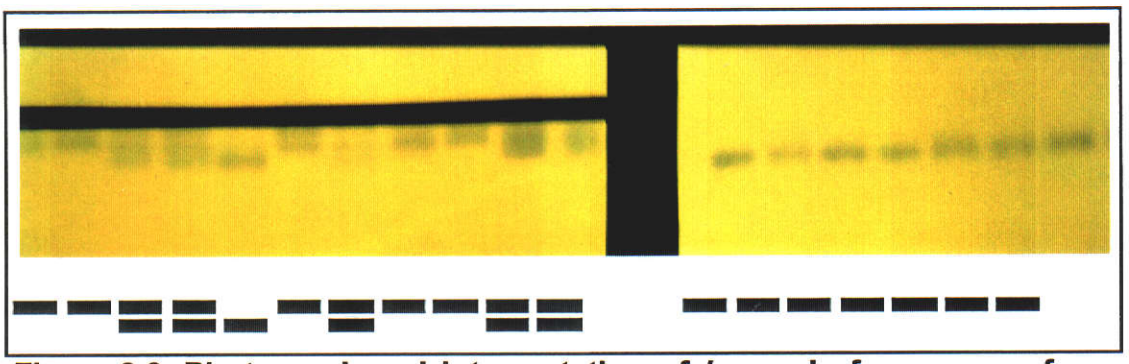


Figure 3.6: Photograph and interpretation of *Lap* gels for progeny from one Meanarra Hill plant (left) and one Arinya plant (right) showing segregation of alleles.

Discussion

The apparent absence of pollinators, stigma receptivity prior to anther dehiscence, and style/anther movement during flower opening and closing, suggest that *G. verrucosa* is a protogynous facultative selfer. Each form, however, responds differently to the various pollination treatments. The small form sets higher levels of open- and self-pollinated seed than the large form, similar levels when pollinated using other plants from within the population, and significantly less when pollinated from outside the population. The ISI values further contrast differences between the forms as well as highlighting the importance of pollen source on seed set.

Both the small and large forms are partially self-compatible when pollinated from within the population. The large form is also partially self-compatible following pollination with either small or intermediate form pollen from outside the population. In contrast, the small form is preferentially self-pollinating when pollinated with the large form. Interestingly, although pollination from outside both the large form populations elicited the same partially self-compatible response, more seed is set following pollination by the small form than by the intermediate form. Extremely poor seed set accompanies pollination of the small and intermediate forms with pollen from the large form.

The partial self-compatibility of the small form when crossed within populations, and preferentially self-pollinating when crossed from outside populations, strongly suggest this form is oriented towards selfing. The production of seed despite a non-preferred pollen source indicates that reproductive isolation is not complete and some ability to outcross is preserved. The similar levels of seed set by the large form, regardless of pollen source, are suggestive of a mixed mating system. The estimates of outcrossing (t) from the mating system analysis indicate that although outcrossing is occurring, selfing is significant within the Meanarra Hill population. This interpretation does, however, require further qualification due to the small sample size and lack of suitable polymorphic loci.

Differences in the timing and length of flowering between the different *G. verrucosa* forms also suggest differences in reproductive strategy. The small form generally flowers later than the other forms, and at Ajana West 2, where the small and intermediate forms co-occur, flowering in the latter form is finished prior to commencement in the small form. At Pleshkes, however, considerable overlap occurs in the flowering patterns of the co-occurring small and large forms. The proximity of these two forms provides an opportunity for gene exchange and introgression to occur (Chapter 6). The incorporation of genetic material from one form to another may account for the shift in flowering pattern observed in the small form at this population.

Plant breeding systems are thought to have evolved from obligate outbreeding to predominant self-fertilisation (Stebbins, 1957, 1970; Grant, 1975; Jain, 1976; Barrett and Shore, 1987). The chief barrier to such a shift is the presence of deleterious genes in many outcrossers which, upon selfing, can contribute to inbreeding depression. This can be circumvented, however, if self fertilisation occurs gradually through a series of mixed mating stages (Stebbins, 1957). In addition, highly fecund perennial plants, such as *G. verrucosa*, can better tolerate such reproductive shifts since the production of occasional weak or inviable zygotes does not seriously

impede overall reproductive success (Stebbins, 1957). It is also becoming apparent than reverse shifts of breeding strategy do occur and are known in the Western Australian flora (James, 1996). As such, any interpretation of evolutionary relationships should be cautious.

Shifts in reproductive strategy(ies) are often mediated through decreased flower size, alterations to floral morphology, reduced energetic cost per flower and decreased pollen:ovule ratios (Stebbins, 1970; Cruden, 1977; Schoen, 1977; Wyatt, 1984; Cruden and Lyon, 1985; Barrett and Eckert, 1990). Although relationships between reproductive strategy and flower size should be cautiously interpreted (Stebbins, 1970; Schoen, 1982), it is noteworthy that the small form of *G. verrucosa* is characterised by reduced floral attractants, while those of the mixed-mating large form are much larger and more attractive (Chapter 2).

The differences in reproductive strategy employed by the *G. verrucosa* forms suggest the large mixed-mating form is the progenitor to the small selfing form, with the intermediate form perhaps forming an evolutionary link. Responses elicited by this latter form to the various pollination treatments are, however, more indicative of hybridisation than of a transitional evolutionary stage. The intermediate form exhibits the highest levels of natural seeds set, 87% of that possible, significantly higher selfing rates than other forms, and a strong preference for self-pollination. This propensity towards selfing repudiates this form as a transitional stage where intermediate values between the small and large form would be expected, and a mixed-mating system more evident. A selfing mechanism in hybrid populations would ensure reproductive success and maintain hybrid fidelity following hybridisation and possible reproductive isolation from parental types.

Differences in breeding strategies themselves are not necessarily indicative of systematic separation. However, speciation often follows events which prevent gene flow and promote strong natural selection

(Grant, 1981; MacDonald *et al.*, 1987; Purdy *et al.*, 1994). *G. verrucosa* occurs in the transitional-rainfall zone (Chapter 1) which is characterised by the evolution of small, disjunct populations (Hopper, 1992). When pollen movement is limited, morphological and physiological traits which promote selfing may be favoured, particularly where plant density is low or pollinators scarce (Jain, 1976; Solbrig and Rollins, 1977; Lloyd, 1980; Schemske and Lande, 1985). Self-fertilisation is commonly associated with ecological radiation into temporary, pioneer habitats (Stebbins, 1957; Jain, 1976; Rick *et al.*, 1977; Solbrig and Rollins, 1977; Lloyd, 1980; Ramsey *et al.*, 1993). Many selfing populations also inhabit the geographically peripheral habitats of closely related outcrossing or predominantly outcrossing taxa (Rick *et al.*, 1977; Lloyd, 1980; Wyatt, 1984).

The peripheral distribution of the small selfing form of *G. verrucosa* to the large outcrossing form may be further evidence of a progenitor-derivative relationship between them. This form also occurs in drier, more marginal habitats and populations are considerably more disjunct than those of the two other forms (Chapter 1, Figure 1.6). A shift towards selfing in the small form would ensure reproductive success in a difficult and unpredictable environment, particularly given the periodic drought experienced in Mediterranean climates. Retaining some ability to outcross may be significant during range expansions and contractions facilitated by environmental fluctuation, and lead to gene exchange between populations and forms.

Although the pollination treatments produce distinct response patterns in the three forms, variability between populations within forms is also apparent. The comparable treatment responses within populations indicate factors influencing seed set are consistent. The variability observed between populations within the small and large forms probably reflect differences in environmental factors associated with a large distribution. In any given year the success of reproduction depends upon interactions between genetic variables and environmental factors (Clegg,

1980; Delouche, 1980; Stephenson, 1981; Barrett and Eckert, 1990), and plant breeding systems can vary between years, within species and between closely related taxa (Antonovics, 1968; Rick *et al.*, 1977; Schoen, 1982; Wyatt, 1984; Schemske and Lande, 1985; MacDonald *et al.*, 1987; Lyons and Antonovics, 1991; Ramsey *et al.*, 1993; Pickering, 1995).

Reproductive strategy(ies) differ in the three *G. verrucosa* forms. The large form employs a mixed-mating system while the small form has a selfing strategy. These forms probably represent different taxa with the large form being the progenitor to the small form. The intermediate form exhibits a strong self-compatible mechanism suggesting it may be the product of hybridisation between the small and large forms. Reproductive isolation following such events could prevent the production of viable offspring through outcrossing, either with other derived or with parental types. A selfing strategy, however, would be a useful mechanism for overcoming outcrossing barriers, particularly those associated with temporal separation or genomic incompatibility. Understanding the reproductive strategy(ies) employed by the three forms is important for interpreting the population genetic structure of, and genetic differentiation between the three *G. verrucosa* forms.

CHAPTER 4

Allozyme variation and population genetic structure

Introduction

Populations consist of individuals distributed throughout the geographic range of a species (Richardson *et al.*, 1986). The sedentary nature of plants tends to generate non-random clusters of genes and genotypes known as the 'genetic structure' of a population (Nei, 1975; Hamrick and Holden, 1979; Hamrick, 1982, 1989; Richards, 1997). This genetic structure exists both spatially and temporally (Levin and Kerster, 1974; Jain, 1975; Loveless and Hamrick, 1984), and variability may be distributed between geographically distinct populations, local groups and even at the microsite level (Loveless and Hamrick, 1984; Hamrick, 1989). Little is known about the population genetic structure of most plant species (Hamrick and Godt, 1996).

Population genetic structure reflects the interaction of many variables including selection, gene flow and isolation, as well as ecological variables which influence reproduction and dispersal (Hamrick, 1982; Cole and Biesboer, 1992; Carthew, 1993). In natural situations, gene frequencies may progressively diverge between populations and be reflected by either differences in gene frequency, or gene fixation (Bradshaw, 1975; Nei, 1975; Loveless and Hamrick, 1984). Geographic patterns and levels of genetic variation can be useful for evaluating modes of speciation and the underlying causes of morphological variation (Watson *et al.*, 1994).

Geographically restricted species are often characterised by lower genotypic diversity than more widely distributed taxa (Karron, 1987; Hamrick and Godt, 1989). Small population size, reduced gene flow, inbreeding, and founder effect all contribute to the lower genetic diversity of endemic taxa (Hamrick *et al.*, 1979; Hamrick and Godt, 1989; Coello *et al.*, 1993; Baskauf

et al., 1994; Purdy *et al.*, 1994; Watson *et al.*, 1994). Reduced habitat heterogeneity driving selection and adaptation may also influence genetic variability in endemic populations (Baskauf *et al.*, 1994). Some endemic taxa, however, exhibit high levels of variation (Hiebert and Hamrick, 1983; Gottlieb *et al.*, 1985; Karron, 1987; Karron *et al.*, 1988; Murawski and Bawa, 1994; Lewis and Crawford, 1995).

The Western Australian flora, particularly that of the southwest, is distinguished by high species diversity and endemism (Hopper, 1979; James and Hopper, 1981). Many species are rare and locally endemic, the product of a long and complex evolution that has generated many diverse genetic systems (Hopper, 1979). Some of the known mechanisms include intraspecific chromosome variation (James, 1965; Coates and James, 1979; James *et al.*, 1983), polyploidy (Bousfield and James, 1976; Hopper *et al.*, 1987), dysploidy (James, 1981; Stace, 1995), clonality (Kennington *et al.*, 1996), and complex hybridity (James, 1970; James *et al.*, 1983).

Although endemic taxa often exhibit lower levels of genetic diversity, it is generally apportioned in much the same manner as that of more widespread species (Hamrick and Godt, 1989). Among Western Australian endemics, the distribution of genetic diversity is not always well correlated with endemism. For example, while some rare and localised species such as *Banksia cunneata* (Coates and Sokolowski, 1992) and *Eucalyptus caesia* (Moran and Hopper, 1983) exhibit low levels of genetic diversity, while other narrow endemics, such as *Acacia anomala* (Coates, 1988) and *Stylidium coroniforme* (Coates, 1992), do not. Genetic diversity in Western Australian endemics has also been found apportioned both within populations (Coates and Hnatiuk, 1990; Byrne and James, 1991), and between (Moran and Hopper, 1983; Sampson *et al.*, 1988). This genetic variability probably reflects the complex evolutionary history of the area, where recently evolved taxa co-occur with more ancient relictual taxa.

Allozymes are well suited to addressing questions at the population,

subspecies and species level, particularly when phenotypic plasticity makes morphological classification difficult (Crawford, 1989; Brown, 1990). Systematic relationships in several Australian species have been resolved using allozyme differentiation (Coates and Hnatiuk, 1990; Moran *et al.*, 1990). The usefulness of allozymes can be attributed to codominance and polymorphism, Mendelian inheritance, complete penetrance and the absence of epistatic, pleiotropic or environmental effects; the low cost, ease of use and rapidity of assays are also highly advantageous (Brown and Weir, 1983; Hamrick, 1989; Weeden and Wendel, 1989). Allozyme investigations can be restricted, however, by their inability to readily detect mutational differences and the limited number of enzyme markers available (Brown and Weir, 1983; Clegg *et al.*, 1984; Clegg, 1990; Liu and Furnier, 1993).

Aims of this investigation

This investigation aimed to examine the patterns of genetic diversity within and between populations of *G. verrucosa* and, to determine whether separate taxa could be recognised using allozyme markers.

Materials and methods

Sampling strategy

Plants at each population were sampled as outlined in Chapter 1. Inflorescences were collected from these plants, placed in sealed plastic bags and transported to the laboratory at 4°C where buds of a similar age were frozen at -72°C until analysed. Allozyme electrophoresis was carried out on anther material from unopened buds collected during flowering in 1995 (June-October).

Electrophoresis

Electrophoresis was undertaken using the Helena Laboratories (Beaumont TX) cellulose acetate system (Hebert and Beaton, 1989). Anthers of 18-20 plants from each population except Pleshkes (see Table

1.1) were ground with 30 µL of modified extraction buffer (Systma and Schaal, 1985) (50 mg mL^{-1} PVP, 0.5 mM NAD, 0.3 mM NADP, 1.7 mM EDTA, 1.2 mM ascorbic acid, 0.1% BSA, 10% sucrose w/v and 0.7 mg mL^{-1} dithiothreitol in 0.1 M Tris HCl pH7). The extract was centrifuged for 1.5 min, 12 µL transferred to sample wells from which 1 µL was loaded onto pre-soaked cellulose acetate plates. The plates were transferred to running tanks maintained at 5°C and electrophoresed at 5 mA/gel. Running times varied from 30-50 min depending upon the enzyme system under analysis.

Eleven enzyme systems were assayed (Table 4.1). All systems were run in an 80 mM Tris-EDTA-malaeic acid buffer (80 mM Tris, 1 mM Na_2EDTA, 1 mM $MgCl_2$, 6.4 mM histidine, 13.3 mM glycine, 3.4 mM glutamic acid, 3.7 mM aspartic acid, pH to 7.6 with maleic acid followed by pH to 8.1 with sodium hydroxide), except *Aat* which was run in Tris citrate buffer pH 8.1 (100 mM Tris, 10 mM citric acid). Staining procedures followed Richardson *et al.* (1986), except 4 mL of stain buffer was used with stain ingredients proportionally increased. Sixteen zones of activity were scored for nine enzyme. Each zone was assumed to represent a single enzyme locus: *Est*-1, *Aat*-1, *Aat*-2, *Aat*-3, *Aat*-4, *Aat*-5, *Idh*-1, *Lap*-1, *Gpi*-1, *Mdh*-1, *Mdh*-2, *Mdr*-1, *Mdr*-2, *Mdr*-3, *Pgm*-1 and *Skd*-1.

Table 4.1: Enzyme systems examined for allozyme electrophoresis.

Enzyme	Abbreviation	EC code
Acid phosphatase	*Acp*	3.1.3.2
Aspartate aminotransferase	*Aat*	2.6.1.1
Esterase	*Est*	3.1.1.1
Glucose-6-phosphate isomerase	*Gpi*	5.3.1.9
Isocitrate dehydrogenase	*Idh*	1.1.1.42
Leucine aminopeptidase	*Lap*	3.4.11.1
Malate dehydrogenase	*Mdh*	1.1.1.37
Menadione reductase	*Mdr*	1.6.99.22
Phosphoglucomutase	*Pgm*	5.4.2.2
Phosphogluconate dehydrogenase	*6pgd*	1.1.1.44
Shikimate dehydrogenase	*Skd*	1.1.1.25

Genetic analyses

The mean number of alleles per locus (*A*), proportion of polymorphic loci (*P*), observed (H_o) and expected heterozygosity (H_e) and Wright's (1978) F_{IS} were calculated for each population using BIOSYS-1 (Swofford and Selander, 1981) and POPGENE Version 1.2 (Yeh *et. al.*, 1997). These measures were also calculated after pooling individuals in each form and overall. Total genetic diversity, and its distribution within and between populations and forms was estimated using Nei's (1973) gene diversity statistics, and calculated using GENESTAT (Whitkus, 1985). Total gene diversity (H_T) was partitioned into mean gene diversity within (H_S) and between populations (D_{ST}). The proportion of interpopulation differentiation (G_{ST}) was determined by $G_{ST} = D_{ST}/H_T$. Nei's (1972) genetic distance was used to produce the cluster analysis with standard error bars, based on the unweighted pair-group average method using arithmetic means (UPGMA) with Ritland's (1989) Genetic Distance and Clustering (GD) program.

Results

Genetic variation within populations

Although only three loci were monomorphic (*Idh*-1, *Mdh*-2 and *Mdr*-1), many populations were dominated by monomorphic loci (Table 4.2). In those populations where the common allele was not fixed, the alternative alleles were often observed at very low frequencies (*Aat*-1^{c} [1] at Nanns, *Gpi*-1^{c} at Coorow 1, *Gpi*-1^{d} at Ajana West 2, and *Mdr*-3^{b} at Hutt River 2). The distribution of alleles at Pleshkes where the small and large forms co-occurred, strongly suggested a zone of hybridisation. The small form at this site exhibited unique alleles *Aat*-1^{b} and *Aat*-3^{c}, as well as other alleles restricted to the large form (*Aat*-2^{a}, *Aat*-4^{a}, *Aat*-5^{b} and *Aat*-5^{c}). This is discussed in more detail in Chapter 6.

[1] Abbreviation summarises enzyme system, locus and allele. For example, *Aat*-1^{c} is allele c at locus 1 for aspartate aminotransferase.

Table 4.2: Allele frequencies for the 19 *G. verrucosa* sites. ***n***, **sample size. Populations codes: A, Arinya; C1, Coorow 1; C2, Coorow 2; IR, Indarra Reserve; M, Maiseys; PS, Pleshkes Small; AW1, Ajana West 1; AW2, Ajana West 2; HH, Hawks Head; HR1, Hutt River 1; HR2, Hutt River 2; N, Nanns; WB, West Binnu; GP, Geraldton; MH1, Meanarra Hill 1; MH2, Meanarra Hill 2; PL, Pleshkes Large; RB, Red Bluff; PH, Pleshkes Hybrid.**

		Small						*Intermediate*							*Large*					*Transect*
Locus	Allele	A	C1	C2	IR	M	PS	AW 1	AW 2	HH	HR 1	HR 2	N	WB	GP	MH 1	MH 2	PL	RB	PH
Aat-1	*n*	17	19	20	20	20	10	19	18	18	18	20	20	19	20	19	18	10	20	20
	a	1.00	1.00	1.00	1.00	1.00	0.20	1.00	1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	0.88
	b	-	-	-	-	-	0.80	-	-	-	-	-	-	-	-	-	-	-	-	0.15
	c	-	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-	-	-	-
Aat-2	*n*	17	20	20	19	20	10	19	18	19	20	20	21	19	20	19	18	10	20	20
	a	-	-	-	-	-	0.20	-	-	-	-	-	-	-	1.00	1.00	0.83	1.00	0.35	0.75
	b	-	-	-	0.30	-	0.30	-	-	1.00	-	-	-	-	-	-	0.11	-	0.60	0.20
	c	1.00	1.00	1.00	0.70	1.00	0.50	1.00	1.00	-	1.00	1.00	1.00	1.00	-	-	0.06	-	0.05	0.05
Aat-3	*n*	17	20	20	20	20	10	19	18	19	20	20	21	19	20	19	18	10	20	20
	a	-	-	-	0.10	-	0.40	-	-	-	-	-	-	-	1.00	1.00	1.00	0.70	0.45	0.70
	b	1.00	1.00	1.00	0.90	1.00	0.50	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	-	0.30	0.55	0.30
	c	-	-	-	-	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-
Aat-4	*n*	17	20	20	19	20	10	19	18	19	20	20	21	19	20	19	18	10	20	20
	a	-	-	-	-	-	0.50	-	-	-	-	-	-	-	1.00	0.72	0.78	0.80	0.60	0.85
	b	-	-	-	0.21	-	0.30	1.00	-	0.72	-	-	-	-	-	0.28	0.22	0.20	0.35	0.15
	c	1.00	1.00	1.00	0.78	1.00	0.20	-	1.00	0.28	1.00	1.00	1.00	1.00	-	-	-	-	0.05	-
Aat-5	*n*	17	20	20	20	20	10	19	18	19	20	20	21	19	20	19	18	10	20	20
	a	1.00	1.00	1.00	1.00	1.00	0.67	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.90	0.58	0.90	0.88	0.78
	b	-	-	-	-	-	0.11	-	-	-	-	-	-	-	-	0.10	0.31	0.10	0.12	0.14
	c	-	-	-	-	-	0.22	-	-	-	-	-	-	-	-	-	0.11	-	-	0.08
Est-1	*n*	17	20	20	19	20	10	11	17	15	20	20	21	19	19	19	18	10	19	20
	a	1.00	1.00	1.00	1.00	1.00	0.50	1.00	0.88	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	-	1.00	0.35
	b	-	-	-	-	-	0.50	-	0.12	-	-	-	-	-	-	-	1.00	1.00	-	0.65
Gpi-1	*n*	17	19	20	20	20	7	19	18	18	17	16	21	19	20	19	18	7	20	15
	a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.05	-	-	-	-
	b	-	0.05	-	-	-	0.22	-	-	-	0.12	-	0.43	0.42	0.30	0.37	0.50	-	0.15	0.20
	c	1.00	0.95	1.00	1.00	1.00	0.78	1.00	0.94	1.00	0.88	1.00	0.57	0.58	0.60	0.58	0.50	1.00	0.85	0.80
	d	-	-	-	-	-	-	-	0.06	-	-	-	-	-	0.10	-	-	-	-	-
Idh-1	*n*	17	20	20	20	20	10	19	18	18	18	20	20	19	20	19	18	10	20	20
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lap-1	*n*	17	20	20	20	20	10	19	18	18	18	20	20	19	20	19	18	10	20	20
	a	-	-	-	0.10	-	-	0.68	0.83	0.72	0.06	0.15	0.70	0.16	1.00	-	-	-	0.05	-
	b	1.00	1.00	1.00	0.48	1.00	0.75	0.21	0.17	0.17	0.58	0.72	0.18	0.42	-	0.79	0.67	1.00	0.80	0.90
	c	-	-	-	0.42	-	0.25	0.11	-	0.11	0.36	0.13	0.12	0.42	-	0.21	0.33	-	0.15	0.10
Mdh-1	*n*	19	18	17	17	20	9	18	18	18	19	18	20	18	19	18	17	10	20	20
	a	0.11	0.53	0.06	-	-	-	-	-	-	-	-	-	1.00	-	0.03	-	-	-	0.05
	b	0.89	0.47	0.94	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	-	1.00	0.97	1.00	1.00	1.00	0.95
Mdh-2	*n*	17	16	20	19	20	10	14	17	18	17	17	20	19	19	18	17	10	20	18
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdr-1	*n*	17	20	20	20	20	10	19	18	19	20	20	21	19	20	19	18	10	20	20
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mdr-2	*n*	17	19	18	19	19	9	17	18	19	18	20	21	19	20	19	18	9	20	19
	a	0.79	0.84	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.83	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	0.21	0.16	-	-	-	-	-	-	-	-	0.17	0.02	-	-	-	-	-	-	-
Mdr-3	*n*	17	20	18	20	19	9	18	18	19	18	18	21	18	20	19	17	9	20	19
	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.92	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	b	-	-	-	-	-	-	-	-	-	-	0.08	-	-	-	-	-	-	-	-
Pgm-1	*n*	17	20	17	20	20	10	11	18	19	20	19	21	19	20	18	18	10	20	20
	a	-	-	-	-	-	-	0.95	0.39	1.00	-	0.16	0.43	0.37	0.05	-	-	-	0.20	-
	b	1.00	1.00	0.71	1.00	1.00	1.00	0.05	0.61	-	1.00	0.84	0.57	0.63	0.95	0.89	0.94	1.00	0.80	1.00
	c	-	-	0.29	-	-	-	-	-	-	-	-	-	-	-	0.11	0.06	-	-	-
Skd-1	*n*	12	18	20	19	20	9	18	18	19	18	18	20	17	19	18	17	10	20	20
	a	0.33	0.61	0.20	0.26	-	-	-	-	-	-	-	-	-	0.16	0.11	0.06	-	0.15	0.05
	b	0.67	0.39	0.80	0.74	0.87	0.94	1.00	1.00	1.00	0.72	0.44	0.47	0.77	0.84	0.56	0.56	0.75	0.82	0.85
	c	-	-	-	-	0.13	0.06	-	-	-	0.28	0.56	0.53	0.23	-	0.33	0.38	0.25	0.03	0.10

Genetic differentiation between populations and forms

Allele frequencies

Single locus diversity measures were low (Table 4.3a). The mean number of alleles per locus (*A*) was 1.4 (range 1.1 to 1.8), while the percentage of polymorphic loci (*P*) was 29.6% (range 6.3% to 62.5%.). Observed heterozygosity (H_o, 0.055) was considerably less than expected (H_e, 0.097). Populations exhibited marked differences in single locus diversity measures. Heterozygous individuals were absent from populations at Coorow 2, Ajana West 2 and Hawks Head, while very low levels of heterozygosity characterised populations at Arinya (0.011), Coorow 1 (0.017), Maiseys (0.016), Ajana West 1 (0.006) and Hutt River 2 (0.022), where individuals with similar genotypes dominated. In contrast, populations at Meanarra Hill, Red Bluff, Hutt River 1 and West Binnu exhibited higher levels of heterozygosity and approached panmixia. These results were also reflected in the F_{IS} values which varied in a similar manner across populations (range -0.025 to 0.387). The slightly negative F_{IS} values exhibited by the Maiseys, West Binnu and Hutt River 1 populations indicated an excess of heterozygotes.

Forms also exhibited marked differences in single locus diversity measures (Table 4.3a). The percentage of polymorphic loci was lower in the small (26.1%) and intermediate (23.2%) forms than in the large form (36.3%). The large form also approached panmixia, while observed heterozygosity was considerably lower than that expected in the other two forms. This was again reflected by the fixation indices for each form. While pooled data produced higher *A* and *P* values, observed and expected heterozygosites followed similar patterns (Table 4.3b). Trends in the F_{IS} values were also comparable but higher levels of homozygotes were detected. Within the genus, observed heterozygosity (0.056) was considerbaly lower than that expected (0.213) and the F_{IS} values indicated a strong excess of homozygotes (0.580).

Table 4.3a: Single locus diversity measures based on 16 loci at 19 *G. verrucosa* sites. *n*, sample size per locus; *A*, average no. of alleles per locus; *P*, % polymorphic loci per population; H_o, observed heterozygosity; H_e, expected panmictic heterozygosity; F_{IS}, fixation index; standard errors in parentheses.

Form	Population	*n*		*A*		*P*	H_o		H_e		F_{IS}	
Small	Arinya	16.2	(0.6)	1.2	(0.1)	18.8	0.011	(0.011)	0.063	(0.036)	0.154	(0.031)
	Coorow 1	19.4	(0.3)	1.3	(0.1)	25.0	0.017	(0.009)	0.086	(0.044)	0.152	(0.030)
	Coorow 2	19.4	(0.3)	1.2	(0.1)	18.8	0.000	(0.000)	0.054	(0.033)	0.188	(0.041)
	Indarra Reserve	19.5	(0.2)	1.4	(0.2)	31.3	0.053	(0.053)	0.122	(0.051)	0.222	(0.057)
	Maiseys	19.9	(0.1)	1.1	(0.1)	6.3	0.016	(0.016)	0.014	(0.014)	-0.010	(0.000)
	Pleshkes Small	9.5	(0.2)	1.8	(0.2)	56.3	0.047	(0.032)	0.261	(0.068)	0.387	(0.068)
	Mean	**17.3**	**(0.3)**	**1.3**	**(0.1)**	**26.1**	**0.024**	**(0.020)**	**0.100**	**(0.041)**	**0.182**	**(0.038)**
Intermediate	Ajana West 1	17.4	(0.7)	1.2	(0.1)	12.5	0.006	(0.006)	0.036	(0.031)	0.060	(0.016)
	Ajana West 2	17.9	(0.1)	1.3	(0.2)	25.0	0.000	(0.000)	0.069	(0.036)	0.250	(0.050)
	Hawks Head	18.4	(0.3)	1.2	(0.1)	12.5	0.000	(0.000)	0.054	(0.037)	0.125	(0.029)
	Hutt River 1	19.1	(0.3)	1.3	(0.1)	18.8	0.081	(0.049)	0.073	(0.042)	-0.021	(0.003)
	Hutt River 2	19.1	(0.3)	1.4	(0.2)	31.3	0.022	(0.016)	0.105	(0.044)	0.243	(0.039)
	Nanns	20.7	(0.1)	1.4	(0.2)	37.5	0.071	(0.037)	0.133	(0.055)	0.172	(0.030)
	West Binnu	18.8	(0.1)	1.3	(0.2)	25.0	0.135	(0.075)	0.124	(0.057)	-0.025	(0.029)
	Mean	**18.8**	**(0.3)**	**1.3**	**(0.2)**	**23.2**	**0.045**	**(0.026)**	**0.085**	**(0.043)**	**0.115**	**(0.028)**
Large	Geraldton	19.8	(0.1)	1.3	(0.2)	18.8	0.038	(0.038)	0.058	(0.033)	0.118	(0.030)
	Meanarra Hill 1	18.7	(0.1)	1.6	(0.2)	43.8	0.105	(0.056)	0.146	(0.053)	0.150	(0.035)
	Meanarra Hill 2	17.8	(0.1)	1.5	(0.2)	43.8	0.170	(0.082)	0.179	(0.059)	0.050	(0.060)
	Pleshkes Large	9.7	(0.2)	1.3	(0.1)	25.0	0.031	(0.031)	0.085	(0.040)	0.167	(0.044)
	Red Bluff	19.9	(0.1)	1.8	(0.2)	50.0	0.169	(0.053)	0.189	(0.053)	0.011	(0.020)
	Mean	**17.2**	**(0.1)**	**1.5**	**(0.2)**	**36.3**	**0.103**	**(0.052)**	**0.131**	**(0.048)**	**0.099**	**(0.038)**
Transect	Pleshkes Hybrid	19.4	(0.3)	1.8	(0.2)	62.5	0.066	(0.028)	0.193	(0.045)	0.381	(0.060)
Overall Mean		**17.9**		**1.4**		**29.6**	**0.055**		**0.097**		**0.146**	

Table 4.3b: Single locus diversity measures based on 16 loci for pooled *G. verrucosa* forms. Abbreviations outlined in Table 4.3a.

Form	*n*	*A*	*P*	H_o	H_e	F_{IS}
Small	208	2.1 (0.8)	75.0	0.022 (0.056)	0.086 (0.122)	0.614 (0.051)
Intermediate	263	1.8 (0.7)	68.8	0.045 (0.095)	0.170 (0.204)	0.537 (0.048)
Large	172	2.1 (1.0)	62.5	0.110 (0.174)	0.189 (0.186)	0.241 (0.036)
Overall	681	2.4 (0.9)	81.3	0.056 (0.09)2	0.213 (0.206)	0.580 (0.044)

Some loci showed fixed allelic or highly significant allele frequency differences between the observed morphological forms. Although the allele *Aat*-2^a was restricted to all large form populations, it was also observed in the small form at Pleshkes (PS) where both the small and large forms co-occurred (Figure 4.1, left). The small form at this population also exhibited three alleles for this locus while most other small form populations were homozygous for the allele *Aat*-2^c.

The allele *Aat*-3^a, restricted to the large form populations, was observed in two small form populations, Pleshkes Small and Indarra Reserve (Figure 4.1, right). Pleshkes Small also exhibited the three allelic forms at this locus, including the rare allele *Aat*-3^c not observed in any other population. The remaining small and intermediate form populations were homozygous for the allele *Aat*-3^b.

The Pleshkes Small site also exhibited the three *Aat*-4 alleles, including the allele *Aat*-4^a which dominated the large form populations (Figure 4.2, left). Many of the small and intermediate form populations were dominated by the allele *Aat*-4^c.

At the *Lap* locus, the intermediate form populations were characterised by high levels of heterozygosity, exhibiting the three allelic forms (Figure 4.2, right). In contrast, the small and large form populations were mostly either homozygous or heterozygous for two alleles.

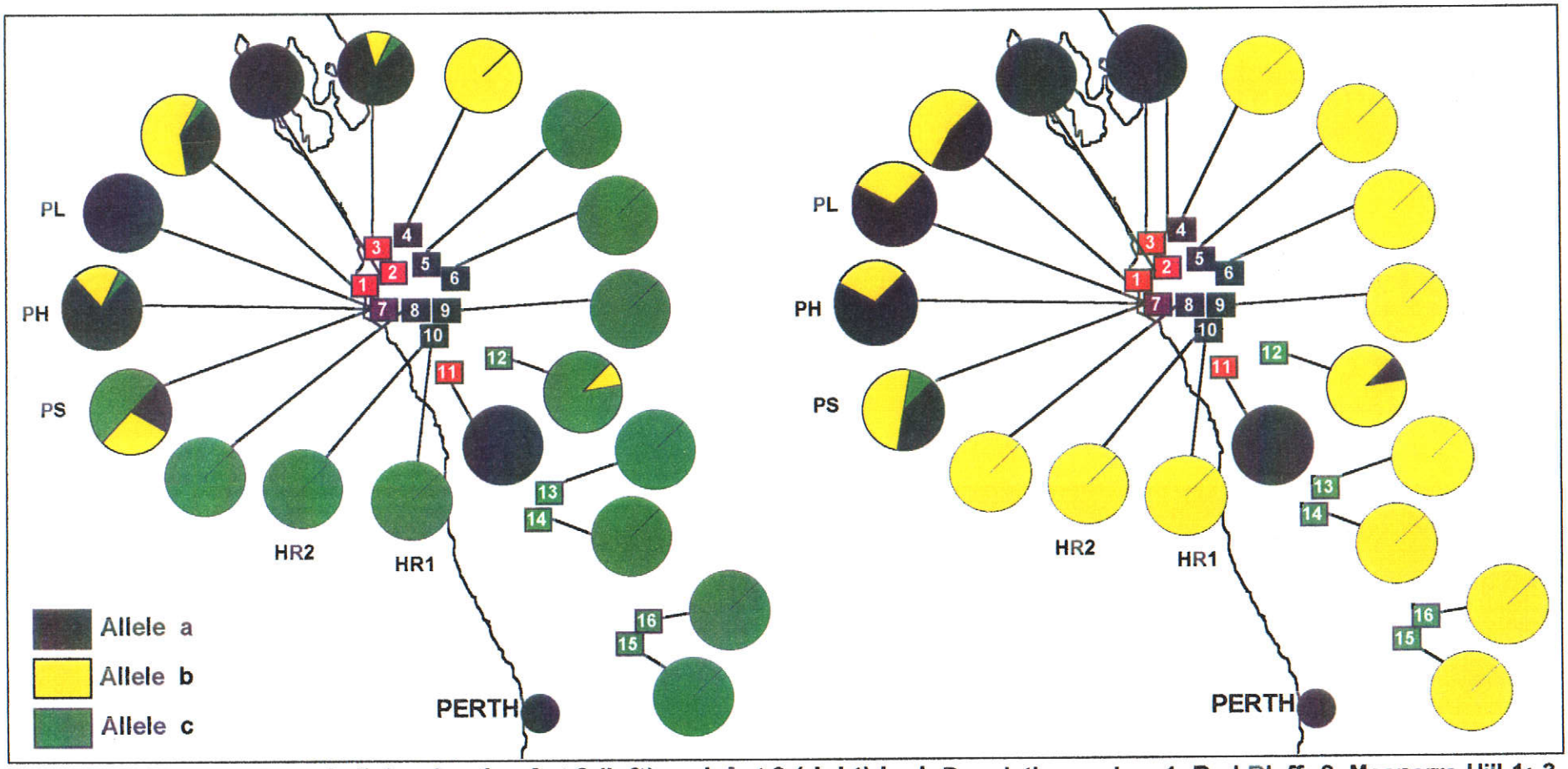


Figure 4.1: Distribution of alleles for the *Aat*-2 (left) and *Aat*-3 (right) loci. Population codes: 1, Red Bluff; 2, Meanarra Hill 1; 3, Meanarra Hill 2; 4, Hawks Head; 5, Ajana West 1; 6, Ajana West 2; 7, Pleshkes (PL, Pleshkes Large; PH, Pleshkes Hybrid; PS, Pleshkes Small); 8, Nanns; 9, West Binnu; 10, Hutt River (sites 1 and 2); 11, Geraldton; 12, Indarra Reserve; 13, Coorow 1;14, Coorow 2; 15, Arinya; 16, Maiseys. Small form; intermediate form; large form; mixed forms.

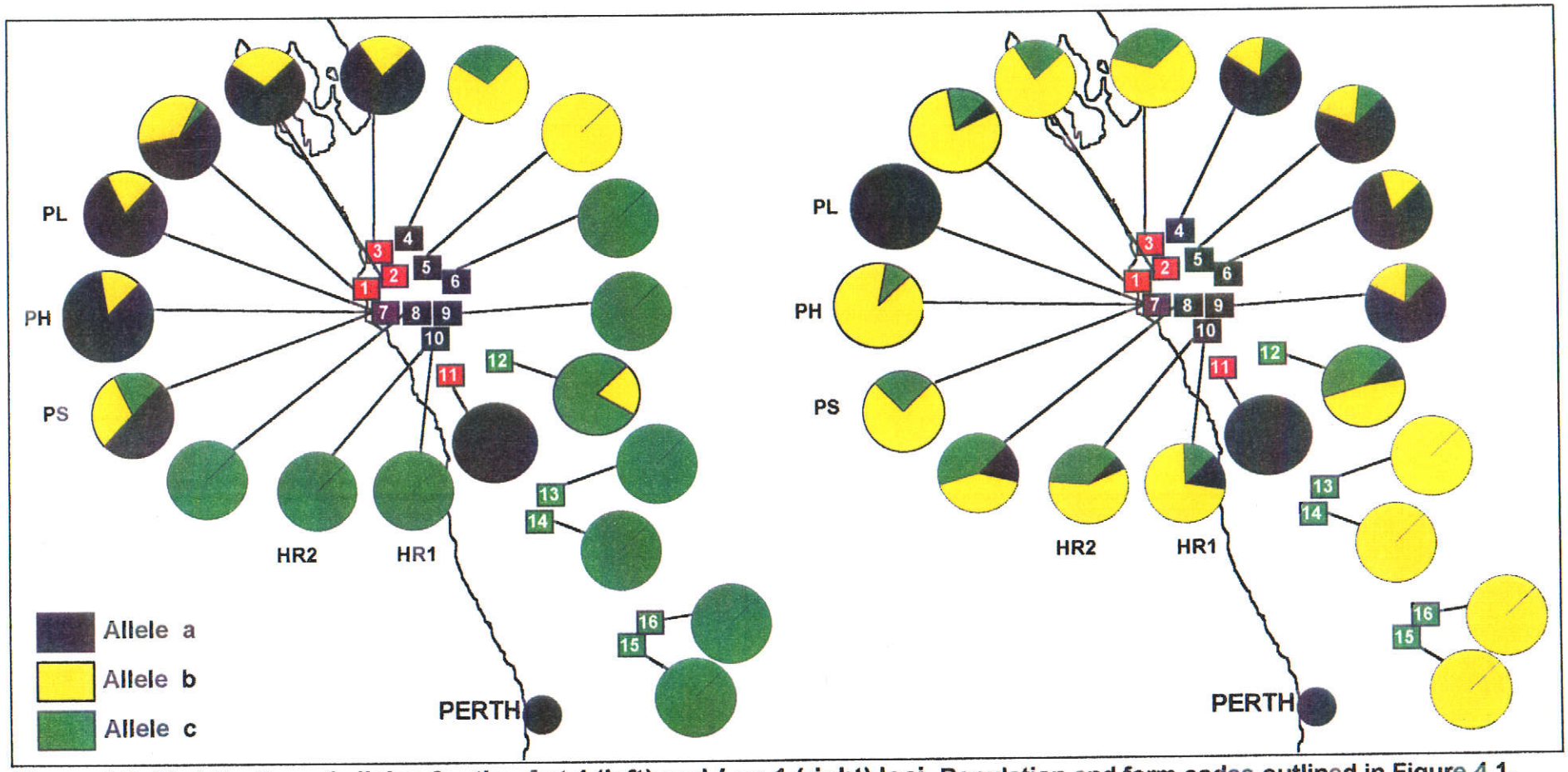


Figure 4.2: Distribution of alleles for the *Aat*-4 (left) and *Lap*-1 (right) loci. Population and form codes outlined in Figure 4.1.

Genetic diversity

The mean total genetic diversity (H_T) was 0.304 with most diversity distributed between populations (D_{ST}, 0.175), rather than within (H_S, 0.129) (Table 4.4). The proportion of genetic diversity between populations (G_{ST}) was very high at 0.576, indicating that 58% of the total diversity was due to interpopulation differentiation. Six loci, *Aat*-1, *Aat*-2, *Aat*-3, *Aat*-4, *Est*-1 and *Mdh*-1 were significant contributors to this high interpopulation differentiation.

Table 4.4: Gene diversity statistics for polymorphic loci for all *G. verrucosa* populations. H_T, total gene diversity; H_S, mean gene diversity within populations; D_{ST}, mean gene diversity between populations; G_{ST}, the proportion of interpopulation differentiation.

Locus	H_T	H_S	D_{ST}	G_{ST}
Aat-1	0.090	0.022	0.068	0.754
Aat-2	0.576	0.101	0.475	0.825
Aat-3	0.429	0.114	0.315	0.734
Aat-4	0.620	0.177	0.443	0.715
Aat-5	0.118	0.097	0.021	0.179
Gpi-1	0.270	0.210	0.060	0.223
Est-1	0.297	0.063	0.234	0.788
Lap-1	0.550	0.299	0.251	0.457
Mdh-1	0.177	0.052	0.125	0.705
Mdr-2	0.057	0.050	0.007	0.129
Mdr-3	0.008	0.008	0.000	0.000
Pgm-1	0.352	0.159	0.193	0.549
Skd-1	0.414	0.327	0.087	0.209
Mean	**0.304**	**0.129**	**0.175**	**0.576**

Gene diversity statistics generated according to morphological form showed the mean total genetic diversity (H_T) of the small form was lower (0.192) than other two forms, with most of this diversity apportioned within populations (H_S, 0.122), rather than between (D_{ST}, 0.070) (Table 4.5). Interpopulational differentiation accounted for 36% of this variation. Although genetic diversity measures for the large form were somewhat higher (H_T, 0.254), this form also partitioned genetic diversity mostly within populations (H_S, 0.164) rather than between (D_{ST}, 0.090), with similar interpopulational differentiation (G_{ST}, 35%). Total gene diversity in the intermediate form (H_T, 0.226) was similar to the large form, but was mostly

apportioned between populations (D_{ST}, 0.121) not within (H_S, 0.105). These intermediate form populations, with the highest interpopulational differentiation (G_{ST}, 54%), contributed significantly to the high overall interpopulation differentiation encountered within the complex, and to the differentiation between the small and large forms. As a genus, genetic diversity was high (H_T, 0.331) which was also apportioned mostly within populations (H_S, 0.197) rather than between (D_{ST}, 0.134). Interpopulational differentiation was also high (G_{ST}, 40.4%).

Table 4.5: Gene diversity statistics over all loci unbiased for sample size and population number for *G. verrucosa* forms. Diversity indices outlined in Table 4.4.

Form	H_T	H_S	D_{ST}	G_{ST}
Small	0.192	0.122	0.070	0.363
Intermediate	0.226	0.105	0.121	0.536
Large	0.254	0.164	0.090	0.354
Overall	0.331	0.197	0.134	0.404

Genetic distance and UPGMA

Average genetic distance (D) within the small and large forms were low, 0.071 and 0.070 respectively, but higher within the intermediate form (0.112) (Table 4.6). Distances between the intermediate and large forms were considerably higher (0.301) than those between the small and large forms (0.223) and the small and intermediate forms (0.122).

Table 4.6: Average genetic distance (D) within and between forms of *G. verrucosa*.

Form	*Small*	*Intermediate*	*Large*
Small	0.071	0.122	0.223
Intermediate	-	0.112	0.301
Large	-	-	0.070

Two distinct clusters were observed based on estimates of genetic distance (D) (Figure 4.3). The initial dichotomy separated the large form populations at Pleshkes, Meanarra Hill, Red Bluff and Geraldton as a distinct group, within which, the small form at Pleshkes was a discrete

entity. The transect at Pleshkes (Pleshkes Hybrid) was also included in this group. The second group consisted of both the small and intermediate forms. All small form populations were clustered, with the intermediate form at Hutt River, within the intermediate form.

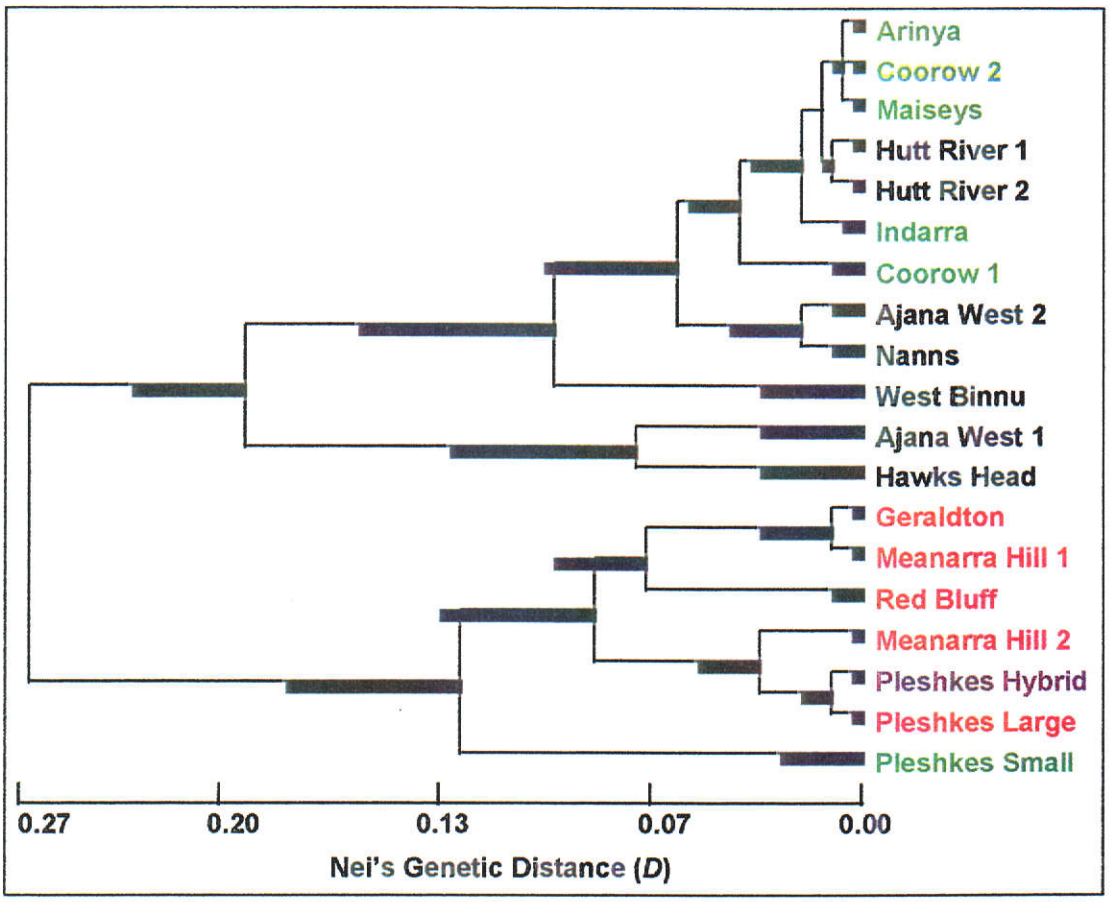


Figure 4.3: Cluster analysis of *G. verrucosa* populations based on Nei's genetic distance (*D*) and UPGMA algorithm. Population clusters are statistically significant if the shaded standard bar is less than half the branch length (Ritland, 1989). Small form; intermediate form, large form; transect.

Discussion

This investigation highlights *G. verrucosa* as an enigmatic species complex. The level of genetic divergence between the large form and the small and intermediate forms, the presence of alleles characterising the various forms, and apportioning of genetic diversity in different ways within the three forms, support this notion. The propensity for the large *G. verrucosa* form to approach panmixia, and the extremely low genetic

diversity observed in the small form, strongly suggest that not only do these forms represent distinct taxa, but that contrasting reproductive strategies are employed. The differences in fixation indices (F_{IS}) between the forms provide further evidence of reproductive distinctiveness. The small form tends to exhibit an excess of homozygotes indicating inbreeding, while heterozygotes are more evident in the large form.

Selfing populations are expected to exhibit low levels of genetic diversity (Hamrick *et al.*, 1979). Morphologically, selfers frequently present smaller, self-compatible flowers, invest fewer resources in floral attractants, and set larger quantities of seed (Solbrig and Rollins, 1977; Cruden and Lyon, 1985; Ritland and Ritland, 1989). Selfing populations are also often located peripherally to the range of closely related outcrossing, or predominantly outcrossing, taxa (Grant, 1975; Lloyd, 1980). In remote, temporary or harsh environments where plant density is low, pollinators scare or where bottlenecks recur, selfing can be highly advantageous (Stebbins, 1970; Solbrig and Rollins, 1977; Lloyd, 1980). In contrast, predominantly outcrossed species exhibit significantly higher levels of genetic diversity (Hamrick and Godt, 1989), possess larger and more abundant flowers but set fewer seeds (Solbrig and Rollins, 1977; Lyons and Antonovics, 1991).

The small *G. verrucosa* form exhibits many of the morphological characters associated selfing. In addition, it occurs inland in drier, harsher environments, and is somewhat peripheral to the range of the large form. The higher levels of genetic diversity and larger and more abundant flowers of the large *G. verrucosa* form, are consistent with the an outcrossing, or at least a mixed mating, reproductive system. Although reproductive strategy is a major influence on genetic diversity, other factors may also explain the levels of diversity observed.

The level of overall genetic diversity exhibited by *G. verrucosa* (*P*, 29.6%; *A*, 1.4) at a population level is consistent with that expected of endemic taxa (*P*, 26.3%; *A*, 1.4) (Hamrick and Godt, 1989), with genetic variation residing

between populations rather than within. Differences in levels of diversity within populations are also evident. Some populations exhibit high levels of heterozygosity while in others, heterozygosity is absent or very low. Such population differentiation can be explained by small population size and population disjunction severely limiting gene flow and promoting genetic drift and fixation (Hamrick, 1982; Loveless and Hamrick, 1984; Schoen and Brown, 1991). Theoretically, occasional gene flow can prevent differentiation but in reality, it is often too low or infrequent to stop divergence, but high enough to maintain diversity (Schoen and Brown, 1991). With the exception of populations at Meanarra Hill, Hutt River and Pleshkes, most other populations are small, disjunct and outside realistic gene flow, promoting genetic isolation and population divergence.

Within a species' range, location can occasionally explain levels of genetic diversity. Peripherally situated populations often exhibit lower genetic diversity than those more centrally located (Butcher *et al.*, 1992; Sherman-Broyles *et al.*, 1992). In *G. verrucosa*, the distribution of genetically depauperate populations across the whole species' range suggests that location alone does not significantly influence the levels of genetic diversity observed. Although somewhat difficult to quantify, differences in genetic variation can also reflect variability in environmental heterogeneity with populations in more challenging environments exhibiting higher genetic diversity (Brown and Schoen, 1992). Being widely distributed, *G. verrucosa* populations are likely to experience considerable ecological variability. In contrast to expectations, however, many of the less genetically diverse populations coincide with environments which are harsher, drier and more unpredictable. In these situations, intense fluctuations in population size can occur, and generate considerable change to their population genetic structure.

Low levels of allozyme polymorphism within some populations and significant population differentiation, suggest that bottlenecks and/or founder effects may play a significant role in the evolution of *G. verrucosa*.

The Hawks Head, Geraldton and Nanns populations occur in disturbed habitats, while the Ajana West, Coorow, Indarra Reserve, Red Bluff and West Binnu populations are located on road verges. At Pleshkes and Hutt River, commercial harvesting is undertaken annually and when production falls, areas are disturbed to stimulate germination. A recent investigation has confirmed this species is a disturbance opportunist, and that little seed remains in the seed soil bank after one year (Roche, 1998). Under these conditions, it is likely that many of these populations experience periodic bottlenecks.

Seedling recruitment following any disturbance is likely to be from limited seed resources produced in the previous season. This not only generates natural bottlenecks, but also restricts the genetic contribution of previous generations. Not all disturbed populations, however, exhibit low levels of genetic diversity. The West Binnu population for example, exhibits higher levels of heterozygosity. Although bottlenecks may substantially reduce genetic variability and contribute to the loss of rare alleles, the resultant genetic structure largely depends upon genetic variation in the original stock (Taggart *et al.*, 1990). The levels of diversity in the West Binnu population may reflect a more diverse founder stock.

The patterns of allelic distribution observed within and between *G. verrucosa* populations appear to stem from recent evolutionary events such as isolation promoting limited gene flow, and bottlenecks. The levels of genetic diversity within and between the different forms suggest that hybridisation is an important evolutionary event within this complex. The moderate total genetic diversity of both small and large forms is mostly apportioned within populations rather than between. That of the intermediate form is generally apportioned between populations, not within, although relatively high levels of diversity within populations do exist. In this latter form, 54% of the total genetic diversity is due to interpopulational differences, approximately twice that of the other forms. The higher levels of population differentiation in the intermediate *G. verrucosa* form may reflect

hybridisation between the small and large forms Similarly, hybrid populations between *Carpobrotus edulis* and *C. chilensis* exhibit higher levels of population differentiation than their parental types (Gallagher *et al.*, 1997).

The relationships between some populations based on genetic distances further support the intermediate form being of hybrid origin (Figure 4.3). The intermediate form populations at Ajana West are less than 3 km apart. Despite this, Ajana West 1 is more closely related to Hawks Head, located 3 km west, than to Ajana West 2, which clusters with Nanns some 50 km to the south. These latter populations occur close to plants belonging to the small form and exhibit the small form *Aat*-4^{c} allele. In contrast, the Ajana West 1 and Hawks Head populations exhibit the *Aat*-4^{b} allele commonly observed in the large form. These differences in allele distribution possibly reflect independent hybridisation events between the small and large forms followed by isolation and introgression.

The relationship between the intermediate form Hutt River population and the small form populations suggests this population is also of hybrid origin. Although no small form plants occur in close proximity to this population, a relatively inaccessible intermediate form population 3 km away does contain some small form plants (I. Casley, pers. comm.). This population may reflect a hybridisation event which has resulted in a close genetic affinity with the small form parental type, but morphological alignment with the large form.

Plants at the Geraldton populations are likewise intriguing. These closely resemble the small Arinya form in habit, but exhibit larger and more abundant flowers characteristic of the large form. Allozymically, these plants are allied with the large form. Such mixed characters may denote a hybridisation event similar to that at Hutt River, but which resulted in morphologically and genetically different hybrid offspring.

G. verrucosa occurs within the transitional-rainfall zone, a species-rich region with a high number of infraspecific variants, cryptic species complexes and natural hybrids (Hopper, 1979; 1992). Plant evolution in this region has largely been driven by small population size, induced by recurrent late Tertiary-Quaternary climatic and sea level fluctuations, in a heterogeneous soil mosaic (Hopper, 1992). Range expansion and contraction of the small and large forms during such perturbations could generate a series of temporally and spatially distributed hybrid populations. The novel genetic arrays created by each hybridisation event would readily apportion genetic diversity between populations rather than within, as observed in the intermediate *G. verrucosa* form. In addition, differential selection pressures at each hybrid population are likely to further contribute to population differentiation.

Evolution of the *G. verrucosa* species complex has largely been directed by recurrent climatic and geological events creating a heterogenous environment. While population disjunction has promoted divergence, small and large forms within the complex exhibit levels of genetic variability and differentiation indicative of distinct taxa. Higher levels of population divergence within the intermediate form suggest that this form is of hybrid origin. More specifically, it has arisen through a series of hybrid events between the small and large forms. Assessing relationships using an alternative molecular marker may provide further evidence of hybridisation as well as examining whether similar relationships between the forms exist.

CHAPTER 5

Variation identified by Randomly Amplified Polymorphic DNA (RAPDs)

Introduction

Polymerase chain reaction (PCR) technology has led to the development of several techniques for analysing genetic variation within and between populations (Clegg and Durbin, 1990; Hillis *et al.*, 1990). Assessing diversity using these techniques provides a direct examination of genomic DNA, in contrast to those based on more indirect methods, such as allozymes. The Randomly Amplified Polymorphic DNA (RAPDs) procedure amplifies genomic DNA using single short oligonucleotide primers of an arbitrary sequence (Williams *et al.*, 1993). The use of short primers increases the likelihood of finding many complementary sites within any complex eukaryotic genome (Grattapaglia *et al.*, 1992; Waugh and Powell, 1992). The resultant amplified products are then electrophoretically separated and visualised.

Increasingly, RAPDs have been utilised to investigate population genetic structure, systematic relationships, hybridisation, pedigree analysis, and to aid in plant breeding (Williams *et al.*, 1990; Rafalski *et al.*, 1991; Rieseberg *et al.*, 1993; Wang *et al.*, 1994; Rieseberg, 1996). RAPDs can also fingerprint individual plants, enhancing the management of rare and/or vulnerable taxa (Rossetto *et al.*, 1995; James and Ashburner, 1997; Waycott, 1998). Recently, studies which compare genetic variation observed at enzyme and RAPD loci have provided important insights into the levels and distribution of genetic variability both within and between individuals and populations (Liu and Furnier, 1993; Peakall *et al.*, 1995; Waycott, 1995; 1998; Szmidt *et al.*, 1996).

Although a valuable research tool, some difficulties are associated with RAPDs. Being dominant markers, heterozygotes can be difficult to detect

(Williams *et al.*, 1990; Liu and Furnier, 1993) while fragment interpretation and homology can also be problematic (Liu and Furnier, 1993; Rieseberg, 1996). Other difficulties with respect to reproducibility, artefact production and DNA template contamination can often be overcome by careful optimisation of protocols and reaction conditions (Hillis *et al.*, 1990; Giovannoni *et al.*, 1991; Bell and DeMarini, 1991; Riedy *et al.*, 1992; Weeden *et al.*, 1992; Ellsworth *et al.*, 1993; Ragot and Hoisington, 1993; Bachmann, 1994).

Aims of this investigation

Morphological, reproductive and allozyme analyses clearly suggest that the *G. verrucosa* complex consists of at least two taxa with hybrid-derived intermediates. This research undertook to determine whether the taxa could also be differentiated using the more direct genomic assessment afforded by RAPDs.

Materials and methods

Sampling strategy

Six populations representing the three forms were selected from across the species range (Table 5.1). Fresh vegetative material was collected from ten plants within each population, placed in sealed plastic bags, transported to the laboratory at 4°C and frozen at -20°C until analysis was undertaken.

Table 5.1: *G. verrucosa* populations sampled for RAPD analysis

Form	Population	Code
Small	Arinya	A
	Coorow 1	C1
Intermediate	Ajana West 1	AW1
	Hutt River 2	HR2
Large	Geraldton	G
	Meanarra Hill 1	MH1

DNA extraction

DNA extraction proved extremely problematic. Very low yields of poor quality DNA were achieved using modified SDS (Milligan, 1992) and CTAB (Doyle and Doyle, 1987) methods. Marginally higher yields (5-100 ng/5-8 gm of fresh material), were obtained using a modified method of Byrne *et al.* (1993): fresh vegetative material (5-8 gm) was ground in liquid nitrogen, 25 mL of extraction buffer was added and the mix passed through a cloth sieve. The resultant liquid was centrifuged (5 min, 2000 g), the supernatant removed and the pellet resuspended in 2.5 mL of wash buffer. To this, 350 µL of 5M NaCl, 400 µL of 8.6% CTAB/0.7m NaCl and 800 µL of 10% sarcosyl were added and the mix incubated (60°C, 15 min). Four mL of chloroform/isoamyl alcohol (24:1v/v) were added, gently shaken (30 min, 3000 rpm) and the mix again centrifuged (10 min, 2000 rpm). The supernatant was transferred to a clean tube, 3 mL of cold isopropynol added, incubated (-20°C, 1 h) and centrifuged (10 min, 2000 g) to pellet the DNA. The supernatant was removed, 1 mL of 50% isopropynol/0.3M ammonium acetate (NH_4Ac) added, incubated (25°C, 30 min) and centrifuged (5 min, 2000 g). Following removal of the supernatant, the pellet was air dried for 2 h then resuspending in 20 µL of TE.

Amplification

Initial primer amplification indicated further purification of the DNA was required. Phenol/chloroform/isoamyl alcohol (25:24:1 v/v) (40 µL) was added to each sample, gently shaken, then centrifuged and the supernatant removed. The pellet was washed twice with ether to remove residual phenol and chloroform, and suspended in 20 µL of TE. The unexpected difficulties involved in obtaining sufficient quantities of good quality DNA exhausted all vegetative material collected and cost prevented further collection of material. This limited amplification to 4-5 plants from each population and also prevented replication.

Eleven primers were initially tested, of which five produced amplifiable and interpretable bands (Table 5.2). All reactions were set up using standard

sterile conditions and solutions. Each 25 µL reaction mixture contained 2.5 µL 10X Buffer II reaction mix (Perkin Elmer), 3.0 µL 25 mM magnesium chloride, 2.0 µL 5 mM dNTP, 1.0 µL primer (0.2 µM), 0.2 µL (5 units/µL) *Taq* polymerase (Perkin Elmer) and 1 µL genomic DNA template (10-50 ng). Amplification was performed using a Hybaid Touchdown Thermocycler with a protocol of one cycle at 94°C for 2 min, followed by 45 cycles of 94°C for 1 min, 34°C for 30 secs and 72°C for 1 min, with a final extension of 72°C for 5 min.

Table 5.2: Primers used for PCR fingerprint analysis on the six populations of *G. verrucosa*. Source codes: UBC, University of British Columbia; KPBG, Kings Park and Botanic Gardens.

Primer No.	Sequence	Length	Source
264	TCC ACC GAG C	10	UBC
243	GGG TGA ACC G	10	UBC
854	TCT CTC TCT CTC TCT CRG	18	UBC
B82	TGA GCG GAC A	10	KPBG
B88	CCC ACC AAC	9	KPBG

Amplified samples (10 µL) were loaded onto 1.5% agarose and run in a 1X TBE buffer system for 2 h. Gels were visualised using ethidium bromide, photographed with a digital camera and images produced using Adobe Photoshop Version 3.0. Bands were scored 'blind', that is, without knowledge of the plant, population or form, on a presence/absence (1/0) basis.

Each band was assumed to represent a single locus comprised of two alleles. A data matrix composed of the presence/absence of scored bands for individuals in each population for each primer was constructed and used to examine the relationships between populations. Nei's (1978) genetic distance (*D*) was calculated using dominant diploid marker parameters of the POPGENE program (Yeh and Boyle, 1997) and a UPGMA dendogram of populations constructed.

The number of RAPD phenotypes within each population was determined and used to calculate the multilocus diversity statistic D_G:

$$D_G = 1 - \Sigma[(n_i(n_i-1))/(N(N-1))]$$

where n_i is the number of individuals with genotype i and N is the total sample size (Ellstrand and Roose, 1987; Murawski and Hamrick, 1990).

The presence/absence of each polymorphic RAPD band within a population was used to calculate a frequency matrix for nonmetric multi-dimensional scaling. Euclidean distances between populations were calculated and a two-dimensional Euclidean model produced. Relationships between populations were visualised in two dimensions based on the Euclidean distances.

Results

The five RAPD primers generated considerable diversity with 34 unambiguous and scorable bands produced (Figure 5.1). Each plant surveyed exhibited a unique genotype resulting in a mean multilocus genotype diversity value (D_G) of 1 and a mean of 4.7 RAPD genotypes across the populations sampled (Table 5.3). In contrast, the mean D_G value for allozymes was 0.767.

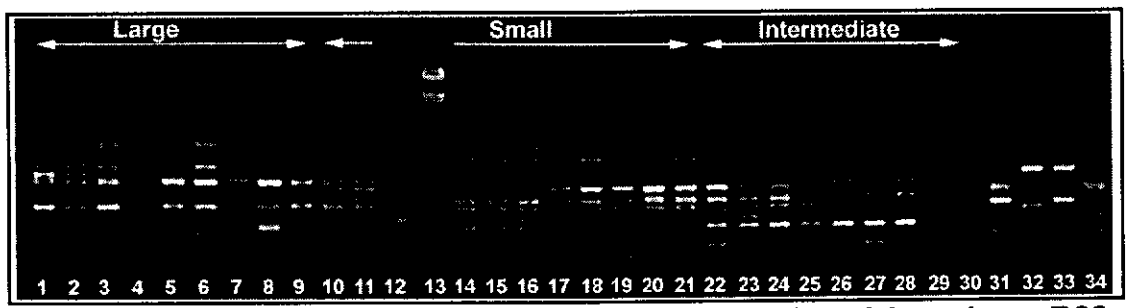


Figure 5.1: Gel-electrophersis of RAPD bands produced by primer B82. ***Lanes* 1-4, Geraldton; *5-9*, Meanarra Hill 1; *10-11* and *14-16*, Coorow 1; *17-21*, Arinya; *22-25*, Ajana West 1; *26-30*, Hutt River 2; *31-33*, *Drummondita hasselli*; *12* and *19*, pUC19/Hpa II DNA marker; *13*, λ EcoRI & Hind III DNA marker.**

Table 5.3: RAPD and allozyme multilocus genotype diversity statistics for the six *G. verrucosa* populations. *n*, number of individuals; *N*, number of genotypes.

Form	Population	*RAPDs*			*Allozymes*		
		n	*N*	D_G	*n*	*N*	D_G
Small	Arinya	5	5	1	17	5	0.61
	Coorow 1	5	5	1	20	12	0.92
Intermediate	Ajana West 1	4	4	1	19	4	0.57
	Hutt River 2	5	5	1	20	14	0.95
Large	Geraldton	4	4	1	20	5	0.61
	Meanarra Hill 1	5	5	1	19	16	0.95
Mean		**4.7**	**4.7**	**1**	**19.2**	**9.3**	**0.77**

D_G values for such a small sample size were uninformative and provided no evidence of fixed geographic or morphological variation. Relationships between forms in the UPGMA dendogram (Figure 5.2) were similar to those found in morphological (Chapter 2, Figure 2.3) and allozyme (Chapter 4, Figure 4.3) analyses. Forms were readily separated. The large form populations at Geraldton and Meanarra Hill 1 were clearly divergent from the small and intermediate form populations which grouped together. The intermediate form was nested within the small form.

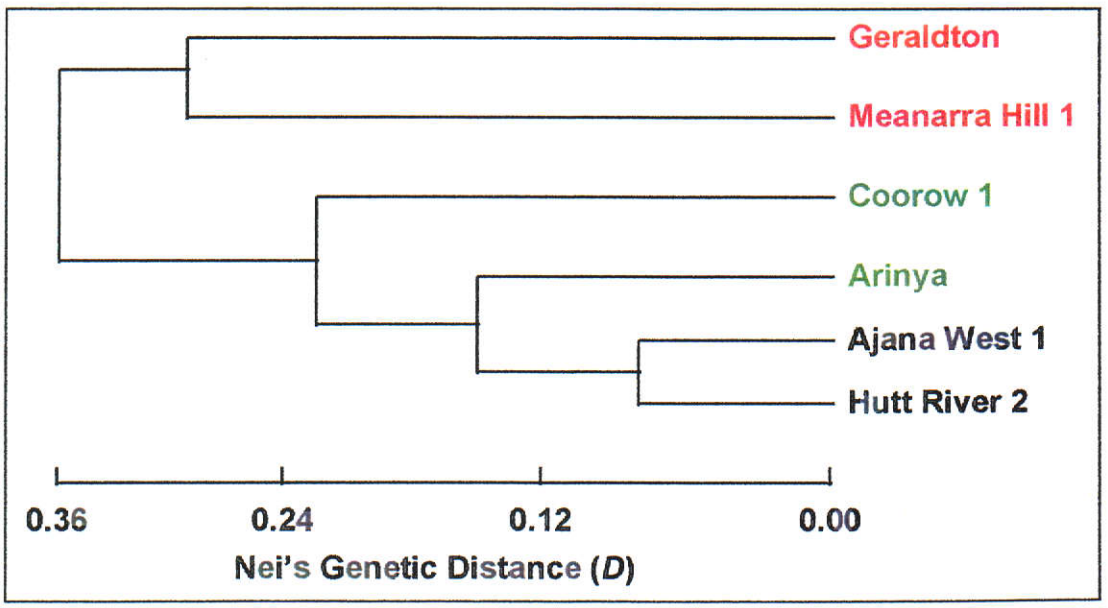


Figure 5.2: Cluster analysis of *G. verrucosa* populations based on Nei's genetic distance (*D*) and UPGMA algorithm. Small form; intermediate form; large form.

Nonmetric multidimensional scaling of populations was not as informative as the cluster analysis (Figure 5.3). Populations of the large form at Geraldton and Meanarra Hill 1 were quite separate as were the small form populations at Coorow 1 and Arinya. The intermediate form populations of Ajana West 1 and Hutt River 2 were placed to the centre of all populations.

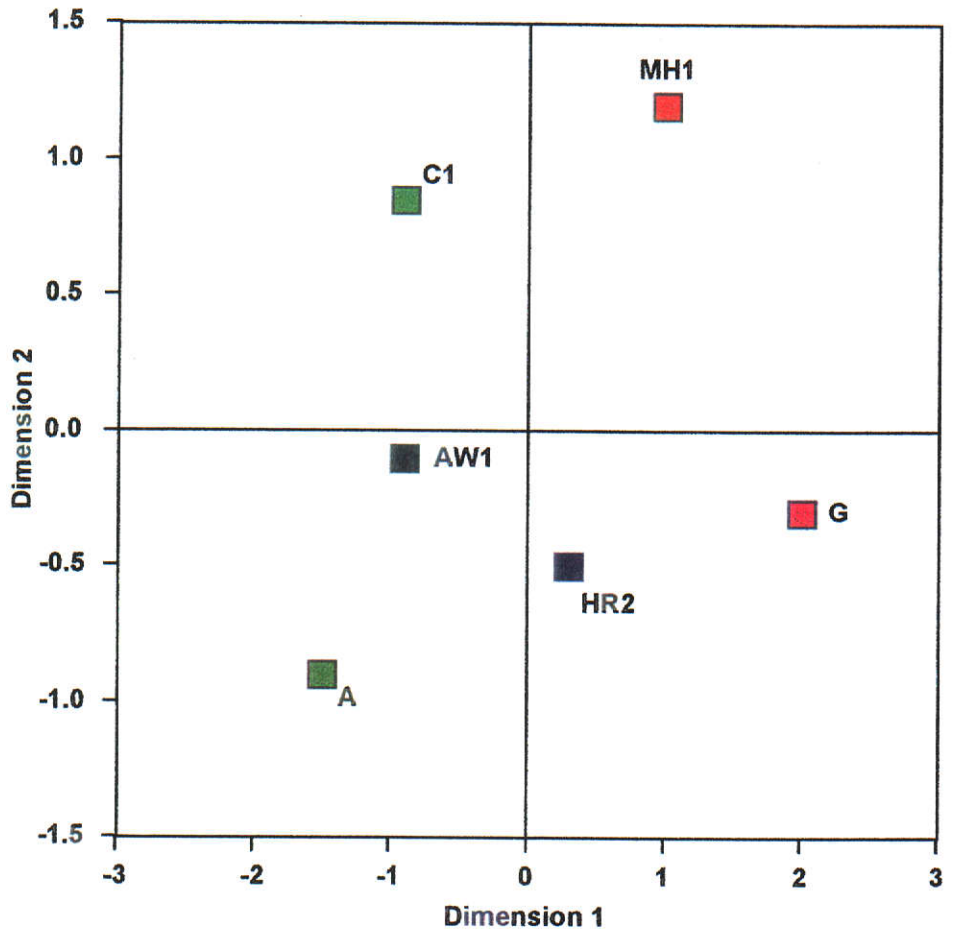


Figure 5.3: Nonmetric multidimensional scaling of *G. verrucosa* populations. Population codes in Table 5.1. Small form; intermediate form; large form.

Discussion

Although sample sizes in this investigation are lower than those used for the allozyme analysis (Chapter 4), RAPDs do detect a greater level of genetic diversity. This increased diversity is apparent in the unique multilocus genotypes identifying each individual. An increasing number of investigations which have also detected higher levels of diversity using RAPDs (Liu and Furnier, 1993; Peakall *et al.*, 1995; Waycott, 1995; Szmidt

et al., 1996; Sonnante *et al.*, 1997; Waycott *et al.*, 1997). This investigation also highlights the utility of RAPDs to fingerprint individual plants. Differences in the levels of diversity revealed by these techniques may be explained by the type of polymorphism each method detects. Allozymes are a functional group of enzymes and polymorphisms reflect changes to the coding sequence of genes. In contrast, DNA polymorphisms, such as those detected by RAPDs, occur in both coding and non-coding regions (Bachmann, 1994).

Investigations comparing RAPD and allozyme markers generally indicate good congruence between analyses. Increased levels of genetic diversity associated with RAPDs did not alter the patterns of diversity in *Populus tremuloides* and *P. grandidenata* (Liu and Furnier, 1993), while heterozygosities and population fixation indices also agree in *Pinus mariana* (Isabel *et al.*, 1995). In *Posidonia australis,* multilocus genotypes are similarly distributed regardless of the molecular marker employed (Waycott, 1995). Comparable RAPD and allozymic relationships exist between *Abies alba* and *A. nebrodensis* (Vicario *et al.*, 1995) but RAPDs are a better reflection of systematic relationships between *Vigna luteola* and *V. marina* than allozymes (Sonnante *et al.*, 1997).

Notwithstanding the small sample size and lack of replication in this investigation, the patterns of morphological and allozyme diversity exhibited by the *G. verrucosa* complex are also reflected by RAPD variation. The large form is clearly resolved from the other two forms by cluster analysis, indicating the morphological and genetic divergence of this form. Although nonmetric multidimensional scaling does not clearly resolve relationships between forms, the separation between the large form populations at Meanarra Hill 1 and Geraldton and placement of this latter form near the small form at Arinya may be significant.

Plants at the Geraldton population have a similar habit to those in the Arinya population (Chapter 1, Figure 1.5), but flower size and abundance is

reminiscent of the large form. Relationships indicated by nonmetric multidimensional scaling suggest a closer evolutionary relationship between the Geraldton and intermediate form populations than revealed by the other analyses. The Geraldton population may result from a hybridisation event between the small and large forms creating a population of mixed morphological characters (Chapters 2 and 4). As such, this population might be expected to exhibit molecular and morphological characteristics common to both these forms.

Relationships between the small and intermediate forms based on RAPD markers differ to those based on allozymes. In this investigation, the intermediate form is placed within the small form while allozymes nest the small form within the intermediate form (Chapter 4, Figure 4.3). Although this suggests these forms are not divergent, evidence from other sources indicates this may not be the case. At Ajana West 2, where the small and intermediate forms co-occur, morphological and phenological integrity remains intact despite their close proximity (Chapters 2 and 3).

Although the morphological characters of widely distributed species can form distinct geographic patterns (Thorpe, 1976), evidence from this investigation supports previous analyses (Chapters 2 and 4), which indicate that morphological divergence in the *G. verrucosa* complex has a strong genetic basis. This also provides further support for systematic revision of the genus. The somewhat blurred boundaries between the small and intermediate forms do, however, make systematic clarification difficult, especially given the possibility that this form is the product of multiple hybridisation events.

CHAPTER 6

Variation across a zone of hybridisation

Introduction

Many organisms do not fit traditional species concepts (Hopper, 1995). This lack of conformity has been attributed to geographic distribution, differences in reproductive strategy, environmental heterogeneity and founder effect (Gottlieb, 1972; Endler, 1977). More frequently, however, hybridisation and the subsequent stabilisation of hybrid derivatives, has been found to obscure systematic boundaries (Gottlieb, 1972).

When genetically distinct groups of individuals meet, offspring of mixed ancestry, or hybrids, are sometimes produced (Harrison, 1990). Although many related taxa are reproductively isolated by physical and physiological mechanisms, in reality, these barriers are often semi-permeable, permitting localised hybridisation between sympatric, but non-interbreeding taxa (Grant, 1981; Harrison, 1986). Hybridisation is thought to proceed through either the development of clines between contiguous populations (primary intergradation) or, convergence between allopatrically divergent populations (secondary contact) (Mayr, 1942; Endler, 1977; Harrison and Rand, 1989). Since both processes produce similar patterns of variation, it is often impossible to determine which of the two has influenced the evolution of a hybrid zone (Endler, 1977; Harrison, 1993).

Hybridisation is common within Angiosperms (Mayr, 1970). Plant hybrid zones are usually comprised of complex hybrid swarms consisting of parental forms, F_1 hybrids, backcross types and segregation products (Mayr, 1970; Grant, 1981; Harrison, 1993). The sedentary nature of plants places many hybrids in close proximity to their progenitors, providing an opportunity for crossing and backcrossing with the parental types. Occasionally introgression, the permanent incorporation of genes from one set of differentiated populations into another, occurs (Rieseberg and

Wendel, 1993). Introgression is associated with increased genetic diversity through recombination and increased mutation, the origin and transfer of adaptations, the origin of new genotypes and the breakdown or reinforcement of reproductive isolating mechanism (Harrison, 1990; Rieseberg and Wendel, 1993).

The complex patterns of variation associated with hybridisation and introgression are often dependent on the allele/locus under consideration (Barton and Gale, 1993; Harrison, 1993). Repeated crossing between hybrids and one parental type can produce a population resembling the recurrent parent, but varying in the direction of the other (Grant, 1981). Alternatively, offspring may be recombinants exhibiting properties different to both parents (Harrison, 1993). Differential selection can result in a universally favourable allele spreading throughout the population while other loci favoured according to environmental or genetic backgrounds, can be maintained despite random mating (Barton and Gale, 1993). Female- and male-dispersed traits may introgress differentially through asymmetric mating success (Young, 1996). Further, random events which have no appreciable effect on fitness, can also be reflected in the genetic structure of hybrid zones (Barton and Gale, 1993).

The role of hybridisation and introgression in speciation and the eventual fate of hybrids zones is the subject of considerable debate (Harrison and Rand, 1989; Rieseberg, 1995). Although hybridisation can produce ecologically or behaviourally inviable or sterile individuals, it may also perfect isolating mechanisms (assuming hybrids are rare and inviable), create new species and/or increase genetic variability (Mayr, 1970; Richards, 1997). Endler (1977) suggests hybrid zones will either broaden through introgressive hybridisation or disappear through completion of speciation. Hybridisation may also threaten parental integrity, as well as blurring systematic boundaries by creating 'semispecies' (Grant, 1981; Rieseberg and Wendel, 1993). In some cases, species distinctiveness is unaffected by hybridisation (Grant, 1981).

Hybridisation within the flora of the southwest of Western Australia appears to be less pronounced than other regional floras (Hopper, 1995). The characteristic small disjunct populations have experienced extended periods of isolation (Hopper, 1995), suggesting opportunities for hybridisation are rare. Mechanisms which prevent hybridisation should taxa again come into contact, may be further perfected following population disjunction. Within the transitional-rainfall zone, high species diversity, allopatric replacement of closely related species, cryptic species complexes and hybridity are known (Hopper, 1979; 1992). The floral antiquity within this region suggests that hybridisation events are probably ancient and possibly, are more common than previously thought. With the advent of molecular markers, the difficulties detecting such events may now be overcome.

Aims of this investigation

The wide distribution of *G. verrucosa* throughout the transitional-rainfall zone and morphological intermediacy of some populations strongly suggest that hybridisation has occurred. Patterns of allelic distribution and genetic differentiation between the three forms (Chapter 4), support the view that hybridisation is a significant evolutionary event within the complex. Hybridisation is well known within other members of Rutaceae, in particular the genera *Phebalium*, *Boronia*, *Correa* and *Eriostemon* (Hopper, 1995). The occurrence of both large and small forms at the Pleshkes site provided an opportunity to investigate the patterns of morphological and allozymic variation within each form, and within what appeared to be a zone of hybridisation.

Materials and Methods

Sampling strategy

Two sampling sites approximately 500 m apart were selected to represent the small and large forms at Pleshkes and were designated Small (S) and Large (L) respectively. Within each site ten plants were tagged, numbered

and mapped. Where the two forms converged, twenty plants were similarly treated along a 100 m transect at right angles to the zone of integration. This site was designated as Transect (T). Each transect plant was assigned to a form and those which could not be readily classified were described as "hybrids" (Table 6.1).

Table 6.1: Morphological classification of individuals within the Transect at Pleshkes.

Plant No.	Form	Plant No.	Form
T1	Small	**T11**	Small
T2	Small	**T12**	"Hybrid"
T3	Small	**T13**	Large
T4	"Hybrid"	**T14**	Large
T5	Small	**T15**	"Hybrid"
T6	"Hybrid"	**T16**	Large
T7	"Hybrid"	**T17**	Large
T8	Small	**T18**	Large
T9	"Hybrid"	**T19**	Large
T10	"Hybrid"	**T20**	Large

This population represented a complex mix of individuals and forms located at one of three sites. To clearly illustrate associations between individuals, forms and sites, a coding system of numbers, abbreviations and symbols was adopted. Each individual was coded by the site letter and plant number. To denote the form to which each individual belonged, the small form was represented by ▲, the large form by ■ and the "hybrid" form by ●. For example, T4● designated the "hybrid" form Plant 4 from the Transect.

Morphometric analyses

The morphological character means for each plant within the three sites were standardised by zscores and nonmetric multidimensional scaling and cluster analysis conducted as described in Chapter 2.

Allozyme analysis

Inflorescences were collected, transported and processed as outlined in

Chapter 4. For this analysis, each plant was considered as a single population and missing data coded with the most common allele. In the absence of any other suitable measure of genetic relatedness, Nei's (1972) genetic distance was used to produce the cluster analysis based on the unweighted pair-group average method using arithmetic means (UPGMA). All computations were undertaken using BIOSYS-1 (Swofford and Selander, 1981).

Results

Morphometric analyses

Kruskal's stress measure (0.159) and the squared correlation coefficient (r^2 = 0.876) for nonmetric multidimensional scaling indicated the two dimensional Euclidean model described the data reasonably well. Although no clear dichotomy between the forms was observed, most plants tended to group within their designated form (Figure 6.1). The "hybrid" form plants were variously placed. A small grouping of these plants (T4, T6, T9 and T15) occurred between the small and large forms while the plants T7 and T12 were placed nearer to the small form plants. The plant T10 had stronger affinities with the large form than the small.

Some plants, however, did not fall with their expected grouping. The small form plant T1, fell within the "hybrid" cluster while another small form plant, T2, was placed some distance to all other plants. The large form plant L5 was also more closely allied with the small form and associated with the "hybrid" plant T7.

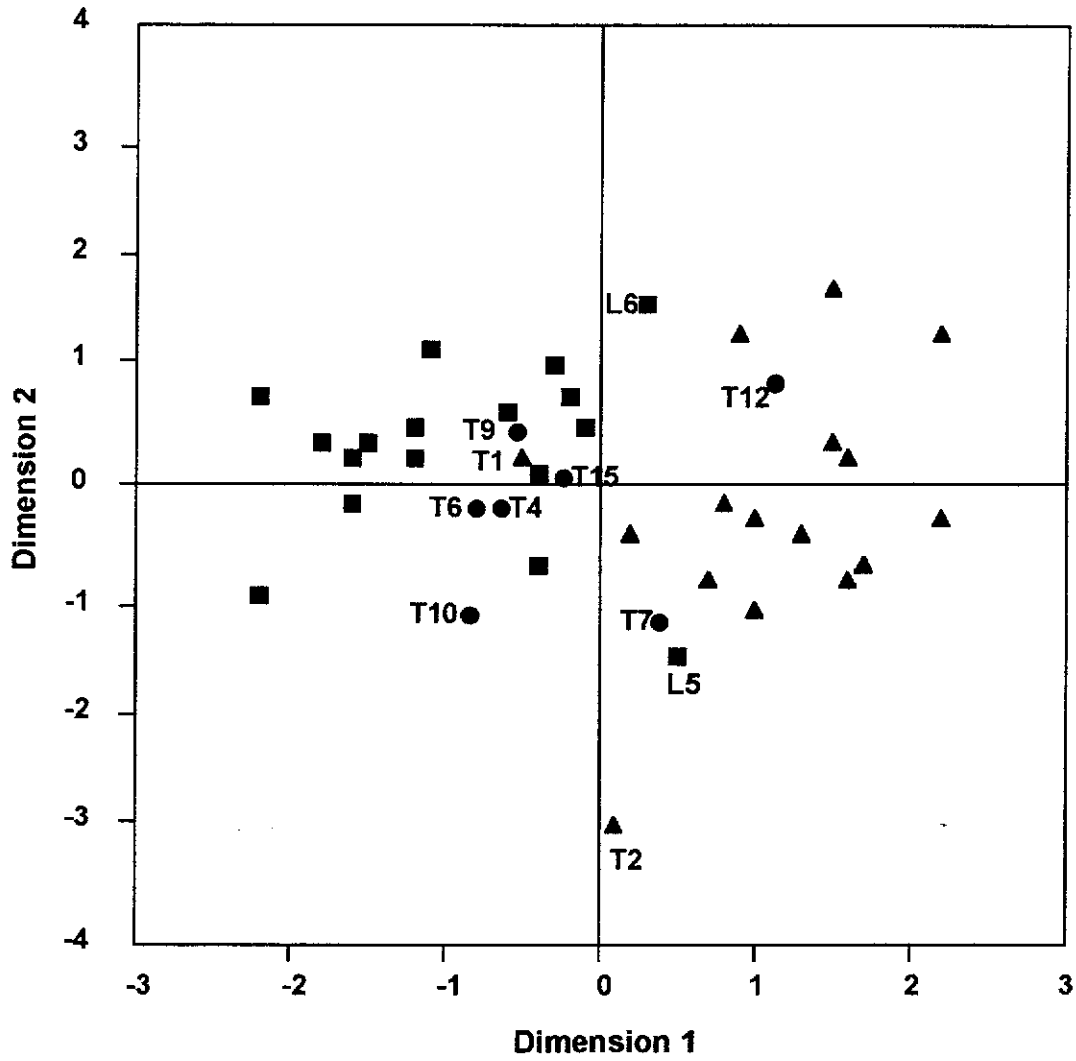


Figure 6.1: Nonmetric multidimensional scaling of individuals at Pleshkes based on Euclidean distances and UPGMA algorithm. ▲ small form, ● "hybrid" form; ■ large form. Only plants specifically referred to in the text are numbered.

Cluster analysis produced two groups which coincided with the small and large forms (Figure 6.2). With the exception of T1 and T2, which fell within the large form, all small form plants grouped together. Again plants from within the transect did not always fall with their expected form. The "hybrid" plants T7 and T12 were classified as small form plants, while the remaining "hybrid" plants were grouped with the large form in the second cluster.

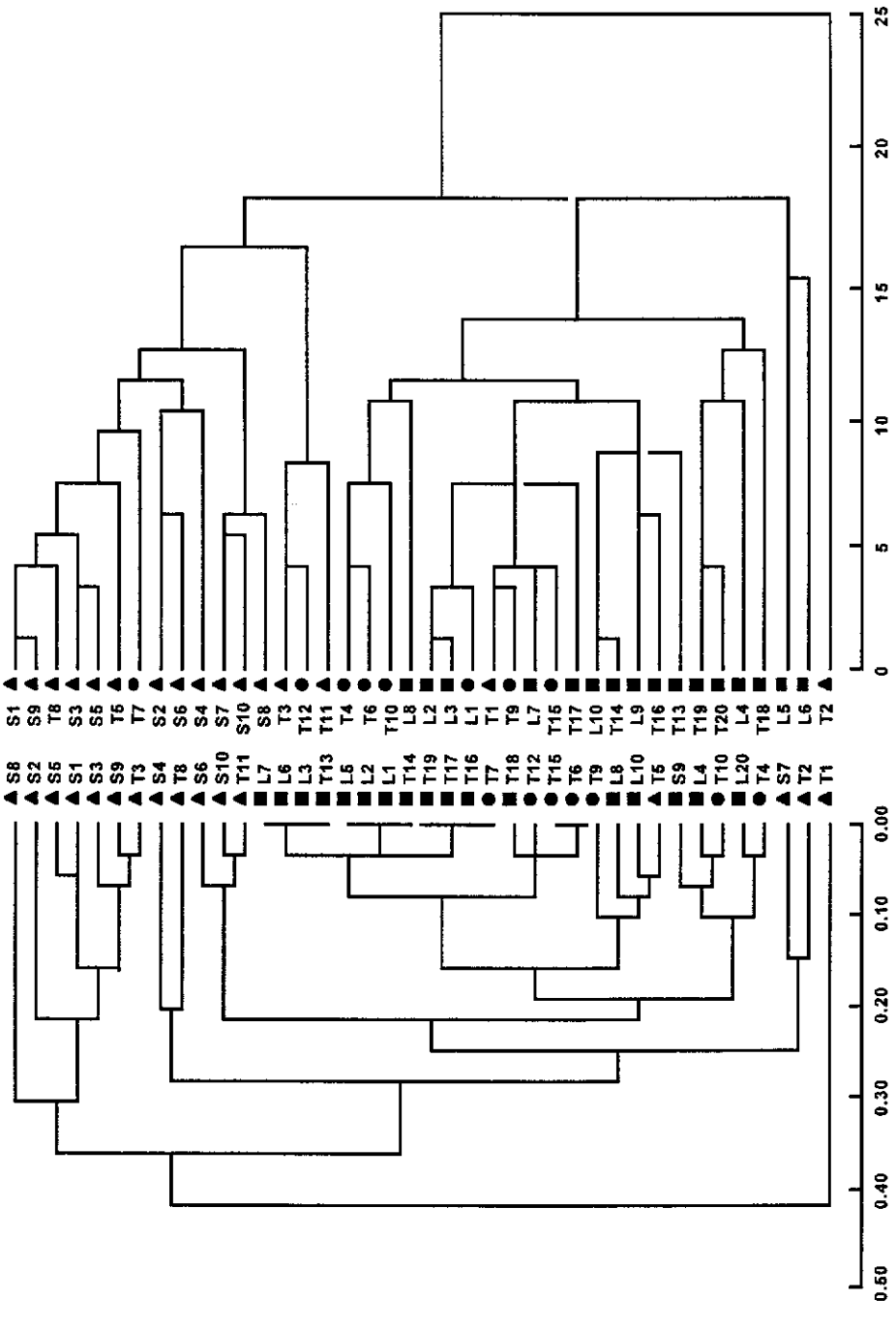


Figure 6.2: Comparison of allozyme (left) and morphometric (right) cluster analyses. ▲, small form; ●, "hybrid" form; ■, large form. S, small form site; T, transect; L, large form site. Number is plant number within site.

Allozyme analysis

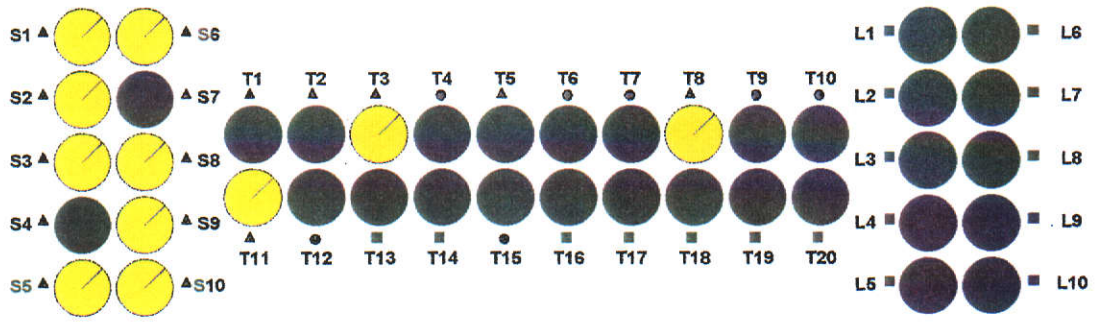
Six loci were monomorphic within the site: *Est*-1, *Idh*-1, *Mdh*-1, *Mr*-2, *Mr*-3 and *Pgm*-1, and heterozygous individuals were rare at most other loci. Allelic distributions within the five *Aat* loci were interesting and suggested patterns consistent with hybridisation and introgression.

The allele *Aat*-1^a [1] in other *G. verrucosa* populations surveyed, was either fixed, or at very high frequencies (Chapter 4, Table 4.2). At the Pleshkes population, all the large form plants, and those designated as being the "hybrid" form within the transect, were homozygous for this allele (Figure 6.3, top). Although five small form plants also exhibited this allele (S4, S7, T1, T2 and T5), the alternate and unique allele, *Aat*-1^b, was prevalent in this form.

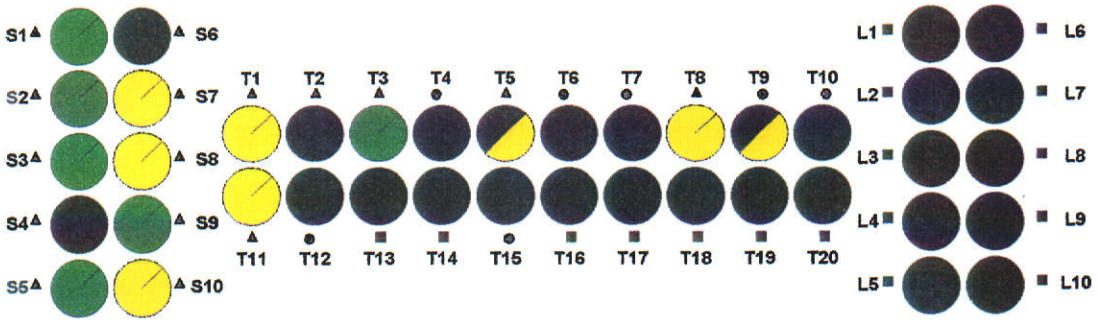
Three small form Pleshkes individuals (S4, S6 and T2), exhibited the allele *Aat*-2^a which was otherwise restricted to the large form populations (Figure 6.3, middle). Two other individuals from within the transect, the small form plant T5 and the "hybrid" form plant T9, were heterozygous for the alleles *Aat*-2^a and *Aat*-2^b. The allele *Aat*-2^c was restricted to the small form.

All three *Aat*-3 alleles were exhibited by the small form plants at Pleshkes (Figure 6.3, bottom). One of these alleles, *Aat*-3^c, was unique and rare to this population, being observed in only one small form plant (S2). The alleles *Aat*-3^a and *Aat*-3^b were observed in both the small and large forms, but were unevenly distributed between them. The *Aat*-3^b allele was more prevalent in the small form, while the allele *Aat*-3^a was more common in the large. Most of the "hybrid" form plants in the transect exhibited the allele *Aat*-3^a. Two transect individuals, the small form T5 and the "hybrid" form T9, were heterozygous for the alleles *Aat*-3^a and *Aat*-3^b.

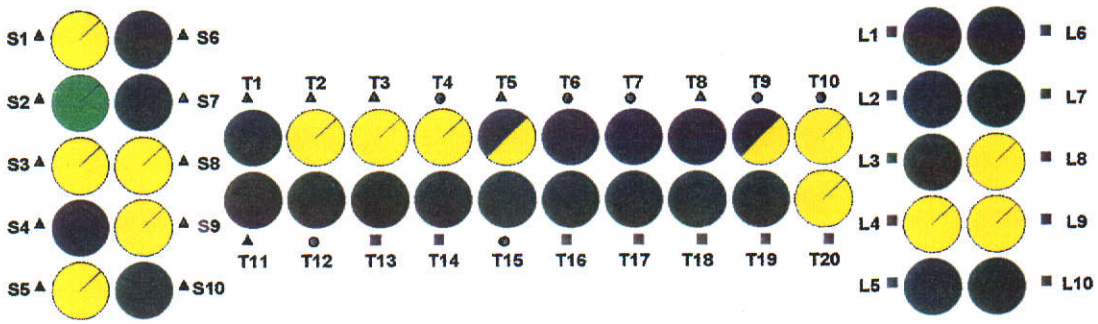
[1] Abbreviation summarises enzyme system, locus and allele. For example, *Aat*-1^a is allele a at locus 1 for aspartate aminotransferase.



Aat-1



Aat-2



Aat-3

Figure 6.3: Allelic distribution within the *Aat*-1, *Aat*-2 and *Aat*-3 loci at Pleshkes. ▲ small form; ● "hybrid" form; ■ large form. S, small form site ; T, transect; L, large form site. Number indicates plant within site.

The allele *Aat*-4^{a} dominated in the large form *G. verrucosa* populations, while the alternate allele *Aat*-4^{c}, was prevalent in most of the small and intermediate form populations (Chapter 4, Table 4.2). At Pleshkes, the large form allele (*Aat*-4^{a}), was observed in over half of the small form plants (S1, S4, S6, S7, S10, T1, T2, T8 and T11) and all the "hybrid" form plants (Figure 6.4, top).The remaining small from plants exhibited the alternate alleles *Aat*-4^{b} and *Aat*-4^{c}.

All three *Aat*-5 alleles were also observed in the small form at Pleshkes (Figure 6.4, bottom). The alleles *Aat*-5^{b} and *Aat*-5^{c} were observed elsewhere only in the large form populations. The "hybrid" form plant T5, was heterozygous for the allele *Aat*-5^{a} observed in all populations, and *Aat*-5^{c}, observed only in the large form population at Meanarra Hill 2.

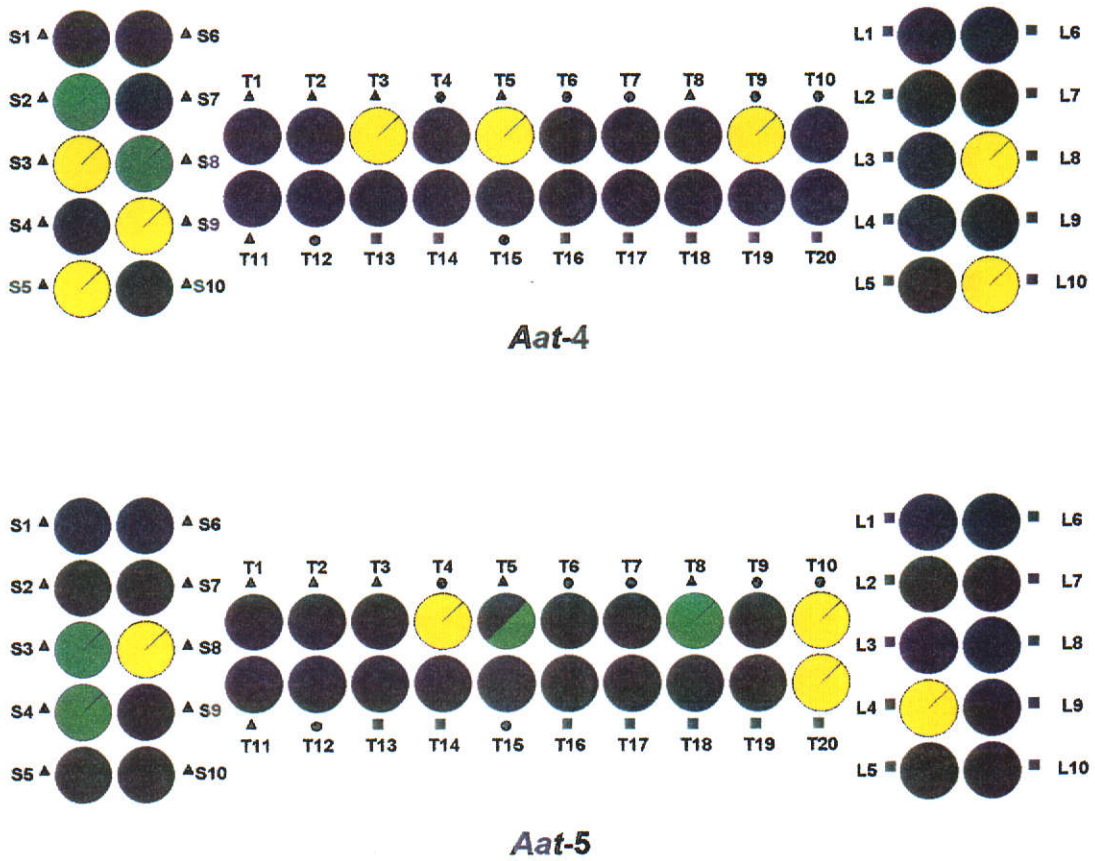


Figure 6.4: Allelic distribution within the *Aat*-4 and *Aat*-5 loci at Pleshkes. ▲ small form; ● "hybrid" form; ■ large form. S, small form site ; T, transect; L, large form site. Number indicates plant within site.

The relationships between individuals based on estimates of genetic distance (*D*) are summarised in Figure 6.2 (left). All the small form plants within Pleshkes, with the exception of the transect plant T5, clustered together. The placement of this plant is likely to be result from it being heterozygous for three of the five *Aat* loci, and exhibiting the large form allele *Aat*-1^b. The remaining large and "hybrid" form plants were also grouped together.

Discussion

Distribution patterns of morphological and genetic variation within the small and large forms, and across the intergradtion zone at Pleshkes, strongly suggest hybridisation and introgression has, and probably is, still occurring. Although the relationships between individuals in both the morphometric and allozyme analyses differed, plants do group within their respective forms. The small form is morphologically distinguishable from the large form, exhibits alleles not observed in any other population, as well as alleles which are present elsewhere in populations of the large form only.

The presence of the unique alleles *Aat*-1^b and *Aat*-3^c in the small form, suggests these have arisen independently within this population. The finding of novel alleles, or 'hybrizimes', is important. Not only are these indicative of hybridisation, hybrizimes are relatively unknown from within plant hybrid zones (Rieseberg and Wendel, 1993). Novel alleles have also been observed in another transitional-rainfall zone hybrid population between *Stylidium affine and S. caricifolium* (Coates and James, 1996). The possibility of a further example here highlights the complexity of floral evolution within this region and within *G. verrucosa*.

In addition to exhibiting novel alleles, allelic movement between the small and large forms at Pleshkes appears to be asymmetric. The allelic distribution at the *Aat*-1 locus suggest a movement of alleles from the large

form to the small form. This latter form also exhibits several alleles, *Aat*-2[a], *Aat*-4[a], *Aat*-5[b] and *Aat*-5[c], observed elsewhere only in the large form populations. The presence of rare heterozygous individuals within the transect suggests these may have arisen following hybridisation and gene exchange between the forms. The patterns of allelic distribution observed strongly suggest introgression is occurring from the large form into the small form. Such asymmetric hybridisation is well known from studies of several hybrid zones (Harrison, 1986; Potts and Reid, 1988; Keim *et al.*, 1989; Bacilieri *et al.*, 1996; Edwards-Burke *et al.*, 1997). Given the outcrossing nature of the large form and apparent selfing within the small form (Chapter 3), it is highly likely that allelic movement between the large and small forms is asymmetric.

Although reproductive barriers between the small and large form appear to be asymmetric, some seed is set, irrespective of pollen parent (Chapter 3). If the intermediate form has arisen from hybridisation events between the small and large forms, the crossing of this form back to the parental types is an important indicator of its possible hybrid origins. Experimental crosses between the intermediate and large forms show that reasonable seed set occurs when the paternal parent is the intermediate form, but the reciprocal cross using the large form as the paternal parent produces a strong preferential self-pollinating response in the intermediate form. Such differences in reproductive response cannot be readily explained but may be accounted for by selection for or against certain genetic combinations. Unfortunately time and resources have prevented experimental reproductive crosses between the small and intermediate forms, but clearly, such information would further elucidate relationships between the forms.

The introgression of alleles from one form to the other may also contribute to the morphological placement of the small Pleshkes form with the intermediate form populations (Chapter 2, Figures 2.1 - 2.4). "Genetic swamping" through uneven introgression of large form alleles into the

small form, could be commensurate with an increase in physical size. Such an increase would more closely align this form with the intermediate form, than with the small form populations. Despite gene flow between the forms within the Pleshkes population, however, the small form still retains sufficient morphological integrity to distinguish it from the large form.

The shift in flowering phenology of the small form at Pleshkes further supports the hypothesis of gene flow between the forms. At this site, the small form flowers considerably earlier than at any other comparable population (Chapter 3, Table 3.3). It is unusual that this form at Pleshkes does not follow a similar pattern. Further north, at Ajana West 2, where the small and intermediate forms co-occur, flowering in the small form was consistent with that of other small form populations. The variation in flowering phenology of the small form at Pleshkes may be due to the incorporation of some genetic component which controls flowering in the large form, into the small form. The processes which explain the two forms at Pleshkes, however, remain unclear.

Since primary intergradation and secondary contact can both produce similar patterns of variation (Endler, 1977; Harrison, 1993), it is not possible to determine which has influenced the observed variation at Pleshkes. Forms may have either met and hybridised at this population or, have radiated into new habitats following speciation. The presence of alleles which coincide with the different forms (Chapter 4), as well as alleles unique to the small form within this population, do suggest that differences between forms have occurred allopatrically, and that this population represents a zone of secondary contact (Young, 1996). The possibility that allopatric forms of *G. verrucosa* have come into secondary contact is high. The genus occurs in a region influenced by recurrent late Tertiary-Quaternary climatic fluctuations (Hopper, 1992), implying range expansions and contractions have occurred, and on more than one occasion. Under these circumstances, hybridisation events may be both spatially and temporally distributed.

Allelic distribution and the partitioning of genetic variability within and between forms (Chapter 4), suggests hybridisation is a significant evolutionary feature within the *G. verrucosa* complex. This is further supported by the allelic distribution between the forms within the Pleshkes population and the presence of novel alleles. The distribution of most intermediate form populations between those of the small and large forms (Chapter 1, Figure 1.6) further indicates these are the product of hybridisation events following secondary contact.

CHAPTER 7

General Discussion

This investigation examines morphological, reproductive and genetic relationships within and between populations and forms of the enigmatic species, *Geleznowia verrucosa.* The three observed forms exhibit morphological and genetic separation, differences in the levels and distribution of genetic diversity, and contrasting reproductive strategies. This disparity between the forms strongly supports the view that this is a species' complex comprised of two taxa, and a series of hybrid derivatives. The small taxon is characterised by smaller leaf and flower characters with fewer flowers per inflorescence, while the large taxon exhibits larger morphological characters and more abundant flowers. Between these two taxa are a series of intermediate form populations which are morphologically allied with the large taxon, but genetically, are more closely related to the small taxon. It is now apparent that restricted geographic range, a fragmented population structure and hybridisation are significant evolutionary features within this complex.

G. verrucosa is geographically restricted and endemic to the southwest of Western Australia. At a population level, endemic taxa are expected to exhibit fewer polymorphic loci (*P*, 26%), with lower genetic diversity apportioned between populations rather than within (Hamrick and Godt, 1989; 1996). Selfing endemic taxa often exhibit even lower levels of genetic diversity (H_e, 0.074) and greater population differentiation (G_{st}, 0.510) (Hamrick and Godt, 1996). Many factors contribute to the levels of genetic diversity observed in endemic taxa. Small population size, restricted gene flow, genetic drift, inbreeding, and bottlenecks and founder effects are often significant contributors to reduced genetic diversity (Hamrick *et al.*, 1979; Loveless and Hamrick, 1984; Hamrick and Godt, 1989, 1996).

As expected of endemic taxa, both the small and large *G. verrucosa* taxa

apportion most of the genetic diversity within populations. Within these taxa, however, the levels of genetic diversity differ considerably. The mean percentage of polymorphic loci and expected heterozygosity in the large taxon (*P*, 36.3%, H_e, 0.131) are higher than levels reported for endemic taxa (*P*, 26.3%, H_e, 0.063) (Hamrick and Godt, 1989) while those of the small taxon are consistent (*P*, 26.1%, H_e, 0.100). Most *G. verrucosa* populations, irrespective of taxon, are small and disjunct, predisposing them to limited gene flow, drift and fixation, as well as to intense fluctuations in size and associated founder effects (Hamrick *et al.*, 1979; Loveless and Hamrick, 1984; Karron, 1987; Hamrick and Godt, 1989). Such conditions are highly conducive to producing both the population genetic structure and low levels of genetic diversity observed within each of these taxa.

Bottlenecks and/or associated founder effects may contribute to the genetic structure of many *G. verrucosa* populations. Being a disturbance opportunist, populations characterised by diminished genetic diversity possibly reflect intense fluctuations in size. Although the soil seed bank can significantly influence the evolutionary potential of populations, and act as a genetic buffer against small population size (McCrue and Holtsford, 1998), the genetic diversity of founder stock is an important contributor to population genetic structure and genetic diversity (Taggart *et al.*, 1990). In *G. verrucosa*, resources in the soil seed bank are rapidly turned over (Roche, 1998), effectively restricting the amount of seed available for seedling recruitment following disturbance, as well as limiting the genetic contribution of previous generations. While intense fluctuations in population size may be important in individual populations, these are unlikely to explain the differences in genetic diversity observed between the small and large taxa.

Differences in the levels of genetic diversity between the taxa also reflect the contrasting reproductive strategy(ies) employed. The significantly lower level of natural seed set in the large taxon (43%), compared to that of the small taxon (75%), provides evidence of different reproductive strategies

(Schoen, 1977; Solbrig and Rollins, 1977). Following experimental pollination treatments, the small taxon exhibits strong selfing mechanisms while a mixed mating system exists within the large taxon. The predominantly selfing nature of the small taxon probably maintains the high levels of observed homozygosity whereas the increase in heterozygosity associated with the large taxon reflects the higher levels of outcrossing (Levin, 1978; Brown, 1979; Hamrick *et al.*, 1979; Gottlieb, 1981; Hamrick and Godt, 1989; Richards, 1997). In the small taxon, a selfing reproductive strategy is concomitant with the investment of fewer resources in floral attractants, but higher seed production (Jain, 1976; Schoen, 1977, 1982; Solbrig and Rollins, 1977; Wyatt, 1984; Cruden and Lyon, 1985; Barrett, 1988; Ritland and Ritland, 1989). This higher fecundity may also compensate for a continually depleted soil seed bank. In the large form, depletion and lower natural seed set are likely to be offset by mass flowering.

The contrasting reproductive strategies, morphological integrity and differences in levels of genetic diversity between the small and large *G. verrucosa* taxa, are suggestive of a progenitor-derivative relationship. Derivative taxa are often self-fertilising, exhibit lower levels of genetic diversity and are distributed peripherally to their progenitors (Stebbins, 1970; Gottlieb, 1973, 1974; Grant, 1975; Gottlieb and Pilz, 1976; Jain, 1976; Lloyd, 1980; Purdy *et al.*, 1994). In this complex, the higher levels of allelic diversity and outcrossing in the large taxon, indicate this may be the progenitor of the small self-fertilising taxon which exhibits lower levels of allelic diversity, and is peripherally distributed to the large taxon.

The evolutionary processes giving rise to the small taxon are likely to be associated with movement into new niches not inhabited by the large taxon. The location of this latter taxon in the northwest, would mean a movement of the small *G. verrucosa* taxon to the southeast, into a harsher and more unpredictable environment. As might be expected of newly evolved taxa, a shift towards selfing in this taxon would ensure reproductive success,

particularly following colonisation into new environments where cohorts and pollinators may be absent (Stebbins, 1970; Jain, 1976; Wyatt, 1984; Cruden and Lyon, 1985).

An alternative evolutionary hypothesis would place the intermediate form as the progenitor to both the small and large taxa. In contrast to these latter taxa, genetic diversity in the intermediate form was mostly apportioned between populations (D_{ST}, 0.121), although relatively high variation within populations (H_S, 0.105) exists. In addition, increased heterozygosity of the *Lap* locus, and the considerably higher interpopulation differentiation (G_{st}, 58%), indicate these populations are more likely to be of hybrid origin, rather than a progenitor to the small and large taxa.

The presence of this intermediate form indicates hybridisation is an important evolutionary event within the complex. Hybridisation can be prevented by reproductive isolation, but in reality, many reproductive barriers are semi-permeable, permitting hybridisation between distinct and sympatric taxa (Grant, 1981). In *G. verrucosa*, pollination experiments indicate that reproductive barriers between taxa are incomplete and where different taxa co-occur, hybridisation can take place. The patterns of allelic distribution between the small and large taxa at Pleshkes reveal limited asymmetric gene exchange. Although gene exchange at this population is probably ongoing, disjunction precludes gene flow between most other populations, suggesting that the hybrid origins of the intermediate form populations are more ancient.

Although the disjunction of extant *G. verrucosa* populations precludes gene flow in most cases, this does not necessarily reflect historical distribution (Karron, 1987; Karron *et al.*, 1988). This species' complex occurs in a floristically rich and diverse region of Western Australia, where many species have restricted, but fragmented, distributions. No major extinctions events such as glaciation, geological uplifting or volcanic activity, have occurred since the Permium. In more recent times, frequent unpredictable

Quaternary climatic fluctuations have occurred in the transitional-rainfall zone where the *G. verrucosa* taxa reside (Hopper, 1979; 1992), and the region is now characterised by a mosaic of relictual and recently speciated taxa. Range expansions and contractions during climatic perturbations, could have brought the small and large *G. verrucosa* taxa together, facilitating hybridisation across a wide distribution and on several different occasions.

Spatially and temporally distributed hybridisation events between the small and large *G. verrucosa* taxa have been governed by genetic exchange and recombination on two levels. Within each taxon, distinct genetic lineages exist. Limited gene flow between populations and self-fertilisation in the small form, are likely to perpetuate such lineages. Hybridisation events would not only reflect the meeting of divergent taxa, but also that of the genetic lineages within them. A series of intermediate, but highly distinctive, populations appears to be the result. The temporal separation of hybridisation events, has further exaggerated population differentiation between the intermediate form populations. Recent and ancient introgression following hybridisation between *H. annus* and *Helianthus petiolaris*, has generated diverse hybrid derivatives, giving rise to three new species (Rieseberg, 1991).

The higher levels of genetic diversity between intermediate form populations probably reflect a diverse array of hybrid products created by meetings between the small and large taxa. This genetic distinctiveness is probably further perpetuated by the strong preferential selfing mechanism of the intermediate form preventing introgression. Indeed, selfing is a useful strategy for preserving novel and adaptive genotypes, while also preventing the incorporation of "foreign" genes into the genome through outcrossing (Richards, 1997). In the intermediate form, selfing would ensure reproductive success and maintain hybrid fidelity.

This investigation has served to highlight several important evolutionary

events and forces within the *G. verrucosa* species' complex. It has also systematically clarified the complex, despite the existence of hybrid populations. Being a heterogeneous mix of parental types and derivatives, hybrid populations can be difficult to identify, and hence, resolve systematically. In *G. verrucosa*, this is even more difficult given that the intermediate populations probably result from both recent and ancient hybridisation events. It is unlikely such elucidation would have been possible without the use of complementary morphological and molecular approaches.

The morphological disparity between the small and large forms provides strong evidence of systematic divergence. The close morphological association between the intermediate and large forms is suggestive of phenotypic plasticity across a wide species' range. Since morphological differentiation between hybrids and one parental type are not always clear (Edwards-Burke *et al.*, 1997), the use of molecular markers can be extremely diagnostic. Following allozyme analysis, morphological intermediacy between *Festuca baffinensis* and *F. brachyphylla* has been ascribed to phenotypic plasticity, rather than hybridisation (Aiken *et al.*, 1994) while putative hybrids between *Carpobrotus edulis* and *C. chilensis* have been clearly characterised using a similar approach (Albert *et al.*, 1997; Gallagher *et al.* 1997).

Such comparisons between morphological and molecular data can be problematic given that these characters can have different environmental sensitivities, and may evolve independently (Harrison, 1986; Hillis, 1987; Patterson *et al.*, 1993; Michaud *et al.*, 1995). Pleiotropy, dominance and epistasis can also alter the relationship between genotype and morphological phenotype (Gallez and Gottlieb, 1982). Disparity between morphological and molecular markers in *Triticum turgidum* is probably explained by differential selection for agronomic traits (Tsegaye *et al.*, 1996), while that between *Layia discoridea* and *L. glandulosa* is attributable to recent divergence (Gottlieb *et al.*, 1985). The strong correlation between

morphological and molecular markers in other studies, however, has allowed systematic clarification (Wolff and Jefferies, 1987; Hoey *et al.*, 1996). In *G. verrucosa*, utilisation of contrasting, but complementary approaches, has served not only to clarify systematic relationships, but also to accentuate the complexity of the intermediate form and its hybrid origins.

Conclusions and comments

Species complexes are well documented within the highly speciated Western Australian flora. Evidence from this investigation has highlighted *G. verrucosa* to be a complex genus comprising of three morphologically recognisable forms. The high degree of morphological and genetic differentiation between the small and large forms supports the proposal that these represent distinct taxa and should be formally recognised as such. The intermediate form consists of a series of populations exhibiting mixed characters and are probably of hybrid origin.

Three events appear to be significant in the evolution of *G. verrucosa*. Firstly, the large taxon is the progenitor to the small taxon. Associated with this divergence, is a shift by the small taxon to a self fertilising reproductive strategy concomitant with a reduction in floral attractants and increased investment in seed production. This shift is also been associated with movement into a harsher and more unpredictable environment. Secondly, climatic events during the Tertiary and Quaternary have promoted range expansions and contractions of the small and large taxa, bringing them back into contact and facilitating both temporally and spatially distributed hybridisation. Finally, the morphological and genetic diversity exhibited by the intermediate form reflects the integration of genotypes from divergent taxa, as well as different genetic lineages within these taxa. The result is a series of distinctive hybrid populations. More recent evolution within the complex has been driven by endemism, small population size, isolation, bottlenecks and founder effects.

It is now apparent that formal recognition of the small and large taxa within the *G. verrucosa* complex is warranted. Revision will require reference to both the original descriptions of Bentham and Mueller (1863), the type specimens held at Kew Gardens and the guidance the foremost Rutaceae taxonomist Dr Paul Wilson, from the Western Australian Herbarium. The type specimens for *G. verrucosa* and *G. macrocarpa* are extremely similar, exhibiting the small leaves and flowers characteristic of the small taxon; the type specimen for *G. calycina* clearly represents the large taxon (Chapter 1, Figure 1.4). In the short term and for simplicity, it is recommended the small form be retained as *G. verrucosa* and the large form be reinstated as *G. calycina*. At this point, the intermediate hybrid form does not warrant formal recognition, and is best left as *G. verrucosa*.

Future directions

As with many investigations, this research has succeeded in posing more questions than it has been able to answer. It has, however, provided a valuable insight into the forces which have driven evolution in the *G. verrucosa* complex. Of particular interest is the probability of hybridisation between the small and large taxa. The mechanisms involved in such events are largely unexplored within the Western Australian flora since population disjunction provides little opportunity for extant populations to hybridise. The co-occurrence and integration between small and large taxa at Pleshkes provides an extremely valuable resource for future investigations into hybridisation and gene exchange within this population. Further assessment of genetic relatedness between the taxa and intermediate form using cpDNA and/or rDNA markers would be invaluable for further understanding the mechanisms of evolution within the genus, as well as hybridisation between them.

Research should also be directed towards establishing whether chromosomal races exist and if they do, whether these reflect the systematic boundaries as suggested by this examination. The mating

system and reproductive biology of the two taxa and intermediate form would also benefit from further examination and clarification.

It is also worth noting that *G. verrucosa* is a species under threat from habitat loss and commercial over-exploitation. Systematic clarification following the morphological, genetic, and reproductive insights obtained from this investigation, will provide invaluable information for the successful management of this important natural resource. Since few populations of the small and large taxa remain, these will require careful management, and perhaps a stronger conservation strategy, following formal systematic revision.

References

Aiken, S.G., Consaul, L.L., Spidle, A. and May, B. 1994. Allozyme and morphological observations on *Festuca hyperborea*, compared with *F. baffinensis* and *F. brachyphylla* (Poaceae) from the Canadian Arctic. ***Nordic Journal of Botany*** 14, 137-143.

Albert, M.E., D'Antonia, C.M. and Schierenbeck, K.A. 1997. Hybridization and introgression in *Carpobrotus* spp. (Aizoaceae) in California. I. Morphological evidence. ***American Journal of Botany*** 84(7), 896-904.

Alexander, M.P. 1980. A versatile stain for pollen, fungi, yeast and bacteria. ***Stain Technology*** 55(1), 13-18.

Antonovics, J. 1968. Evolution in closely adjacent plant populations V. Evolution of self-fertility. ***Heredity*** 23, 219-238.

Armstrong, J.A. 1983. Rutaceae In: Morley, B.D. and Toelken, H.R. (eds) **Flowering Plants in Australia.** Rigby Publishers, Adelaide. pp. 194-198.

Bachmann, K. 1994. Molecular markers in plant ecology. ***New Phytologist*** 126, 403-418.

Bacilieri, R., Ducousso, A., Petit, R.J. and Kremer, A. 1996. Mating system and asymmetric hybridization in a mixed stand of European oaks. ***Evolution*** 50(2), 900-908.

Baines, J.A. 1981. **Australian Plant Genera**. The Society for Growing Australian Plants, Sydney, Australia. 406 pp.

Baldwin, B.G., Kyhos, D.W. and Dvorak, J. 1990. Chloroplast DNA evolution and adaptive radiation in the Hawaiian silversword alliance (Asteraceae-Madiinae). ***Annals of the Missouri Botanical Gardens*** 77, 96-109.

Banyard, B.J. and James, S.H. 1979. Biosystematic studies in the *Stylidium crassifolium* species complex (Stylidiaceae). ***Australian Journal of Botany*** 27, 27-37.

Barrett, S.C.H. 1988. Evolution of breeding systems in *Eichhornia* (Pontederiaceae): A review. ***Annals of the Missouri Botanic Gardens*** 75, 741-760.

Barrett, S.C.H. and Eckert, C.G. 1990. Variation and evolution of mating systems in seed plants In: Kawano, S. (ed) **Biological Approaches and Evolutionary Trends in Plants.** Academic Press, London. pp.229-254.

Barrett, S.C.H. and Shore, J.S. 1987. Variation and evolution of breeding systems in the *Turnera ulmifolia* L. complex (Turneraceae). ***Evolution*** 41(2), 340-354.

Barrett, S.C.H., Harder, L.D. and Worley, A.C. 1996. The comparative biology of pollination and mating in flowering plants. ***Philosophical Transactions of the Royal Society of London*** 351, 1271-1280.

Barton, N.H. and Gale, K.S. 1993. Genetic analysis of hybrid zones In: Harrison, R.G. (ed) **Hybrid Zones and the Evolutionary Processes.** Oxford University Press, New York. pp. 13-45.

Baskauf, C.J., McCauley, D.E. and Eickmeier, W.G. 1994. Genetic analysis of a rare and widespread species of *Echinaceae* (Asteraceae). ***Evolution*** 48(1), 180-188.

Baum, D.A., Systma, K.J. and Hoch, P.C. 1994. A phylogenetic analysis of *Epilobium* (Onagraceae) based on nuclear ribosomal DNA sequences. ***Systematic Botany*** 19(3), 363-388.

Bell, D.A. and DeMarini, D.M. 1991. Excessive cycling converts PCR products to random length higher molecular weight fragments. ***Nucleic Acids Research*** 19(18), 5079.

Bentham, G. and Mueller, F. 1863. **Flora Australiensis: A Description of the Plants of the Australian Territory**. Lovell Reeve & Co., London. 508 pp.

Bogler, D.J. and Simpson, B.B. 1995. A chloroplast DNA study of the Agavaceae. ***Systematic Botany*** 20(2), 191-205.

Bogler, D.J. and Simpson, B.B. 1996. Phylogeny of Agavaceae based on ITS rDNA sequence variation. ***American Journal of Botany*** 83(9), 1225-1235.

Bousfield, L.R. and James, S.H. 1976. The behavior and possible cytoevolutionary significance of B chromosomes in *Dampiera linearis* (Angiospermae: Goodeniaceae). ***Chromosoma*** 55, 309-323.

Bradshaw, A. 1965. Evolutionary significance of phenotypic plasticity in plants. ***Advances in Genetics*** 13, 115-155.

Bradshaw, A.D. 1975. Population structure and the effects of isolation and selection In: Frankel, O.H. and Hawkes, J.G. (eds) **Crop Genetic Resources for Today and Tomorrow.** Cambridge University Press, London. pp. 37-51.

Broadhurst, L.M. 1995. ***Geleznowia verrucosa* Turcz. (Rutaceae): the health and status of recently surveyed populations.** A Report to the Department of Conservation and Land Management, Western Australia (Wildlife Branch). 38 pp.

Brown, A.H.D. 1979. Enzyme polymorphism in plant populations. ***Theoretical Population Biology*** 15, 1-42.

Brown, A.H.D. 1990. The role of isozyme studies in molecular systematics. ***Australian Systematic Botany*** 3, 39-46.

Brown, A.H.D. and Allard, R.W. 1970. Estimation of the mating system in open-pollinated maize populations using isozyme polymorphisms. ***Genetics*** 66, 133-145.

Brown, A.H.D. and Schoen, D.J. 1992. Plant population genetic structure and biological conservation In: Sanderlund, O.T., Hindar, K. and Brown, A.H.D. (eds) **Conservation of Biodiversity for Sustainable Development.** Scandinavian University Press, Oslo. pp. 88-104.

Brown, A.H.D. and Weir, B.S. 1983. Measuring genetic variability in plant populations In: Tanksley, S.D. and Orton, T.J. (eds) **Isozymes in Plant Genetics and Breeding** Part A. Elsevier Science Publishers B.V., Amsterdam. pp. 219-239.

Brown, A.H.D., Barrett, S.C.H. and Moran, G.F. 1985. Mating system estimation in forest trees: Models, methods and meanings In: Gregorius, H.-R. (ed) **Population Genetics in Forestry.** Springer-Verlag, Berlin. pp. 33-49.

Brown, A.H.D., Burdon, J.J. and Jarosz, A.M. 1989. Isozyme analysis of plant mating systems In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology.** Dioscorides Press, Portland, Oregon. pp. 73-86.

Butcher, P.A., Bell, J.C. and Moran, G.F. 1992. Patterns of genetic diversity and nature of the breeding system in *Melaleuca alternifolia* (Myrtaceae). ***Australian Journal of Botany*** 40, 365-375.

Byrne, M. 1996. Application of DNA markers to the genetics of the Australian flora In: Hopper, S.D., Chappill, J.A., Harvey, M.S. and George, A.S. (eds) **Gondwanan Heritage : Past, Present and Future of the Western Australian Biota.** Surrey Beatty & Sons, Chipping North, NSW. pp. 259-270.

Byrne, M. and James, S.H. 1991. Genetic diversity in the cycad *Macrozamia riedlei*. ***Heredity*** 67, 35-39.

Byrne, M. and Moran, G.F. 1994. Population divergence in the chloroplast genome of *Eucalyptus nitens*. ***Heredity*** 73, 18-28.

Byrne, M., Moran, G.F. and Tibbits, W.N. 1993. Restriction map and maternal inheritance of chloroplast DNA in *Eucalyptus nitens*. ***The Journal of Heredity*** 84(3), 218-220.

Carthew, S.M. 1993. Population genetic structure of *Banksia spinulosa*. ***Heredity*** 70, 566-573.

Castiglione, S., Wang, G., Damiani, G., Bandi, C., Bisoffi, S. and Sala, F. 1993. RAPD fingerprints for identification and for taxonomic studies of elite poplar (*Populus* spp.) clones. ***Theoretical and Applied Genetics*** 87, 54-59.

Chalmers, K.J., Waugh, R., Sprent, J.I., Simons, A.J. and Powell, W. 1992. Detection of genetic variation between and within populations of *Gliricidia sepium* and *G. maculata* using RAPD markers. ***Heredity*** 69, 465-472.

Chappill, J.A. and Ladiges, P.Y. 1992. Geographical variation in morphology of *Eucalyptus mannifera* and allied taxa. ***Australian Systematic Botany*** 5, 359-372.

Clegg, M.T. 1980. Measuring plant mating systems. ***BioScience*** 30(12), 814-818.

Clegg, M.T. 1990. Molecular diversity in plant populations In: Brown, A.H.D., Clegg, M.T., Kahler, A.L. and Weir, B.S. (eds) **Plant Population Genetics, Breeding and Genetic Resources**. Sinauer Associates Inc., Sunderland MA. pp. 98-115.

Clegg, M.T. and Allard, R.W. 1973. Viability versus fecundity selection in the slender wild oat, *Avena barbata* L. ***Science*** 181, 667-668.

Clegg, M.T. and Durbin, M.L. 1990. Molecular approaches to the study of plant biosystematics. ***Australian Systematic Botany*** 3, 1-8.

Clegg, M.T., Brown, A.H.D. and Whitfeld, P.R. 1984. Chloroplast DNA diversity in wild and cultivated barley: implications for genetic conservation. ***Genetic Research*** 43, 339-343.

Coates, D.J. 1981. Chromosome, morphometric and breeding system studies in the *Stylidium caricifolium* species complex (Stylidiaceae). ***Australian Journal of Botany*** 29, 397-417.

Coates, D.J. 1988. Genetic diversity and population genetic structure in the rare Chittering Grass Wattle, *Acacia anomala* Court. ***Australian Journal of Botany*** 36, 273-286.

Coates, D.J. 1992. Genetic consequences of a bottleneck and spatial genetic structure in the triggerplant *Stylidium coroniforme* (Stylidiaceae). ***Heredity*** 69, 512-520.

Coates, D.J. and Hnatiuk, R.J. 1990. Systematic and evolutionary inferences from isozyme studies in the genus *Eremaea* (Myrtaceae). ***Australian Systematic Botany*** 3, 59-74.

Coates, D.J. and James, S.H. 1979. Chromosome variation in *Stylidium crossocephalum* and the dynamic coadaption of its lethal system. ***Chromosoma*** 72, 357-376.

Coates, D.J. and James, S.H. 1996. Chromosome repatterning, population genetic structure and local speciation in southwestern Australian triggerplants (*Stylidium*) In: Hopper, S.D., Chappill, J.A., Harvey, M.S. and George, A.S. (eds) **Gondwanan Heritage : Past, Present and Future of the Western Australian Biota.** Surrey Beatty & Sons, Chipping North NSW. pp. 276-286.

Coates, D.J. and Sokolowski, R.E.S. 1992. The mating system and patterns of genetic variation in *Banksia cuneata* A.S. George (Proteaceae). ***Heredity*** 69, 11-20.

Coello, G., Escalante, A. and Soberon, J. 1993. Lack of genetic variation in *Lacandonia schimatica* (Lacandoniaceae: Triuridales) in its only known locality. ***Annals of the Missouri Botanical Garden*** 80(4), 898-901.

Cole, C.T. and Biesboer, D.D. 1992. Monomorphism, reduced gene flow, and cleistogamy in rare and common species of *Lespedeza* (Fabaceae). ***American Journal of Botany*** 79(5), 567-575.

Conn, B.J. 1984. A taxonomic revision of *Prosanthera* Labill. Section *Klanderia* (F.v. Muell.) Benth. (Labiatae). ***Journal of the Adelaide Botanic Gardens*** 6(3), 207-348.

Cook, I.O. and Ladiges, P.Y. 1991. Morphological variation within *Eucalyptus nitens* s. lat. and recognition of a new species, *E. denticulata*. ***Australian Systematic Botany*** 4, 375-390.

Crawford, D.J. 1983. Phylogenetic and systematic inferences from electrophoretic studies In: Tanksley, S.D. and Orton, T.J. (eds) **Isozymes in Plant Genetics and Breeding Part A.** Elsevier Science Publishers B.V., Amsterdam. pp. 257-287.

Crawford, D.J. 1989. Enzyme electrophoresis and plant systematics In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology.** Dioscorides Press, Portland, Oregon. pp. 146-164.

Crowden, R.K. and Menadue, Y. 1990. Morphometric analysis of variation in the '*Epacris tasmanica* complex' (Epacridaceae). ***Australian Journal of Botany*** 3, 253-264.

Cruden, R.W. 1977. Pollen-ovule ratios: A conservative indicator of breeding systems in flowering plants. ***Evolution*** 31, 32-46.

Cruden, R.W. and Lyon, D.L. 1985. Patterns of biomass allocation to male and female functions in plants with different mating systems. ***Oecologia*** 66, 299-306.

Dafni, A. 1992. **Pollination Ecology - A Practical Approach**. Oxford University Press, Oxford. 250 pp.

Davis, P.H. and Heywood, V.H. 1991. **Principles of Angiosperm Taxonomy**. Today & Tomorrow's Printers & Publishers, New Delhi. 558 pp.

Delouche, J.C. 1980. Environmental effects on seed development and seed quality. ***HortScience*** 15(6), 775-780.

Dixon, K.W., Roche, S. and Pate, J.S. 1995. The promotive effect of smoke derived from burnt native vegetation on seed germination of Western Australian plants. ***Oecologia*** 101, 185-192.

Dowling, T.E., Moritz, C., Palmer, J.D. and Rieseberg, L.H. 1996. Nucleic Acids III: analysis of fragments and restriction sites In: Hillis, D.M., Moritz, C. and Mable, B.K. (eds) **Molecular Systematics.** Sinauer Associates, Inc., Sunderland MA. pp. 249-320.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. ***Phytochemical Bulletin*** 19(1), 11-15.

Doyle, J.J., Doyle, J.L. and Brown, A.H.D. 1990. Analysis of a polyploid complex in *Glycine* with chloroplast and nuclear DNA. ***Australian Systematic Botany*** 3, 125-136.

Doyle, J.J., Doyle, J.L., Ballenger, J.A., Dickson, E.E., Kajita, T. and Ohashi, H. 1997. A phylogeny of the chloroplast gene *rbc*L in the Leguminosae: taxonomic correlations and insights into the evolution of nodulation. ***American Journal of Botany*** 84(4), 541-580.

Duvall, M.R., Clegg, M.T., Chase, M.W., Clark, W.D., Kress, W.J., Hills, H.G., Eguiarte, L.E., Smith, J.F., Gaut, B.S., Zimmer, E.A. and Learn, G.H. 1993. Phylogenetic hypotheses for the monocotyledons constructed from *rbc*L sequence data. *Annals* ***of the Missouri Botanical Gardens*** 80, 607-619.

Edwards-Burke, M.A., Hamrick, J.L. and Price, R.A. 1997. Frequency and direction of hybridization in sympatric populations of *Pinus taeda* and *P. echinata* (Pinaceae). ***American Journal of Botany*** 84(7), 879-886.

Elliot, G. 1990. **Australian Plants Identified**. Hyland House, Melbourne. 232 pp.

Elliot, W.R. and Jones, D.L. 1986. **Encyclopaedia of Australian Plants**. Lothian Publishing Company Pty Ltd, Melbourne. 447 pp.

Ellis, M.F. and Sedgley, M. 1992. Floral morphology and breeding system of three species of *Eucalyptus*, section *Bisectaria* (Myrtaceae). ***Australian Journal of Botany*** 40, 249-262.

Ellstrand, N.C. and Roose, M.L. 1987. Patterns of genotypic diversity in clonal plant species. ***American Journal of Botany*** 74(1), 123-131.

Ellsworth, D.L., Rittenhouse, K.D. and Honeycutt, R.L. 1993. Artifactual variation in randomly amplified polymorphic DNA banding patterns. ***BioTechniques*** 14(2), 214-217.

Endler, J.A. 1977. **Geographic variation, speciation, and clines**. Princeton University Press, Princeton, New Jersey. 246 pp.

Galen, C. and Kevan, P.G. 1980. Scent color, floral polymorphisms and pollination biology in *Polemonium viscosum* Nutt. ***The American Midland Naturalist*** 140(2), 281-289.

Gallagher, K.G., Schierenbeck, K.A. and D'Antonia, C.M. 1997. Hybridization and introgression in *Carpobrotus* spp. (Aizoaceae) in California. I. Allozyme evidence. ***American Journal of Botany*** 84(7), 905-911.

Gallez, G.P. and Gottlieb, L. 1982. Genetic evidence for the hybrid origin of the diploid plant *Stephanomeria diegensis*. ***Evolution*** 36, 1158-1167.

Giovannoni, J.J., Wing, R.A., Ganal, M.W. and Tanksley, S.D. 1991. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. ***Nucleic Acids Research*** 19(23), 6553-6558.

Gottlieb, L.D. 1972. Levels of confidence in the analysis of hybridization in plants. ***Annals of the Missouri Botanical Garden*** 59, 435-446.

Gottlieb, L.D. 1973. Genetic differentiation, sympatric speciation, and the origin of a diploid species of *Stephanomeria*. ***American Journal of Botany*** 60(6), 545-553.

Gottlieb, L.D. 1974. Genetic confirmation of the origin of *Clarkia lingulata*. ***Evolution*** 28, 244-250.

Gottlieb, L.D. 1981. Electrophoretic evidence and plant populations. *Progress* ***in Phytochemistry*** 7, 1-46.

Gottlieb, L.D. 1982. Conservation and gene duplication of isozymes in plants. ***Science*** 216, 373-380.

Gottlieb, L.D. and Pilz, G. 1976. Genetic similarity between *Gaura longiflora* and its obligately outcrossing derivative *G. demareei*. ***Systematic Botany*** 1, 181-187.

Gottlieb, L.D., Warwick, S.I. and Ford, V.S. 1985. Morphological and electrophoretic divergence between *Layia discoidea* and *L. glandulosa*. ***Systematic Botany*** 10(4), 484-495.

Grant, V. 1975. **Genetics of Flowering Plants**. Columbia University Press, New York. 514 pp.

Grant, V. 1981. **Plant Speciation**. Columbia University Press, New York. 563 pp.

Grattapaglia, D., Chaparro, J., Wilcox, P., McCord, S., Werner, D., Amerson, H., McKeand, S., Bridgwater, F., Whetten, R., O'Malley, D. and Sederoff, R. 1992. Mapping in woody plants with RAPD markers: Application to breeding in forestry and horticulture. **Applications of RAPD Technology to Plant Breeding.** Crop Science Society of America, Minneapolis, Minnesota. pp. 37-40.

Hair, J.F., Anderson, R.E., Tatham, R.L. and Black, W.C. 1992. **Multivariate Data Analysis**. Macmillan Publishing Company, N.Y. 544 pp.

Hamilton, M.B. and Mitchell-Olds, T. 1994. The mating system and relative performance of selfed and outcrossed progeny in *Arabis fecunda* (Brassicaceae). ***American Journal of Botany*** 81(10), 1252-1256.

Hamrick, J.L. 1982. Plant population genetics and evolution. ***American Journal of Botany*** 69(10), 1685-1693.

Hamrick, J.L. 1989. Isozymes and the analysis of genetic structure in plant populations In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology**. Dioscorides Press, Portland, Oregon. pp. 87-105.

Hamrick, J.L. and Godt, M.J.W. 1989. Allozyme diversity in plant species In: Brown, A.H.D., Clegg, M.T., Kahler, A.L. and Weir, B.S. (eds) **Plant Population Genetics, Breeding, and Genetic Resources.** Sinauer Associates Inc., Sunderland MA. pp. 43-63.

Hamrick, J.L. and Godt, M.J.W. 1996. Conservation genetics of endemic plant species In: Avise, J.C. and Hamrick, J.L. (eds) **Conservation Genetics.** Chapman & Hall, New York. pp. 281-304.

Hamrick, J.L. and Holden, L.R. 1979. Influence of microhabitat heterogeneity on gene frequency distribution and gametic phase disequilibrium in *Avena barbata*. ***Evolution*** 33(2), 521-533.

Hamrick, J.L., Linhart, Y.B. and Mitton, J.B. 1979. Relationships between life history characteristics and electrophoretically detectable genetic variation in plants. ***Annual Review of Ecology and Systematics*** 10, 173-200.

Harbourne, J.B. and Turner, B.L. 1984. **Plant Chemosystematics.** Academic Press, London. 562 pp.

Harrison, R.G. 1986. Pattern and process in a narrow hybrid zone. ***Heredity*** 56, 337-349.

Harrison, R.G. 1990. Hybrid zones: windows on evolutionary process. ***Oxford Surveys in Evolutionary Biology*** 7, 69-128.

Harrison, R.G. 1993. Hybrids and hybrid zones: Historical perspective In: Harrison, R.G. (ed) **Hybrid Zones and the Evolutionary Processes.** Oxford University Press, New York. pp. 3-12.

Harrison, R.G. and Rand, D.M. 1989. Mosaic hybrid zones and the nature of species boundaries In: Otte, D. and Endler, J.A. (eds) **Speciation and Its Consequences.** Sinauer, Sunderland MA. pp. 111-133.

Hebert, P.D.N. and Beaton, M.J. 1989. **Methodologies for allozyme analysis using cellulose acetate electrophoresis.** Helena Laboratories, Beaumont TX. 32 pp.

Hedberg, O. 1988. Classical taxonomy versus modern systematic studies in African botany. ***Monographs in Systematic Botany Missouri Botanical Gardens*** 25, 567-573.

Heslop-Harrison, J. and Heslop-Harrison, Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; Intracellular hydrolysis of fluorescein diacetate. ***Stain Technology*** 45(3), 115-120.

Heywood, V.H. 1967. **Plant Taxonomy**. Edward Arnold (Publishers) Ltd., London. 60 pp.

Hiebert, R.D. and Hamrick, J.L. 1983. Patterns and levels of genetic variation in Great Basin bristlecone pine, *Pinus longaeva*. ***Evolution*** 37(2), 302-310.

Hignight, K.W., Bashaw, E.C. and Hussey, M.A. 1991. Cytological and morphological diversity of native apomictic buffelgrass, *Pennisetum ciliare* (L.) Link. ***Botanical Gazette*** 152(2), 214-218.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. ***Annual Review of Ecology and Systematics*** 18, 23-42.

Hillis, D.M., Larson, A., Davis, S.K. and Zimmer, E.A. 1990. Nucleic Acids III: Sequencing In: Hillis, D.M. and Moritz, C. (eds) **Molecular Systematics.** Sinauer Associates, Inc., Sunderland MA. pp. 318-370.

Hillis, D.M., Mable, B.K. and Moritz, C. 1996. Applications of molecular systematics: the state of the field and a look to the future In: Hillis, D.M., Moritz, C. and Mable, B.K. (eds) **Molecular Systematics.** Sinauer Associates, Inc., Sunderland MA. pp. 515-543.

Hnatiuk, R.J. 1990. **Census of Australian Plants**. Australian Flora and Fauna Series Report No. 11. 650 pp.

Hoelzel, A.R. and Dover, G.A. 1991. **Molecular Genetic Ecology**. Oxford University Press, New York. 75 pp.

Hoey, B.K., Crowe, K.R., Jones, V.M. and Polans, N.O. 1996. A phylogenetic analysis of *Pisum* based on morphological characters, and allozyme and RAPD markers. ***Theoretical and Applied Genetics*** 92, 92-100.

Holsinger, K. 1996. Pollination biology and the evolution of mating systems in flowering plants. ***Evolutionary Biology*** 29, 107-149.

Hopper, S.D. 1979. Biogeographic aspects of speciation in the southwestern Australian flora. ***Annual Review of Ecology and Systematics*** 10, 399-422.

Hopper, S.D. 1992. Patterns of plant diversity at the population and species levels in south-west Australian mediterranean ecosystems In: Hobbs, R.J. (ed) **Biodiversity of Mediterranean Ecosystems in Australia.** Surrey Beatty & Sons, Chipping North NSW. pp. 27-46.

Hopper, S.D. 1995. Evolutionary networks: natural hybridization and its conservation significance In: Saunders, D.A., Craig, J.L. and Mattiske, E.M. (eds) **Nature Conservation 4: The Role of Networks**. pp. 51-66.

Hopper, S.D., Purdie, R.W., George, A.S. and Patrick, S.J. 1987. *Constylis*. **Flora of Australia** Volume 45, 57-110.

Isabel, N., Beaulieu, J. and Bousquet, J. 1995. Complete congruence between gene diversity estimates derived from genotypic data at enzyme and random amplified polymorphic DNA loci in black spruce. ***Proceedings of the National Academy of Science of the United States of America*** 92, 6369-6373.

Jain, S.K. 1975. Population structure and the effects of breeding system In: Frankel, O.H. and Hawkes, J.C. (eds) **Crop Genetic Resources for Today and Tomorrow.** Cambridge University Press, Cambridge. pp. 15-36.

Jain, S.K. 1976. The evolution of inbreeding in plants. ***Annual Review of Ecology and Systematics*** 7, 469-495.

James, E.A. and Ashburner, G.R. 1997. Intraspecific variation in *Astelia australiana* (Liliaceae) and implications for the conservation of this Australian species. ***Biological Conservation*** 82, 253-261.

James, F.C. and McCulloch, C.E. 1990. Multivariate analysis in ecology and systematics: Panacea or Pandora's box ? ***Annual Review of Ecology and Systematics*** 21, 129-166.

James, S.H. 1965. Complex hybridity in *Isotoma petraea* I. The occurrence of interchange heterozygosity, autogamy and a balanced lethal system. ***Heredity*** 20(3), 341-353.

James, S.H. 1970. Complex hybridity in *Isotoma petraea*. ***Heredity*** 25(1), 53-77.

James, S.H. 1979. Chromosome numbers and genetic systems in the trigger plants of Western Australia (*Stylidium*; Stylidiaceae). ***Australian Journal of Botany*** 27, 17-25.

James, S.H. 1981. Cytoevolutionary patterns, genetic systems and the phytogeography of Australia In: Keast, A. (ed) **Ecological Biogeography of Australia.** Dr W. Junk bv Publishers, The Hague. pp. 763-782.

James, S.H. 1996. Seed abortion and the evaluation of genetic systems in some Australian native plant groups. **Gondwanan Heritage : Past, Present and Future of the Western Australian Biota.** Surrey Beatty & Sons, Chipping North, NSW. pp. 90-99.

James, S.H. and Hopper, S.D. 1981. Speciation in the Australian flora In: Pate, J.S. and McComb, A.J. (eds) **The Biology of Australian Plants.** University of Western Australia Press, Perth. pp. 361-381.

James, S.H., Wylie, A.P., Johnson, M.S., Carstairs, S.A. and Simpson, G.A. 1983. Complex hybridity in *Isotoma petraea* V. Allozyme variation and the pursuit of hybridity. ***Heredity*** 51(3), 653-663.

Karron, J.D. 1987. A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. ***Evolutionary Ecology*** 1, 47-58.

Karron, J.D., Linhart, Y.B., Chaulk, C.A. and Robertson, C.A. 1988. Genetic structure of populations of geographically restricted and widespread species of *Astragalus* (Fabaceae). ***American Journal of Botany*** 75(8), 1114-1119.

Kazan, K., Manners, J.M. and Cameron, D.F. 1993. Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. ***Theoretical and Applied Genetics*** 85, 882-888.

Keighery, G. and Gibson, N. 1993. Biogeography and composition of the flora of the Cape Range Peninsula, Western Australia In: Humphreys, W.F. (ed) **The Biogeography of Cape Range Western Australia** Records of the Western Australian Museum Supplement No. 45. Western Australian Museum, Perth Western Australia. pp. 51-85.

Keim, P., Paige, K.N. Whitham, T.G. and Lark, K.G. 1989. Genetic analysis of an interspecific hybrid swarm of *Populus*: occurrence of unidirectional introgression. ***Genetics*** 123, 557-565.

Kennington, W.J., Waycott, M. and James, S.H. 1996. DNA fingerprinting supports notions of clonality in a rare mallee, *Eucalyptus argutifolia*. ***Molecular Ecology*** 5, 693-696.

Kenrick, J. 1986. A method for estimating self-incompatibility In Williams, E.G., Irvine, D. and Knox, R.B. (eds) Pollination '86. Melbourne University Press, Melbourne. pp. 116-120.

Levin, D.A. 1978. Genetic variation in annual *Phlox*: self-compatible versus self-incompatible species. ***Evolution*** 32(2), 245-263.

Levin, D.A. 1983. Polyploidy and novelty in flowering plants. ***The American Naturalist*** 122(1), 1-25.

Levin, D.A. 1993. Local speciation in plants: the rule not the exception. ***Systematic Botany*** 18(2), 197-208.

Levin, D.A. and Kerster, H.W. 1974. Gene flow in seed plants In: Dobzhansky, T., Hecht, M.K. and Steere, W.S. (eds) **Evolutionary Biology 7**. Plenum Press, New York. pp. 139-220.

Lewis, P.O. and Crawford, D.J. 1995. Pleistocene refugium endemics exhibit greater allozyme diversity than widespread congeners in the genus *Polygonella* (Polygonaceae). ***American Journal of Botany*** 82(2), 141-149.

Linhart, Y.B., Mitton, J.B., Sturgeon, K.B. and Davis, M.L. 1981. Genetic variation in space and time in a population of ponderosa pine. ***Heredity*** 46(3), 407-426.

Liston, J. 1970. Introduction In: Lockhart, W.R. and Liston, J. (eds) **Methods for Numerical Taxonomy.** American Society for Microbiology, Bethesda MA. pp. 1-3.

Liu, Z. and Furnier, G.R. 1993. Comparison of allozyme, RFLP, and RAPD markers for revealing genetic variation within and between trembling aspen and bigtooth aspen. ***Theoretical and Applied Genetics*** 87, 97-105.

Lloyd, D.G. 1980. Demographic factors and mating patterns in angiosperms In: Solbrig, O.T. (ed) **Demography and Evolution in Plant Populations.** Blackwell Scientific Publications, Oxford. pp. 67-88.

Love, A. and Love, D. 1974. **Plant Chromosomes**. J. Cramer, Vaduz. 184 pp.

Loveless, M.D. and Hamrick, J.L. 1984. Ecological determinants of genetic structure in plant populations. ***Annual Review of Ecology and Systematics*** 15, 65-95.

Lyons, E.E. and Antonovics, J. 1991. Breeding system evolution in *Leavenworthia*: Breeding system variation and reproductive success in natural populations of *Leavenworthia crassa* (Cruciferae). ***American Journal of Botany*** 78(2), 270-287.

MacDonald, S.E., Chinnappa, C.C., Reid, D.M. and Purdy, B.G. 1987. Population differentiation of the *Stellaria longipes* complex within Saskatchewan's Athabasca sand dunes. ***Canadian Journal of Botany*** 65, 1726-1732.

Markert, C.L. and Moller, F. 1959. Multiple forms of enzymes: tissue, ontogenetic, and species specific patterns. ***Proceedings of the National Academy of Science*** 45, 753-763.

Martin, F.W. 1958. Staining and observing pollen tubes in the style by means of fluorescence. ***Stain Technology*** 34, 125-128.

Mayr, E. 1942. **Systematics and the Origin of Species**. Columbia University Press, New York. 334 pp.

Mayr, E. 1970. **Populations, Species, and Evolution**. The Belknap Press, Cambridge, MA. 453 pp.

McCrue, K.A. and Holtsford, T.P. 1998. Seed bank influences on genetic diversity in the rare annual *Clarkia springvillensis* (Ongaraceae). ***American Journal of Botany*** 85(1), 30-36.

Metcalf, C.R. 1983. Secretory structures : Cells, cavities and canals In: Metcalf, C.R. and Chalk, L. (eds) **Anatomy of the Dicotyledons.** Clarendon Press, Oxford. pp. 64-67.

Michaels, H.J., Scott, K.M., Olmstead, R.G., Szaro, T., Jamsen, R.K. and Palmer, J.D. 1993. Interfamilial relationships of the Asteraceae: Insights from *rbc*L sequence variation. ***Annals of the Missouri Botanical Gardens*** 80, 742-751.

Michaud, H., Toumi, L., Lumaret, R., Li, T.X., Romane, F. and Di Giusto, F. 1995. Effect of geographic discontinuity on genetic variation in *Quercus ilex* L. (Holm oak). Evidence from enzyme polymorphism. ***Heredity*** 74, 590-606.

Milligan, B.J. 1992. Plant DNA isolation In: Hoelzel, A.R. (ed) **Molecular Genetic Analysis of Populations - A Practical Approach.** Oxford University Press, Oxford. pp. 59-88.

Mitton, J.B. 1989. Physiological and demographic variation associated with allozyme variation In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology.** Dioscorides Press, Portland, Oregon. pp. 127-145.

Moller, M. and Cronk, Q.C.B. 1997. Origin and relationships of *Saintpaulia* (Gesneriaceae) based on ribosomal DNA internal transcribed spacer (ITS) sequences. ***American Journal of Botany*** 84(7), 956-965.

Mopper, S., Mitton, J.B., Whitham, T.G., Cobb, N.S. and Christensen, K.M. 1991. Genetic differentiation and heterozygosity in pinyon pine associated with resistance to herbivory and environmental stress. ***Evolution*** 45(4), 989-999.

Moran, G.F. and Hopper, S.D. 1983. Genetic diversity and the insular population structure of the rare granite rock species, *Eucalyptus caesia* Benth. ***Australian Journal of Botany*** 31, 161-172.

Moran, G.F., Bell, J.C. and Prober, S. 1990. The utility of isozymes in the systematics of some Australian tree groups. ***Australian Systematic Botany*** 3, 47-57.

Muir, B.G. 1977. Biological survey of the Western Australian wheatbelt. Part 4: Vegetaion of West Bendering. **Records of the Western Australian Museum** Supplement 5, 5-31.

Murawski, D.A. and Bawa, K.S. 1994. Genetic structure and mating system of *Stemonoporus oblongifolius* (Dipterocarpaceae) in Sri Lanka. ***American Journal of Botany*** 81(2), 155-160.

Murawski, D.A. and Hamrick, J.L. 1990. Local genetic and clonal structure in the terrestrial bromeliad, *Aechmea magdelenae*. ***American Journal of Botany*** 77, 1201-1208.

Murphy, R.W., Sites, J.W., Buth, D.G. and Haufler, C.H. 1996. Proteins: Isozyme electrophoresis In: Hillis, D.M., Moritz, C. and Mable, B.K. (eds) **Molecular Systematics.** Sinauer Associates, Inc., Sunderland MA. pp. 51-120.

Nei, M. 1972. Genetic distance between populations. ***The American Naturalist*** 106(949), 283-292.

Nei, M. 1973. Analysis of gene diversity in subdivided populations. ***Proceedings of the National Academy of Sciences of the United States of America*** 70(12), 3321-3323.

Nei, M. 1975. **Molecular Population Genetics and Evolution**. North-Holland Publishing Company, Amsterdam. 288 pp.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. ***Genetics*** 89, 583-590.

Norusis, M.J. 1994. **SPSS: Professional Statistics, Version 6.1**. SPSS Inc., Chicago IL. 385 pp.

Palmer, J.D. 1987. Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. ***The American Naturalist*** 130 (Supp.), S6-S29.

Palmer, J.D., Jansen, R.K., Michaels, H.J., Chase, M.W. and Manhart, J.R. 1988. Chloroplast DNA variation and plant phylogeny. ***Annals of the Missouri Botanical Garden*** 75(4), 1180-1206.

Palumbi, S.R. 1996. Nucleic Acids II: the polymerase chain reaction In: Hillis, D.M., Moritz, C. and Mable, B.K. (eds) **Molecular Systematics.** Sinauer Associates, Inc., Sunderland MA. pp. 205-247.

Patterson, C., Williams, D.M. and Humphries, C.J. 1993. Congruence between molecular and morphological phylogenies. ***Annual Review of Ecology and Systematics*** 24, 153-188.

Paynter, B.H. and Dixon, K.W. 1990. Seed viability and embryo decline in *Geleznowia verrucosa* Turcz. (Rutaceae). ***Scientia Horticulturae*** 45, 149-157.

Paynter, B.H. and Dixon, K.W. 1991. Propagation of yellow bells (*Geleznowia verrucosa* Turcz., Rutaceae) from seed. ***Australian Journal of Agricultural Research*** 42, 901-909.

Peakall, R., Smouse, P.E. and Huff, D.R. 1995. Evolutionary implications of allozyme and RAPD variation in diploid populations of dioecious buffalograss *Buchloe dactyloides*. ***Molecular Evolution*** 4, 135-147.

Pickering, C.M. 1995. Variation in flowering parameters within and among five species of Australian alpine *Ranunculus*. ***Australian Journal of Botany*** 43, 103-112.

Potts, B.M. and Reid, J.B. 1988. Hybridization as a dispersal mechanism. ***Evolution*** 42(6), 1245-1255.

Pryor, L.D., Williams, E.R. and Gunn, B.V. 1995. A morphometric analysis of *Eucalyptus urophylla* and related taxa with descriptions of two new species. ***Australian Systematic Botany*** 8, 57-70.

Purdy, B.G., Bayer, R.J. and MacDonald, S.E. 1994. Genetic variation, breeding system evolution, and conservation of the narrow sand dune endemic *Stellaria arenicola* and the widespread *S. longipes* (Caryophyllaceae). ***American Journal of Botany*** 81(7), 904-911.

Rafalski, J.A., Tingey, S.V. and Williams, J.G.K. 1991. RAPD markers - a new technology for genetic mapping and plant breeding. ***AgBiotech News and Information*** 3(4), 645-648.

Ragot, M. and Hoisington, D.A. 1993. Molecular markers for plant breeding : comparisons of RFLP and RAPD genotyping costs. ***Theoretical and Applied Genetics*** 86, 975-984.

Ramsey, M., Prakash, N. and Cairns, S. 1993. Breeding systems of disjunct populations of Christmas Bells (*Blandfordia grandiflora* R. Br., Liliaceae): variation in self-fertility and an ovular mechanism regulating self-fertilisation. ***Australian Journal of Botany*** 41, 35-47.

Richards, A.J. 1997. **Plant Breeding Systems**. Chapman & Hall, London. 529 pp.

Richardson, B.J., Baverstock, P.R. and Adams, M. 1986. **Allozyme Electrophoresis: A Handbook for Animal Systematics and Population Studies**. Academic Press, Sydney. 410 pp.

Rick, C.M., Fobes, J.F. and Holle, M. 1977. Genetic variation in *Lycopersicon pimpinellifolium*: Evidence of evolutionary change in mating systems. ***Plant Systematics and Evolution*** 127, 139-170.

Riedy, M.F., Hamilton, W.J. and Aquadro, C.F. 1992. Excess of non-parental bands in offspring from known primate pedigrees assayed using RAPD PCR. ***Nucleic Acids Research*** 20(4), 918.

Rieseberg, L.H. 1991. Homoploid reticulate evolution in *Helianthus* (Asteraceae): evidence from ribosomal genes. ***American Journal of Botany*** 78(9), 1218-1237.

Rieseberg, L.H. 1995. The role of hybridization in evolution: old wine in new skins. ***American Journal of Botany*** 82(7), 944-953.

Rieseberg, L.H. 1996. Homology among RAPD fragments in interspecific comparisons. ***Molecular Ecology*** 5, 99-105.

Rieseberg, L.H. 1997. Hybrid origins of plant species. ***Annual Review of Ecology and Systematics*** 28, 359-389.

Rieseberg, L.H. and Wendel, J.F. 1993. Introgression and its consequences in plants In: Harrison, R.G. (ed) **Hybrid Zones and the Evolutionary Processes.** Oxford University Press, New York. pp. 70-109.

Rieseberg, L.H., Choi, H., Chan, R. and Spore, C. 1993. Genomic mapping of a diploid hybrid species. ***Heredity*** 70, 285-293.

Ritland, K. 1989. Genetic differentiation, diversity and inbreeding in the mountain monkeyflower (*Mimulus caespitosus*) of the Washington Cascades. ***Canadian Journal of Botany*** 67, 2017-2024.

Ritland, K. and Jain, S. 1981. A model for the estimation of outcrossing rate and gene frequencies using *n* independent loci. ***Heredity*** 47(1), 35-52.

Ritland, C. and Ritland, K. 1989. Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). ***American Journal of Botany*** 76(12), 1731-1739.

Roche, S. 1998. Smoke induced seed germination of selected Australian native plants. PhD Thesis, Botany Department, University of Western Australia, Perth Western Australia.

Roose, M.L. and Gottlieb, L.D. 1976. Genetic and biochemical consequences of polyploidy in *Tragapogon*. ***Evolution*** 30, 818-830.

Rossetto, M., Weaver, P.K. and Dixon, K.W. 1995. Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (Proteaceae). ***Molecular Ecology*** 2, 89-97.

Rutishauser, A., Nogler, G.A. and Schwendener, J. 1969. Apomixis as a cause of polyploidy and polymorphism. ***Chromosome Today*** 2, 202-205.

Sampson, J.F., Hopper, S.D. and James, S.H. 1988. Genetic diversity and the conservation of *Eucalyptus crucis* Maiden. ***Australian Journal of Botany*** 36, 447-460.

Schemske, D.W. and Lande, R. 1985. The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. ***Evolution*** 39(1), 41-52.

Schoen, D.J. 1977. Morphological, phenological, and pollen-distribution evidence of autogamy and xenogamy in *Gilia achilleifolia* (Polemoniaceae). ***Systematic Botany*** 2, 280-286.

Schoen, D.J. 1982. The breeding system of *Gilia achilleifolia*: Variation in floral characters and outcrossing rate. ***Evolution*** 36(2), 352-360.

Schoen, D.J. and Brown, A.H.D. 1991. Intraspecific variation in population gene diversity and effective population size correlated with the mating system in plants. ***Proceedings of the National Academy of Sciences of the United States of America*** 88, 4494-4497.

Sherman-Broyles, S.L., Broyles, S.B. and Hamrick, J.L. 1992. Geographic distribution of allozyme variation in *Ulmus crassifolia*. ***Systematic Botany*** 17(1), 33-41.

Shields, C.R., Orton, T.J. and Stuber, C.W. 1983. An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissue In: Tanksley, S.D. and Orton, T.J. (eds) **Isozymes in Plant genetics and Breeding Part A.** Elsevier Science Publishers B.V., Amsterdam. pp. 443-468.

Skroch, P., Tivang, J. and Nienhuis, J. 1992. Analysis of genetic relationships using RAPD marker data. **Applications of RAPD Technology to Plant Breeding.** Crop Science Society of America, Minneapolis, Minnesota. pp. 26-30.

Slatkin, M. 1985. Rare alleles as indicators of gene flow. ***Evolution*** 39(1), 53-65.

Slatkin, M. and Maruyama, T. 1975. The influence of gene flow on genetic distance. ***The American Naturalist*** 109, 597-601.

Smith-White, S. 1954. Chromosome numbers in the Boronineae (Rutaceae) and their bearing on the evolutionary development of the tribe in the Australian flora. ***Australian Journal of Botany*** 2, 287-303.

Smith-White, S. 1959. Cytological evolution in the Australian flora. ***Cold Spring Harbor Symposium on Quantitative Biology*** 24, 273-289.

Sneath, P.H.A. and Sokal, R.R. 1973. **Numerical Taxonomy**. W.H. Freeman and Company, San Francisco. 573 pp.

Solbrig, O.T. and Rollins, R.C. 1977. The evolution of autogamy in species of the mustard genus *Leavenworthia*. ***Evolution*** 31, 265-281.

Soltis, D.E. and Rieseberg, L.H. 1986. Autopolyploidy in *Tolmiea menziesii* (Saxifragaceae): Genetic insights from enzyme electrophoresis. ***American Journal of Botany*** 73(2), 310-318.

Soltis, D.E. and Soltis, P.S. 1997. Phylogenetic relationships in Saxifragaceae sensu lato : a comparison of topologies based on 18S rDNA and *rbc*L sequences. ***American Journal of Botany*** 84(4), 504-522.

Soltis, D.E., Kuzoff, R.K., Conti, E., Gornall, R. and Ferguson, K. 1996. *mat*K and *rbc*L gene sequence data indicate that *Saxifraga* (Saxifragaceae) is polyphyletic. ***American Journal of Botany*** 83(3), 371-382.

Sonnante, G., Spinosa, A., Marangi, A. and Pignone, D. 1997. Isozyme and RAPD analysis of genetic diversity within and between *Vigna luteola* and *V. marina*. ***Annals of Botany*** 80, 741-746.

Stace, C.A. 1989. **Plant Taxonomy and Biosystematics**. Edward Arnold, London. 264 pp.

Stace, H.M. 1995. Primitive and advanced character states for chromosome number in Gondwanan angiosperm families of Australia, especially Rutaceae and Proteaceae. Kew Chromosome Conference IV, Royal Botanic Gardens, Kew.

Stace, H.M., Armstrong, J.A. and James, S.H. 1993. Cytoevolutionary patterns in Rutaceae. ***Plant Systematics and Evolution*** 187, 1-28.

Stebbins, G.L. 1957. Self fertilization and population variability in the higher plants. ***The American Naturalist*** 91, 337-354.

Stebbins, G.L. 1970. Adaptive radiation of reproductive characteristics in angiosperms, I. Pollination mechanisms. ***Annual Review of Ecology and Systematics*** 1, 307-326.

Stebbins, G.L. 1971. **Chromosomal Evolution in Higher Plants**. Edward Arnold (Publishers) Ltd, London. 216 pp.

Stephenson, A.G. 1981. Flower and fruit abortion: Proximate causes and ultimate functions. ***Annual Review of Systematics and Ecology*** 12, 253-279

Strauss, S.Y. and Karban, R. 1994. The significance of outcrossing in an intimate plant-herbivore relationship. I. Does outcrossing provide an escape from herbivores adapted to the parent plant ? ***Evolution*** 48(2), 454-464.

Swofford, D.L. and Selander, R.B. 1981. BIOSYS-1: A FORTRAN program for the comprehensive analysis of electrophoretic data in populations genetics and systematics. ***Journal of Heredity*** 72, 281-283.

Systma, K.J. and Schaal, B.A. 1985. Genetic variation, differentiation and evolution in a species of complex tropical shrubs based on isozymic data. ***Evolution*** 39, 582-593.

Szmidt, A.E., Wang, X.-R. and Lu, M.-Z. 1996. Empirical assessment of allozyme and RAPD variation in *Pinus sylvestris* (L.) using haploid tissue analysis. ***Heredity*** 76, 412-420.

Tabachnick, B.G. and Fidell, L.S. 1989. **Using Multivariate Statistics.** Harper Collins Publishers, New York. 746 pp.

Taggart, J.B., McNally, S.F. and Sharp, P.M. 1990. Genetic variability and differentiation among founder populations of the pitcher plant (*Sarracenia purpurea* L.) in Ireland. ***Heredity*** 64, 177-183.

Tao, Y., Manners, J.M., Ludlow, M.M. and Henzell, R.G. 1993. DNA polymorphisms in grain sorghum (*Sorghum bicolor* (L.) Moench). ***Theoretical and Applied Genetics*** 86, 679-688.

Thorpe, R.S. 1976. Biometric analysis of geographic and racial affinities. ***Biological Review*** 51, 407-452.

Tingey, S.V., Rafalski, J. and Williams, J.G.K. 1992. Genetic analysis with RAPD markers. **Applications of RAPD Technology to Plant Breeding.** Crop Science Society of America, Minneapolis, Minnesota. pp. 3-8.

Torres, A.M. 1989. Isozyme analysis of tree fruits In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology.** Dioscorides Press, Portland, Oregon. pp. 192-205.

Torres, A.M., Millan, T. and Cubero, J.I. 1993. Identifying rose cultivars using randomly amplified polymorphic DNA markers. ***HortScience*** 28(4), 333-334.

Tsegaye, S., Tesemma, T. and Belay, G. 1996. Relationships among tetraploid wheat (*Triticum trugidum* L.) landrace populations revealed by isozyme markers and agronomic traits. ***Theoretical and Applied Genetics*** 93, 600-605.

van Ham, R.C.H.J. and Hart, H.T. 1998. Phylogenetic relationships in the Crassulaceae inferred from chloroplast DNA restriction site variation. ***American Journal of Botany*** 85(1), 123-134.

Vicario, F., Vendramin, G.G., Rossi, P., Lio, P. and Giannini, R. 1995. Allozyme, chloroplast DNA and RAPD markers for determining genetic relationships between *Abies alba* and the relic population of *Abies nebrodensis*. ***Theoretical and Applied Genetics*** 90, 1012-1018.

Wang, W.Y., Pai, R.C., Lai, C.C. and Lin, T.P. 1994. Molecular evidence for the hybrid origin of *Paulonia taiwaniana* based on RAPD markers and RFLP of chloroplast DNA. ***Theoretical and Applied Genetics*** 89, 271-275.

Watson, L.E., Elisens, W.J. and Estes, J.R. 1994. Genetic variation within and among populations of the *Marshallia graminifolia* complex (Asteraceae). ***Biochemical Systematics and Ecology*** 22(6), 577-582.

Waugh, R. and Powell, W. 1992. Using RAPD markers for crop improvement. ***Trends in Biotechnology*** 10, 186-191.

Waycott, M. 1995. Assessment of genetic variation and clonality in the seagrass *Posidonia australis* using RAPD and allozyme markers. ***Marine Ecology Progress Series*** 116, 289-295.

Waycott, M. 1998. Genetic variation, its assessment and implications to the conservation of seagrasses. ***Molecular Ecology*** (In Press).

Waycott, M., James, S.H. and Walker, D.I. 1997. Genetic variation within and between populations of *Posidonia australia*, a hydrophilous, clonal seagrass. ***Heredity*** 79, 408-417.

Weeden, N.F. 1983. Evolution of plant isozymes In: Tanksley, S.D. and Orton, T.J. (ed) **Isozymes in Plant Genetics and Breeding, Part A.** Elsevier Science Publishers B.V., Amsterdam. pp. 175-205.

Weeden, N.F. and Wendel, J.F. 1989. Genetics of plant isozymes In: Soltis, D.E. and Soltis, P.S. (eds) **Isozymes in Plant Biology**. Dioscorides Press, Portland, Oregon. pp. 46-72.

Weeden, N.F., Timmerman, G.M., Hemmat, M., Kneen, B.E. and Lodhi, M.A. 1992. Inheritance and reliability of RAPD markers. **Applications of RAPD Technology to Plant Breeding.** Crop Science Society of America, Minneapolis, Minnesota. pp. 12-17.

Weising, K., Nybom, H., Wolff, K. and Meyer, W. 1995. **DNA Fingerprinting in Plants and Fungi**. CRC Press, Boca Raton. 293 pp.

Welsh, J., Honeycutt, R.J., McClelland, M. and Sobral, B.W.S. 1991. Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). ***Theoretical and Applied Genetics*** 82, 473-476.

Whiffen, T. 1978. Geographic variation in tropical tree species. Fourth Cabot Symposium, Harvard Forest, Petersham MA, Cambridge University Press. pp. 31-54.

Whitkus, R. 1985. FORTRAN program for computing genetic statistics from allelic frequency data. ***Journal of Heredity*** 76, 142.

Williams, J. and Ladiges, P.Y. 1985. Morphological variation in Victorian, lowland populations of *Eucalyptus pauciflora* Sieb. ex Spreng. ***Proceedings of the Royal Society of Victoria*** 97(1), 31-48.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. ***Nucleic Acids Research*** 18(22), 6531-6535.

Williams, J.G.K., Hanafey, M.K., Rafalski, J.A. and Tingey, S.V. 1993. Genetic analysis using random amplified polymorphic DNA markers In: Wu, R. (ed) **Methods in Enzymology Recombinant DNA Part I.** Academic Press, Inc., San Diego. pp. 704-740.

Williams, J.G.K., Kubelik, A.R., Rafalski, J.A. and Tingey, S.V. 1991. Genetic analysis with RAPD markers In: Bennet, J.W. and Lasure, L.L. (eds) **More Gene Manipulations in Fungi.** Academic Press, San Diego. pp. 431-439.

Wolff, S.L. and Jefferies, R.L. 1987. Morphological and isozyme variation in *Salicornia europaea* (s.l.) (Chenopodiaceae) in northeastern North America. ***Canadian Journal of Botany*** 65, 1410-1419.

Wright, S. 1978. **Variability within and among natural populations**. The Univ. of Chicago Press, Chicago. 580 pp.

Wyatt, R. 1984. The evolution of self-pollination in granite outcrop species of *Arenaria* (Caryophyllaceae). I. Morphological correlates. ***Evolution*** 38(4), 804-816.

Yeh, F.C., Yang, R.-C. and Boyle, T. 1997. POPGENE Version 1.21, Microsoft Window-based Freeware for Population Genetic Analysis. University of Alberta, Edmonton.

Young, N.D. 1996. Concordance and discordance: A tale of two hybrid zones in the Pacific Coast irises (Iridaceae). ***American Journal of Botany*** 83(12), 1623-1629.

Zapata, T.R. and Arroyo, M.T.K. 1978. Plant reproductive ecology of a secondary deciduous tropical forest in Venezuela. ***Biotropica*** 10(3), 221-230.

APPENDIX 1: Root tip chromosome counts of *G. verrucosa* populations.

Form	Site	Population Voucher No.	No.	Count 1	2	3	4	5
Small	Arinya	L. Broadhurst 14	AC1	28	27	28	28	28
			AC2	28	28	28	28	28
			AC3	28	28	28	28	28
			AC4	28	28	28	28	28
			AC5	28	28	28	28	28
	Coorow 1	L. Broadhurst 15	14	28	28	28	28	28
			13	28				
	Coorow 2	L. Broadhurst 16	20	28	28	28	28	28
			17	28	28	28	28	28
	Indarra Reserve	L. Broadhurst 6	1	25	28	28	28	28
			12	28	28	28		
	Pleshkes Small	L. Broadhurst 12	8	25	28	28	28	28
			A1	26	28			
			A2	28	28	28	27	28
Intermediate	Ajana West 1	L. Broadhurst 9	9	28	28	28	28	28
			15	28	28	28	28	28
			5	27	28	28	28	28
			20	28	28	28	28	28
			13	26	28	28	28	28
	Ajana West 2	L. Broadhurst 7	12	28	28	28	28	28
			7	28	28	28		
			17	28				
			3	28	28	28	28	28
			15	28	27	28		
	Hawks Head	L. Broadhurst 13	14	26	28			
			2	28	28	28	28	28
			13	24				
			4	28	28	28	28	
	Hutt River 1	L. Broadhurst 5	9	28	28	28	28	
			5	28				
	Hutt River 2	L. Broadhurst 4	8	28	28	28	28	
			19	26	28	28	28	
	Nanns	L. Broadhurst 10	AC	28	26	25	28	26
	West Binnu	L. Broadhurst 17	16	28	27	28	28	28
			17	28	28	28	28	28
			18	27	28	28	28	28
Large	Geraldton Pit	L. Broadhurst 2	3	28	28	28	28	28
			13	28	28	28	28	28
			9	28	28	28	28	28
			19	27	28	28	28	28
			11	28	28	28	28	28
	Meanarra Hill 1	L. Broadhurst 18	5	28	28	28	28	28
			9	28	28	28	28	28
			15	28	28	28	28	
			20	28	28	28	28	28
			10	28	28	28	28	28
	Meanarra Hill 2	L. Broadhurst 19	15	28	28	28	28	28
			18	28	28	28	28	28
			19	28	28			
	Pleshkes Large	L. Broadhurst 11	10	28	28	28	28	28
			5	28	28	28	28	28
			2	28	28	28	28	28
	Red Bluff	L. Broadhurst 3	11	28	28	28	28	28
			15	28	28	28	28	28
			3	28	28	28	28	28
			4	28	26	28	28	28
Transect	Pleshkes Hybrid	L. Broadhurst 20	1	28	26	28	28	28
			18	28				
			9	26	28	28	28	28

Appendix 2: Manuscripts

Manuscripts from this thesis include the following:

Broadhurst, L.M., Coates, D.J. and Tan, B.H. 1998. Genetic diversity in the endemic species *Geleznowia verrucosa* Turcz. (Rutaceae). ***Heredity*** (in press).

Broadhurst, L.M. Coates, D.J. & Tan, B.H. Patterns of morphological variation and allelic distribution across a hybrid zone of *Geleznowia verrucosa* Turcz. (Rutaceae). ***American Journal of Botany*** (in review).

Broadhurst, L.M., Waycott, M. and Tan, B.H. Patterns of genetic variation in the Western Australian endemic *Geleznowia verrucosa* Turcz. (Rutaceae). ***Plant Systematics and Evolution*** (in review).

Manuscripts in Preparation

Broadhurst, L.M. Some aspects of the floral biology of *Geleznowia verrucosa*.

Broadhurst, L.M. Reproductive differences between forms of *Geleznowia verrucosa* (Rutaceae).

Broadhurst, L.M. Patterns of morphological variation within *Geleznowia verrucosa* Turcz (Rutaceae).