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Genetic, cytogenetic and morphological patterns in a mixed mulga population: evidence for apomixis

Rose L. Andrew[A,B,C], *Joseph T. Miller*[A], *Rod Peakall*[B], *Michael D. Crisp*[B] *and Randall J. Bayer*[A]

[A]Centre for Plant Biodiversity Research, CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2602, Australia.
[B]School of Biology and Zoology, Australian National University, Canberra, ACT 0200, Australia.
[C]Corresponding author; email: Rose.Andrew@anu.edu.au

Abstract. The mulga complex (*Acacia aneura* and closely related taxa) is a widespread group that is dominant in much of arid Australia. The group is taxonomically difficult, due to a complex interaction of sympatry and putative hybridisation between the major species, geographic variation within species and sympatric variation within *A. aneura*. Mulga is highly variable in a wide range of vegetative and reproductive characters and it is not unusual to find five or six distinct forms growing side by side. The aim of this project was to gain a better understanding of the relationships among mulga species and *A. aneura* varieties, as well as the maintenance of this variation. A single site in the Northern Territory, containing *A. ayersiana*, *A. minyura* and two varieties of *A. aneura*, was sampled intensively. Six morphotypes were observed in the field and five were strongly supported by morphometric analysis. Although the mulga complex is generally tetraploid ($2n = 52$), triploid ($2n = 39$) and pentaploid ($2n = 65$) seedlings were produced in the study population. Microsatellite primers developed for *A. mangium* (sect. Juliflorae) were amplified in individuals of each morphotype, resulting in genetic marker patterns consistent with polyploidy. Genetic and morphometric distances were correlated and differences between morphotypes account for 63% of the total genetic variation ($\phi PT = 0.63$, $P < 0.001$). Allele sequences confirmed the presence of genuine heterozygosity and clonality was suggested by the low genotypic diversity and the lack of allele segregation. Seedlings had identical genotypes to the maternal plants and polyembryony was observed in each taxon, consistent with apomictic reproduction. Both apomixis and ploidy level variation may restrict gene flow among morphotypes, playing a role in the maintenance of morphological diversity at the study site. The success of the group in arid and semi-arid Australia may also be due, in part, to these factors.

Introduction

Acacia aneura F.Muell. ex Benth., also known as mulga, is the core species of the mulga complex, a widespread group that is dominant in much of arid Australia (Everist 1949; Johnson and Burrows 1994). This species complex currently contains 10 varieties of the widely distributed *A. aneura* and nine other species of more restricted range (Pedley 2001). Mulga is taxonomically difficult, due to a combination of sympatry and putative hybridisation between the major species (Davies 1976), geographic variation within species and sympatric variation within *A. aneura* (Pedley 1973; Lamont and Fox 1981; Fox 1986; Cody 1989). Mulga is characterised by complex assemblages in mixed populations, but very little is known about the genetic basis of this variation, the origins of the morphotypes or the ecological factors allowing them to coexist. The major obstacle to systematic and ecological studies on the *A. aneura* complex is the lack of information regarding its reproductive biology and population genetics.

The morphological variability of mulga is all the more striking because of the highly adaptive nature of the traits involved and the harsh nature of the habitat. While environmental variation almost certainly contributes to the morphological variability of *A. aneura*, both Fox (1986) and Cody (1991) consider much of the variation to be inherited. Trees in mixed populations tend to breed true with respect to phyllode morphology (Dave Albrecht, pers. comm.), suggesting genetic control of these traits, although environmental and ontogenetic factors may contribute to the complexity of these populations. There has been disagreement over the continuity or otherwise of morphological variation within populations of *A. aneura*. Lamont and Fox (1981) recognised six distinct forms at Yeelirrie, WA, while Cody (1989) found continuous

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variation in phyllode length and width at Yelma, WA. This is of crucial importance because continuous variation would suggest that outbreeding occurs often among morphotypes, whereas strong discontinuities could signify barriers to gene flow. Reproductive isolation among morphotypes could suggest reinforcement or sympatric speciation, both of which are controversial and have been demonstrated in few systems. Gene flow among the morphotypes could be reduced by intrinsic mechanisms, such as hybrid breakdown or cross-incompatibility, or external factors, such as disruptive selection or restricted dispersal. It has been suggested that polyploidy played an important role in the success of mulga during and after the climatic fluctuations of the late Tertiary–early Quaternary (Pedley 1973; Hopper and Maslin 1978). Previously, only tetraploid ($2n = 52$) chromosome numbers have been counted in *A. aneura* (Bukhari 1997), but preliminary flow cytometry results suggest that diploids ($2n = 26$) also occur. Differences in ploidy levels could act as a reproductive barrier between morphotypes, as well as having a role in the origin of the morphotypes.

The mating system of mulga is largely unknown, although clonality is not expected because recruitment occurs mostly through seed, which has a hard coat and germinates mainly following fire or flooding (Preece 1971). Australian arid-zone acacias are thought to be largely outbreeding (Keighery 1982) and Australian acacias are largely self-incompatible (Kenrick and Knox 1989). However, self-fertilisation or apomixis may occur in *A. cowleana* and *A. holosericea* (Moran *et al.* 1992).

This study is the first to examine the morphological variation of the mulga complex in a genetic context. Studying the genetic structure of a mixed mulga population is the first step in understanding the genetic relationships, both past and present, of members of the mulga complex. The aim of this study was to determine where the behaviour of sympatric morphotypes fits along the scale from continuous population to separate species and to identify factors that might be involved. The phenotypic diversity of the population was examined by morphometric analyses and the genetic structure of the population was studied with microsatellites developed for *A. mangium* Willd. (Phyllodinae: Juliflorae). Limited progeny trials were also conducted to examine microsatellite inheritance.

Materials and methods

Study site and sample collection

The site chosen for this study was 3.8 km south along the dirt road exiting the Lasseter Hwy 12 km east of Curtin Springs, near Mt Connor, Northern Territory. The 100 × 100-m plot was marked out in a flat area between two dunes, 50 m west of the road. This area is classed as *A. aneura* tall open shrubland with an *Eragrotis eriopoda* open-grassland understorey (Wilson *et al.* 1990). Also common within the plot were *Solanum* spp. and *Ptilotus* spp. All of the woody shrubs in the plot were members of the mulga complex, with the exception of one *A. kempeana* individual. The sample contained most of the variants found in the region. *A. ayersiana* Maconchie and *A. minyura* Randell were found in the plot, together with *A. aneura* varieties *intermedia* and *tenuis*. A narrow-leaved form of *A. minyura* and a broad-leaved form of *A. aneura* var. *intermedia* were postulated to be hybrids with *A. aneura* var. *tenuis* and *A. ayersiana*, respectively.

Samples were collected in October, when pods were abundant and mature. The shortest pod-bearing plants were approximately 2 m tall and most individuals above this height were reproductively mature, therefore all individuals greater than 2 m were sampled and mapped. Field identifications were made following Pedley (2001).

Morphometric analysis

Preliminary measurements for a range of characters were made on a core group of specimens in order to determine the most useful characters. All morphological measurements were made with electronic callipers, with the exception of seed mass. Most of these characters are self-explanatory (Table 1); however, some require clarification. Phyllode width (P2) was measured at the widest point and the distance of this point from the base of the phyllode was measured where possible. This distance was divided by the phyllode length to give the relative position of the widest point (P6). As a measure of curvature (P4), the maximum deviation of the phyllode axis from the shortest line connecting the base and apex was divided by the length of the phyllode. The relative position of the point at which curvature was measured was determined as for the widest point (P5). The thickness of the pod was measured at two or more mature seeds and averaged to produce Character L4, and between two adjacent seeds to produce Character L5. Pod width was also measured at each adjacent mature seed and between these seeds to enable calculation of the mean constriction from their ratio (L7). The maximum width of the 'wing' or the tissue between the margin of the pod and the intramarginal vein was also measured when present (L3). Five seeds were weighed together on an electronic balance (S1).

Continuous morphological variation has been reported previously, based on measurements of randomly chosen phyllodes, pods and seeds (Cody 1989, 1991). Phyllode morphology depends on the position within the plant (field observation) and a large proportion of the variance within a randomly chosen sample may therefore be due to ontogenetic differences. To minimise this effect, the fifth fully expanded phyllode back from the growing tip on each of five randomly chosen branchlets was measured, except in the case of *A. ayersiana*, whose specimens sometimes had fewer than five phyllodes on each branchlet. In this case, the most basal phyllode was measured. Only pods with at least two adjacent mature seeds were selected for the measurement of pod characters.

On the basis of Cramer values, only the most informative characters were measured for the remaining specimens. Seed characters were also excluded from further analysis because the only character with a high Cramer value (S4) was closely related to pod thickness (L4). Hybrid multi-dimensional scaling (HMDS) in the software package PATN (Belbin 1989) was performed on the morphometric data to assess the morphological patterns within and among morphotypes. This is a sensitive ordination technique that is suitable for analysing similarity among *a priori* groupings, but does not assume them. Hybrid MDS in two dimensions was carried out on a Gower metric distance matrix with the PATN default settings. The effects of including and excluding the morphologically distinct *A. ayersiana* and characters with lower Cramer values from the analysis were also tested.

Seed germination and root-tip squashes for determination of ploidy level

Seeds from 14 individuals were scarified at each end and allowed to imbibe water overnight in a beaker. They were then grown in Petri

dishes on filter paper soaked with water. When their roots were 0.5–2 cm in length, the seeds were pretreated in water or colchicine for 4 h at room temperature and fixed overnight in ethanol : acetic acid (3 : 1).

Attempts to observe ploidy levels were made following the methods of Ostergren and Heneen (1962) and Bayer (1984) and Bukhari (1997), with modifications. The seeds were hydrolysed in 1 M HCl for 8 min, rinsed in milliQ H_2O and the roots separated from the seed. The roots were stained in Feulgen fluid (Ostergren and Heneen 1962) at room temperature, in the dark for 2 h, replacing the Feulgen fluid after 1 h. The roots were transferred to 45% acetic acid for at least 15 min and either squashed immediately or stored at –20°C to prevent destaining. Countable metaphase plates were photographed with an Olympus 35 mm camera and Kodak technical pan film.

DNA extraction, microsatellite amplification and sequence analysis

Although several DNA extraction protocols were trialed, none gave more consistent results than the standard hot CTAB method (Doyle and Doyle 1987). A large viscous layer often appeared above the interphase during the chloroform wash, making it difficult to remove the aqueous phase. The effect of this material, probably polysaccharide, was minimised by splitting the powdered leaf between four separate microfuge tubes or scaling the volume up by a factor of 25 and the amount of tissue by five.

A caylase digestion was used to remove polysaccharides from the DNA preparation (Penny Butcher, pers. comm.). 140 µL sterile milliQ H_2O, 50 mL 1 M KOAc (pH 5.5), 20 µL EDTA (pH 8.0), 1 µL Rnase A (Sigma Aldrich, Castle Hill, NSW Australia) and 50 µL freshly made 20 mg mL^{-1} caylase were added and the solution incubated overnight at 37°C. The enzyme was removed by a phenol–chloroform extraction, followed by a chloroform wash (Sambrook *et al.* 1989) and precipitation in isopropanol.

Three microsatellites were amplified using fluorescently labelled primers (Invitrogen, Mt Waverley, Vic., Australia) according to Butcher *et al.* (2000), with minor modifications. Reactions were scaled to 20 µL and primer concentrations were doubled (0.4 µM instead of 0.2 mM). A sample of 0.5 µL of each product was separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyser. Samples were run with a CXR internal fluorescent size marker (Promega, Annandale, NSW, Australia). Fragment sizes were determined with GENESCAN and GENOTYPER fragment analysis software (Applied Biosystems).

Selected alleles were amplified in 20-µL reactions with unlabelled primers. Bands were cloned (TOPO Cloning Kit, Invitrogen) and sequenced with the Big DYE Terminator RR Kit (Applied Biosystems, Scoresby, Vic., Australia) and an automated ABI PRISM sequencer at CSIRO Plant Industry in Canberra, Australia. Sequences were manually aligned with the use of Sequencher 3.0 (Gene Codes Corporation, Ann Arbour, MI, USA).

Analysis of microsatellites

In diploids, microsatellites are ordinarily single-locus codominant markers, characterised by the presence of one or two alleles per locus per individual and recognisable heterozygosity. However, in polyploids the banding can be much more complex, with both multiple loci and multiple alleles detectable per individual. Without the knowledge of the origin of polyploidy (auto- *v.* allopolyploidy) and extensive study of patterns of inheritance in multiple families, it may not be possible to readily assign bands to specific loci. As a further complication, the number of copies of an allele represented at a given allelic position may not be detectable, in many cases. For this reason, microsatellite profiles were treated as multilocus 'DNA fingerprints' and simply coded by the presence or absence of bands in the DNA profile or given a genotype code for each locus. Subsequent statistical analyses were performed as for standard multilocus analyses (Peakall *et al.* 1995). This included the calculation of pairwise genetic distances among individuals, analysis of molecular variance, principal coordinates analysis (PCA) and Mantel tests, to compare equivalent genetic and morphometric distance matrices with the computer package GenAlEx (Peakall and Smouse 2001). Further background on these statistical procedures can be found in Huff *et al.* (1993), Peakall *et al.* (1995) and Maguire *et al.* (2002). The genotypic diversity of each morphotype was quantified by the Shannon-Weaver index:

$$H_W = -\Sigma\pi_i \ln \pi_i,$$

where π is the frequency of multilocus genotype *i* (Paul *et al.* 1997). This measure is not bounded by 1 and is useful for comparison of genotypic diversity by using presence/absence data, such as AFLP data (Gaudeul *et al.* 2000; Larson *et al.* 2001).

Progeny trials

To test segregation of microsatellite alleles, six seedlings were grown from each of eight individuals of different morphotypes. The second leaf of each plant was removed for DNA extraction and genotyping, as for the material collected in the field.

Results

Morphometric analysis

Strong discontinuities were seen when phyllode length and width alone were considered and these characters had the highest Cramer values (Fig. 1; Table 1). Overall, the morphotypes are non-overlapping, except for the broad form of *A. aneura* var. *intermedia*, which appears to fall within the variation of the narrow form. The separation of morphotypes in ordination space depended on the data set used, but the overall patterns remained the same (Fig. 2). Excluding *A. ayersiana* from the analysis improved separation between the broad and narrow forms of *A. minyura*, but *A. aneura* varieties *tenuis* and *intermedia* were more loosely clustered and harder to differentiate. Including and excluding characters also affected the tightness of the morphological clusters. When *A. ayersiana* was included, the best resolution was obtained when the entire character set was used. Without *A. ayersiana*, however, *A. aneura* varieties *tenuis* and *intermedia* each formed tighter clusters when characters with Cramer values ≥ 0.88 were used.

When phyllode and pod data were analysed separately, discontinuities among morphotypes were present in ordinations of both data sets (results not shown). Distance matrices calculated from the pod and phyllode data sets were also significantly correlated (Table 2).

Although phyllode length and width are useful in distinguishing among morphotypes at the Mt Connor site, these traits appear to be susceptible to differences in developmental stage. Some plants had more than one phyllode form, with shorter phyllodes occurring on lower branches or more basal on the same branchlet. This phenomenon was particularly pronounced in *A. aneura* varieties *tenuis* and *intermedia* and may be partly responsible for the variability of these morphotypes (Figs 1 and 2). Pod length is used in most keys, but its Cramer value of 0.6497 suggests that it is too variable to be used in distinguishing

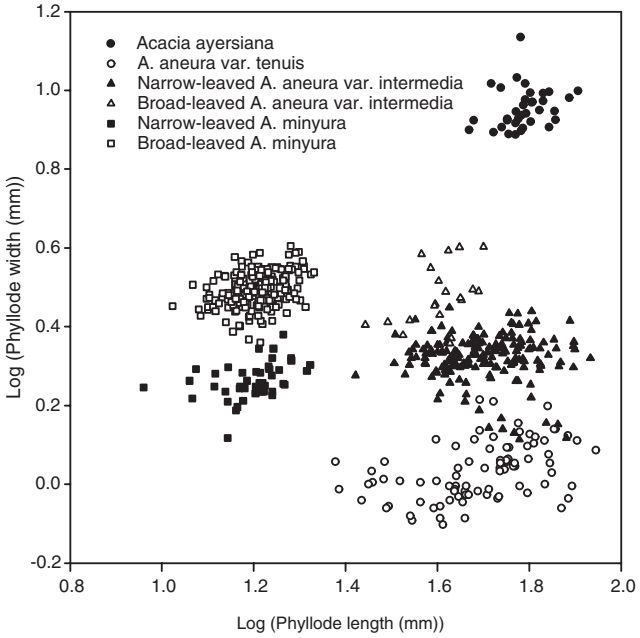

Fig. 1. Discontinuity in phyllode morphology. Points represent log-transformed lengths and widths of individual phyllodes. All *a priori* morphotypes are separated by discontinuities in phyllode morphology, with the exception of the two forms of *Acacia aneura* var. *intermedia* (b and i) and *A. aneura* var. *tenuis* (t). However, the overlap between *A. aneura* var. *intermedia* and *A. aneura* var. *tenuis* is due to 2 of the 56 individuals of these varieties measured.

among closely related morphotypes (Table 1). Phyllode curvature was useful in distinguishing between the narrow and broad forms of *A. minyura*.

Table 1. Cramer values for preliminary character set using *a priori* groupings based on field determinations

*Characters with low Cramer values excluded from Fig. 2*b* **Seed characters excluded from Fig. 2*a*, *b*

Character	Cramer value
Phyllode chraracter	
Length (P1)	0.9039
Width at widest point (P2)	0.9962
Thickness at widest point (P3)	0.9358
Curvature (P4)	0.7541*
Position of point of maximum curvature (P5)	0.5764*
Position of widest point (P6)	0.7244*
Pulvinus length (P7)	0.8913
Legume character	
Length (L1)	0.6497*
Width at widest point (L2)	0.7837*
Wing width (L3)	0.8864
Thickness at seed (L4)	0.8806
Thickness between adjacent seeds (L5)	0.5788*
Apex (L6)	0.5579*
Constriction (L7)	0.5198*
Seed character	
Weight of five seeds (S1)	0.6721**
Length (S2)	0.7605**
Width (S3)	0.6649**
Thickness (S4)	0.8784**

Seedling morphology and chromosome numbers

A number of the germinated seeds grew twin radicles and the seeds contained two or more plumules (Fig. 3*a*). Seven twinned seeds, each of a different morphotype, occurred among 80 seed germinated from 15 individuals. Twenty

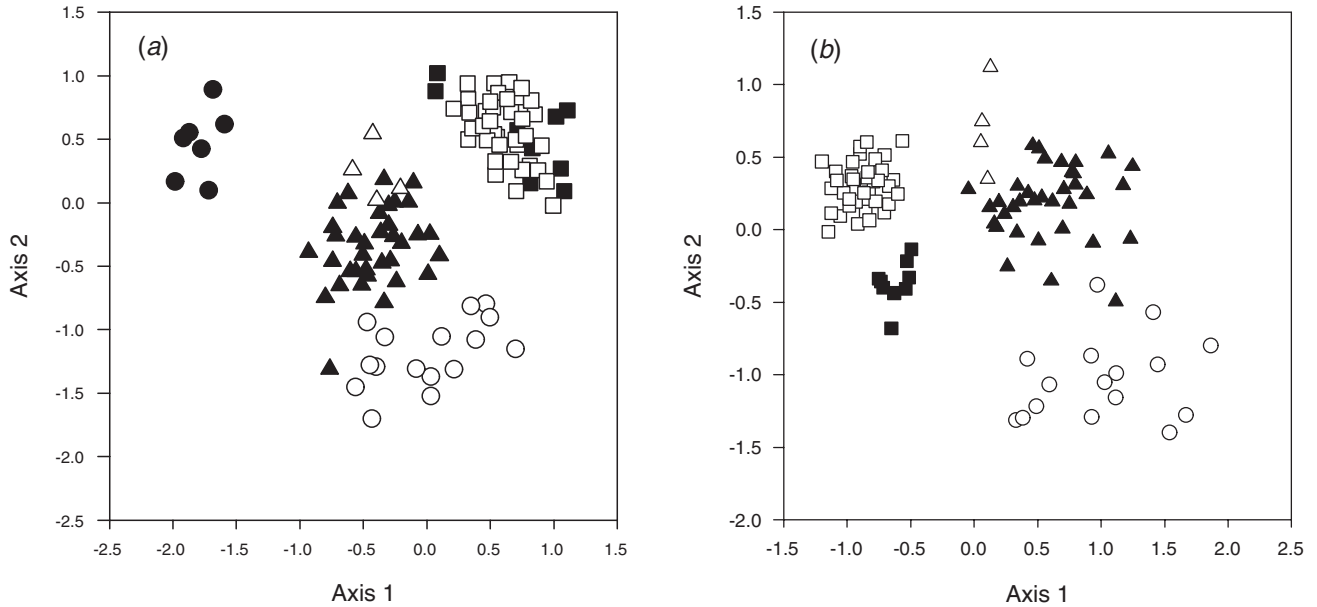

Fig. 2. Hybrid MDS analysis of log-transformed morphometric data. (*a*)Total morphometric data set. (*b*) Data set excluding *Acacia ayersiana* and characters (see Table 1). ●, *A. ayersiana*; ○, *A. aneura* var. *tenuis*; ▲, narrow-leaved *A. aneura* var. *intermedia*; △, broad-leaved *A. aneura* var. *intermedia*; ■, narrow-leaved *A. minyura*; □, broad-leaved *A. minyura*.

Table 2. Mantel-test results
***$P < 0.001$

Distance comparison	Correlation coefficient, *r*
Phyllode *v.* pod characters	0.462***
Morphometric *v.* genetic distance (presence/absence of alleles)	0.374***
Morphometric *v.* genetic distance (genotype)	0.427***
Genetic distance—presence/absence *v.* genotype	0.865***

seeds from each of six individuals of different morphotypes were grown for up to 3 months in the glasshouse for future progeny trials. Several seedlings appeared to have two meristems and three or four cotyledons, despite growing from a single seed (Fig. 3*b*). Between one and three of these twins were found per maternal plant.

Because adequate separation of chromosomes was not achieved, neither aneuploidy nor B chromosomes were possible to detect. Three ploidy levels were observed in the various morphotypes (Table 3). The narrow form of *A. minyura* was triploid ($2n = 3x = 39$; Fig. 4*a*), while *A. ayersiana*, *A. aneura* var. *intermedia* and the broad form of *A. minyura* were tetraploid ($2n = 4x = 52$). Multiple ploidy levels were observed in only one morphotype, *A. aneura* var. *tenuis*, which produced triploid or pentaploid ($2n = 5x = 65$; Fig. 4*b*) progeny; however, additional counts might detect more variation in the other morphotypes.

Microsatellite sequences

As expected for polyploids, complex banding patterns were apparent at each microsatellite locus, with between one and five bands detected per individual per locus. A total of 35 putative alleles were scored, of which 29 were sequenced to confirm whether they were homologous to the original microsatellite locus. All but two of the sequences contained the appropriate priming sites and a similar microsatellite flanking region as the *A. mangium* sequences and were concluded to be homologous. One cloned sequence was not homologous with the microsatellite flanking sequences or simple sequence repeats, but contained both primer-annealing sites. This sequence was likely to have formed by random amplification from a non-target sequence and was considerably shorter than the other sequences for this primer pair. Microsatellite scoring was not affected, however, as it was well outside the expected size range for the primer pair. The other contained only one vector-insertion site, one primer site and a small part of the flanking sequence and may have been formed by recombination with the *Escherichia coli* genome. A 12-bp indel differentiated the mulga Am465 sequences from the *A. mangium* sequence and was the only major flanking region divergence.

Repeat sequences diverged considerably from those of *A. mangium* (Penny Butcher, unpubl. data), with the loss of variable repeats, the appearance of new variable repeats (Fig. 5) and the appearance of homopolymeric stretches (poly-T) with 1–6 bp of length variation among alleles. The latter contributed to size homoplasy among alleles, which was also observed due to mutation of the repeat sequence and different repeat numbers in compound repeats. No evidence was found for homology between alleles of the same size in different morphotypes, although the number of sequences relevant to this question was limited.

Microsatellite length variation

The microsatellites were characterised by profiles containing multiple bands of different heights which often recurred within morphotypes (Fig. 5; Table 4). While multiple bands per microsatellite locus were found, the number of banding combinations was restricted overall. For example, all individuals of the narrow-phyllode form of *A. minyura* shared a three-banded profile for the primer pair Am391 (Table 4). Across the three loci, no two individuals of different morphotypes shared the same DNA profile, although some single-locus profiles occurred in both *A. aneura* var. *tenuis* and *A. aneura* var. *intermedia*. The Shannon-Weaver genotypic diversity index ranged from 0.000 in narrow-leaved *A. minyura* to 0.884 in broad-leaved *A. aneura* var. *intermedia* (Table 5).

In general, morphotypes were genetically non-overlapping (Fig. 6), with the exception of the broad form of *A. aneura* var. *intermedia*, which was interspersed among the narrower individuals. However, the genetic clusters were more diffuse than those seen in the morphometric HMDS (Fig. 2). The narrow form of *A. minyura* is the least variable, followed by *A. ayersiana*, the broad form of *A. minyura* and *A. aneura* var. *tenuis*, whereas *A. aneura* var. *intermedia* is very variable. Principal coordinates analysis does not support the hypothesis of a hybrid origin for the narrow form of *A. minyura*, since it was not placed between *A. minyura* and *A. aneura* var. *tenuis*. Similarly, the *A. aneura* var. *intermedia* specimens were not genetically intermediate between *A. ayersiana* and either *A. aneura* var. *tenuis* or *A. minyura*.

Analysis of molecular variance confirmed that a significant proportion of genetic variance occurred among morphotypes ($\phi PT = 0.632$, $P = 0.001$). Mantel tests showed that genetic and morphological variation in the population were significantly correlated (Table 2). This result is robust across data sets, suggesting that there is very strong congruence of morphological and molecular relationships in the study population.

Progeny trials

The 48 progeny tested were each identical to the maternal parent plant at three loci, with the exception of one individual. The lack of allele segregation shows that the

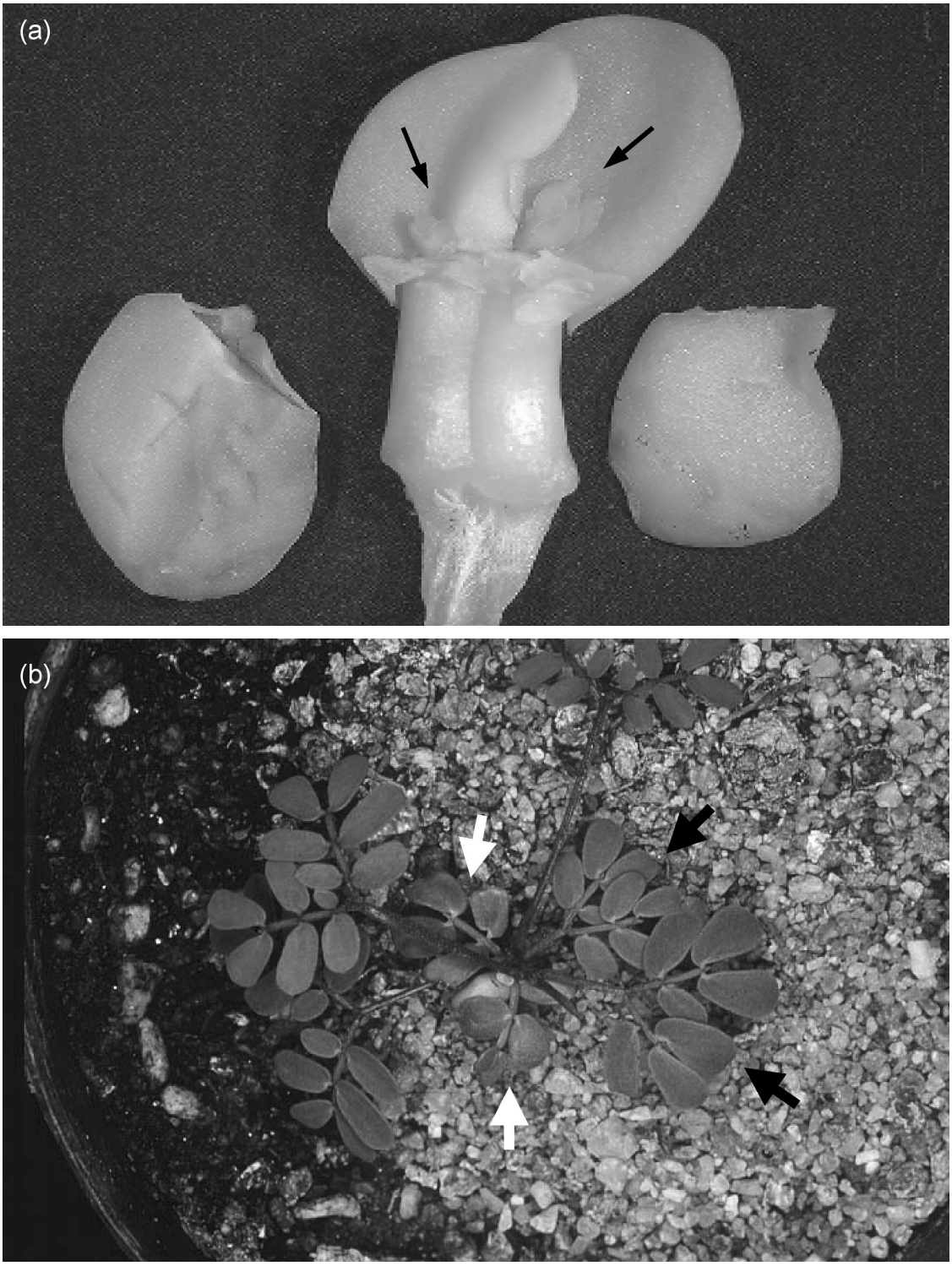

Fig. 3. Twin embryos and seedlings. (*a*) Twin embryos, with two plumules (indicated by black arrows), two radicles and four cotyledons. (*b*) Twin seedlings, showing two meristems, three cotyledons, two first leaves (pinnate; white arrows) and two second leaves (twice pinnate; black arrows).

Table 3. Distribution of ploidy levels among morphotypes and the number of root tips squashed
At least three counts were made from each squash

Morphotype	*n*	3*x*	4*x*	5*x*
Acacia ayersiana	2		2	
Broad *A. minyura*	2		2	
Narrow *A. minyura*	3	3		
A. aneura var. *intermedia*	2		2	
A. aneura var. *tenuis*	3	1		2

mating system of the mulga group does not consist of purely sexual autopolyploids.

Discussion

Genetic basis of morphological variation

Morphometric analysis of the study population provides two lines of evidence to indicate that genetic variation underlies the morphological variation among morphotypes. The first is that variation in a range of traits, which are unlikely to be linked, shows a pattern of population structure that is robust across ordination techniques. Variation in phyllode and pod morphology is congruent, supporting Cody's (1989) finding, as do other vegetative and reproductive characters (Davies 1976; Cody 1991). Second, the results demonstrate that variation among individuals in the Mt Connor mixed population is discontinuous and morphotypes do not overlap. Assuming that these characters are unlinked, both discontinuity in morphology and congruence of pod and phyllode data suggest that intermediate or recombinant forms either rarely survive strong selection, or are not produced due to reproductive barriers.

The finding of discontinuous phyllode variation at Mt Connor contrasts with Cody's (1989) assertion that variation at Yelma, WA, is continuous. Although both sampling strategies and geographic differences may have caused this discrepancy, non-overlapping size classes are not required for differences in these distributions to represent genetic differences among morphotypes (Thiele 1993). Slight overlap of phyllode size ranges does not imply that heritable variation among individuals is continuous if some of the intra-individual variation results from ontogenetic or environmental factors.

Microsatellite and morphometric data also exhibit congruence, demonstrated by the significant Mantel-test correlation (Table 2) and the separation of individuals in the microsatellite ordination space according to morphotype (Fig. 6). Principal components analysis of microsatellite data indicated that most morphotypes are genetically non-overlapping, although the degree of clustering varied among morphotypes. This supports the conclusion, drawn from the morphometric analysis, that reproductive isolating mechanisms are reducing gene flow among morphotypes. Consistent with the morphological evidence, the extensive and significant genetic differentiation revealed by AMOVA confirms that gene flow is restricted among morphotypes.

Microsatellite homaplasy

Variation in allele size was almost entirely due to variation within the repeat, because mutations in the flanking sequence were few and tended not to affect the length of the allele. This contrasts with the observation that multiple bands represent multiple loci in the diploid, predominantly selfing *Glycine* (Peakall *et al.* 1998). Repeat sequences varied in structure, repeat number and repeat motif, producing size homoplasy between morphotypes. Comparison of microsatellite allele sequences from the Mt

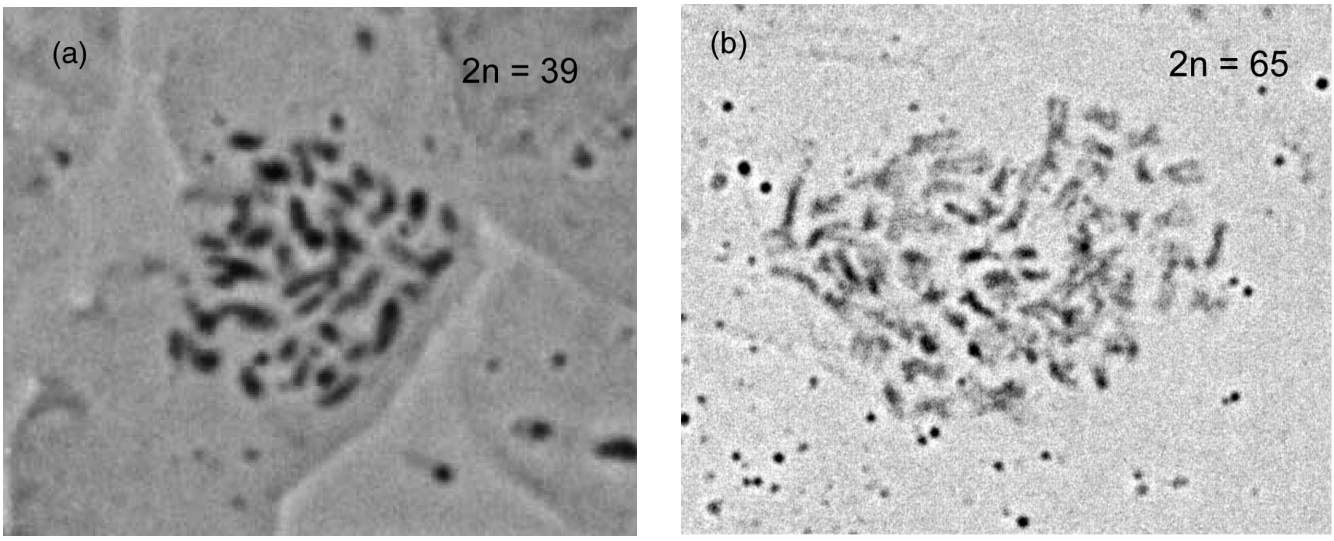

Fig. 4. Mitotic chromosome squashes. Photomicrographs showing (*a*) a triploid cell from the narrow form of *Acacia minyura* and (*b*) a pentaploid cell from *A. aneura* var. *tenuis*. Not all chromosomes are visible, but the difference in the ploidy level can be seen.

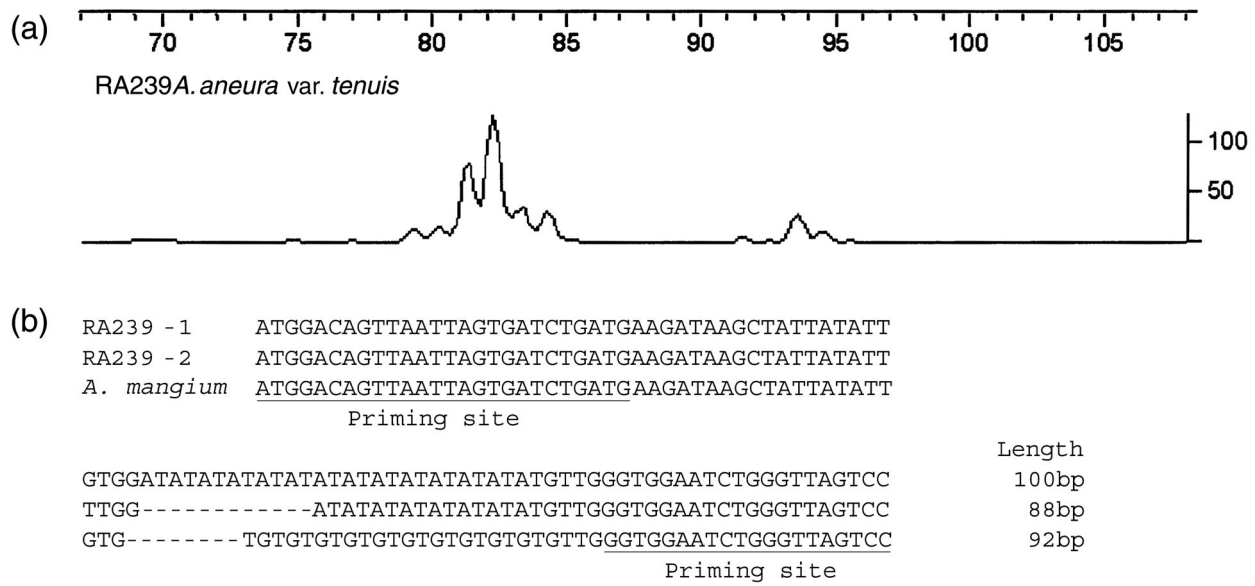

Fig. 5. Complex microsatellite profiles and sequencing of uncertain alleles. (*a*) Fluorescent trace for Am391 PCR products in *Acacia aneura* var. *tenuis*. The very low peak at 94 bp was shown by sequencing to contain the same microsatellite as the taller peak. The vertical scale represents the strength of the fluorescent signal and the horizontal scale represents fragment length. (*b*) Am391 allele sequences, aligned with a sequence from *A. mangium* (Penny Butcher, unpublished data). Fragments 88 and 100 base pairs long were positively identified as microsatellites. Primer-binding sites are underlined in the *A. mangium* sequence and hyphens (-) denote gaps in the sequence alignment. Fragment lengths are given at the end of each sequence, showing that the size marker gave fragment sizes approximately 7 bp below the actual value.

Table 4. Multilocus genotypes and allele sizes for the narrow form of *Acacia minyura* (n1) and *A. aneura* var. *tenuis* (t1–9)

Multilocus genotype code	*n*	Am391 bands present	Am465 bands present	Am503 bands present
n1	11	84, 88, 90	133	146
t1	8	82, 84, 86	133, 139	142, 152
t2	6	82, 94	133	157
t3	4	80, 82, 86	133	142, 146
t4	1	84, 88	133, 136	142, 150
t5	1	80, 82, 86	133	146
t6	1	80, 82, 86	133	142, 152
t7	1	82, 84, 90	133	142
t8	1	82, 84, 86, 98	133, 139	157
t9	1	80, 82, 86	133	142

Connor mulga population with sequences from the source species, *Acacia mangium*, supports most of the observations by other researchers regarding microsatellite cross-transferability and evolution, but also presents some novel results. In general, microsatellite amplification, length and variability decrease with increasing phylogenetic distance from the source species (Orti *et al.* 1997; Huang *et al.* 1998; Peakall *et al.* 1998; Buteler *et al.* 1999). As expected, alleles were shorter in mulga than in *A. mangium* for two of three primer pairs (Butcher *et al.* 2000). However, allele numbers were higher in the mulga morphotypes than in *A. mangium*, possibly due to peculiarities in the cytogenetics and mating system of mulga. The large indel present in all mulga Am465 alleles demonstrates the complications of using microsatellite length variation alone for studies at higher levels. Flanking sequence indels of different sizes have been reported among closely related species in several groups (Orti *et al.* 1997; Huang *et al.* 1998; Peakall *et al.* 1998; Buteler *et al.* 1999). Mulga and *A. mangium* repeat sequences have diverged more strongly than the flanking regions, featuring the disappearance of repeats, the appearance of repeats with new motifs, poly-T single-nucleotide repeat expansions and interruption of repeat motifs. Previous studies have reported alteration of

Table 5. Genotypic diversity of mulga morphotypes

Morphotype	Individuals genotyped	Multilocus genotypes	Shannon-Weaver diversity index, H_W
Acacia ayersiana	9	3	0.936888
A. aneura var. *intermedia* (broad form)	4	4	1.747868
A. aneura var. *intermedia* (narrow form)	37	17	2.42716
A. minyura (broad form)	42	8	0.98563
A. minyura (narrow form)	11	1	0
A. aneura var. *tenuis*	24	9	1.805918

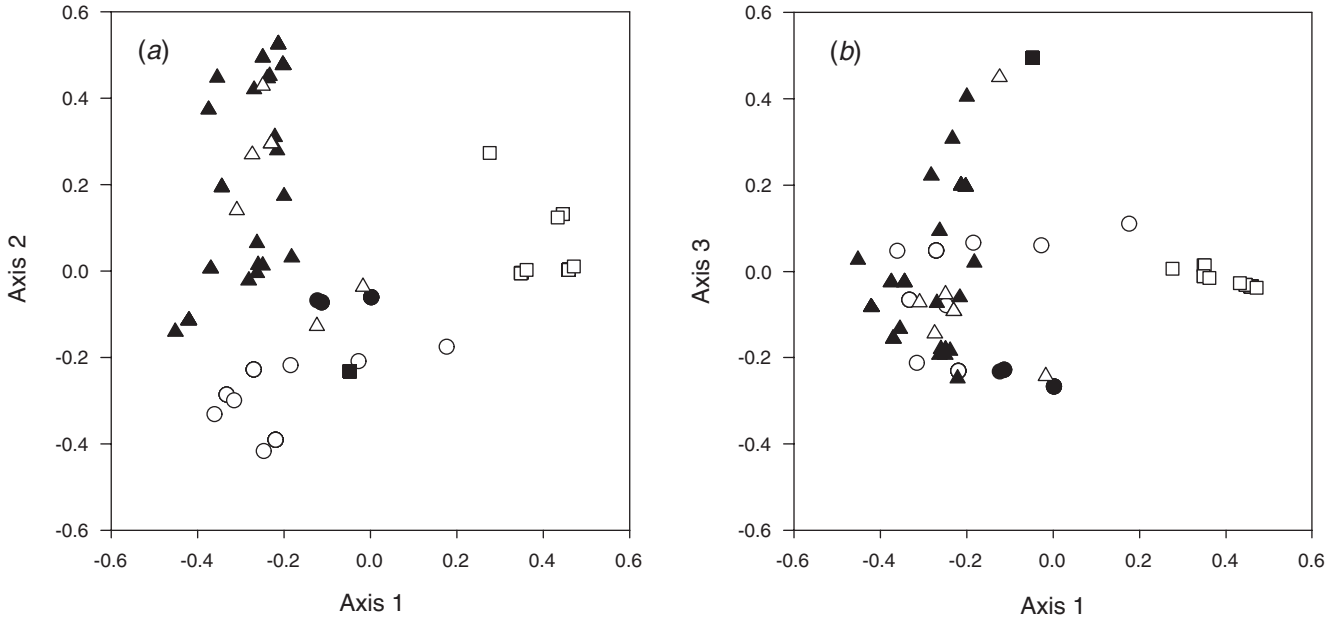

Fig. 6. Principal coordinates analysis of microsatellite data. Individuals are plotted against (*a*) the first two principal axes and (*b*) the first and third principal axes. Note that the ranges of the morphotypes are non-overlapping when all three dimensions are considered and that several points represent a number of individuals. ●, *Acacia ayersiana*; ○, *A. aneura* var. *tenuis*; ▲, narrow-leaved *A. aneura* var. *intermedia*; △, broad-leaved *A. aneura* var. *intermedia*; ■, narrow-leaved *A. minyura*; □, broad-leaved *A. minyura*.

the repeat motif in non-source plant species, although the original repeats were not completely replaced (Huang *et al.* 1998; Buteler *et al.* 1999). Homopolymeric regions have also been reported to complicate microsatellite studies (Orti *et al.* 1997; Huang *et al.* 1998).

Maintenance of mulga morphotypes

Mitotic chromosome counts may provide clues to the maintenance of morphological variation in mixed mulga populations. The only previous reports of ploidy levels in *A. aneura* have been tetraploid ($2n = 4x = 52$) (Bukhari 1997), although variation in ploidy level was suggested by Pedley (1973). Three ploidy levels were shown to occur in the study population, although no diploids ($2n = 26$) were identified (Table 3). Parents and progeny may differ in ploidy level, but it is likely that the seedling ploidy levels are also found in the adult population. Ploidy level can affect many aspects of a plant's life, including physiology, morphology and reproduction. Both pentaploids and triploids have abnormal meiosis, usually producing unreduced or non-functional pollen and embryo sacs (Kirschner and Stepanek 1996). Similarly, differing ploidy levels can act as reproductive barriers between fertile plants, because sterile hybrids with meiotic abnormalities are commonly produced (Quarin 1999).

Several features of the study population suggest apomixis, a reproductive mode that could contribute to restricted gene flow among morphotypes. Apomixis is the asexual production of seeds, producing cloned offspring identical to the maternal plant. The frequency of sexual reproduction is reduced in apomictic species, which tend to consist of groups of genetically identical plants. Apomixis occurs in a range of plant groups and is commonly associated with a clonal genetic structure, polyembryony and polyploidy.

As well as being a possible reproductive barrier maintaining morphological diversity in the study population, the variation in ploidy levels suggests that apomixis is

occurring in the mulga complex. Diploid apomicts and polyploid obligate sexual species both exist, although most taxa containing apomicts also contain polyploids. Since the sexual reproduction of odd-ploids (e.g. triploids and pentaploids) is often compromised, apomixis can be advantageous by providing a means of seed production that is independent of fertilisation (Bonilla and Quarin 1997). This could explain the exceptional seed set observed in the field, which is otherwise incongruent with this type of ploidy variation (Asker and Jerling 1992).

Twin embryos have been observed in seed germinated from each morphotype, suggesting that polyembryony is widespread within the *A. aneura* species complex. Polyembryony has been found in sexual species, due to cleavage of zygotic cells, the presence of multiple embryo sacs in the ovule or the production of embryos from synergids (Asker and Jerling 1992). The latter is often associated with ploidy level variation but odd-ploids (e.g. pentaploids) are rare (Reid Palmer, pers. comm.), suggesting that the production of eggs from synergids is not a cause of polyembryony in mulga. Fox (1979) reported that some seeds produced two radicles, but such seeds always died within 2 weeks of germination. However, he used a less-reliable, but faster and easier method of breaking dormancy. In the present study, seed was not collected in large quantities, so germination rates were required to be as high as possible and the seed coat was broken at each end using nail clippers before overnight imbibition. This may have enabled the twins to break through the seed coat, despite having to share the same amount of endosperm between two plants. Polyembryony was observed in one of 5000 germinated *Acacia karroo* seeds, but analysis of eight isozyme loci indicated that the twins were outcrossed (Oballa 1996).

The presence of fixed heterozygosity in progeny and the stability of highly heterozygous genotypes within morphotypes are suggestive of allopolyploidy, meiotic abnormalities, clonality or a combination of these. Offspring were identical in genotype, with one exception, to the maternal parent in progeny trials, consistent with fixed heterozygosity. Stable meiotic abnormalities result in fixed heterozygosity in *Isotoma petraea* in south-western Western Australia and are thought to have evolved in response to inbreeding (James 1990). This is unlikely to occur in the mulga complex, because self-fertilisation does not appear to occur (Keighery 1982) and complex hybridity is not associated with polyploidy (*Isotoma petraea* has $2n = 14$, James 1990). In allopolyploid species, recombination occurs only within genomes, that is, only among homologous chromosomes from the same parent taxon and fixed heterozygosity may result when there is no allelic variation within a genome. However, much of the heterozygosity in the study population cannot be explained by allopolyploidy, given the lack of band segregation observed (Table 4). The most extreme case is the triploid narrow form of *A. minyura*, which has three alleles for Am391, but even the more genetically diverse morphotypes exhibit less allele segregation than would be expected even under allopolyploidy. For example, *A. aneura* var. *tenuis* allotetraploids with three alleles would be expected to experience some segregation. Clonality is therefore the most likely cause of stable heterozygous genotypes in the population. Although mulga regenerates limbs after lopping for fodder and one form appears to resprout following fire (Peter Latz, pers. comm.), recruitment of mulga plants appears to occur only through seed. The clonal genetic structure of the population could therefore be best explained by facultative apomixis.

While investigation of embryo development is necessary to prove that apomixis is occurring in the mulga complex, it may readily explain the maintenance of morphotypes as discrete, yet coexisting entities. Apomixis produces clones, or groups of genetically identical plants, in which the frequency of sexual reproduction is reduced, sometimes to very low levels (Kirschner and Stepanek 1996). It appears that each morphotype in the study population consists of one or more clones with several ramets, plus a variable number of unique genotypes (Table 4). On the basis of genotypic diversity, the frequency of apomixis appears to vary from no evidence of sexual seed production in the narrow form of *A. minyura*, slight sexuality in *A. ayersiana* and the broad form of *A. minyura* and low levels of apomixis in *A. aneura* varieties *tenuis* and *intermedia* (Table 5) However, given the sample size, it cannot yet be concluded that the narrow *A. minyura* is obligately apomictic. Several studies have demonstrated that even apparently obligate apomicts possess some residual sexuality (Bayer 1990; Kollmann *et al.* 2000). Nevertheless, sexual recombination among clones, of both the same and different morphotypes, is likely to be reduced by their tendency to produce seed asexually. Purely sexual reproduction would erode differences between morphotypes by recombination of the genes controlling divergent traits. In contrast, facultative apomixis could enable morphological variants to persist in sympatry by reducing the rate of homogenisation by sexual recombination.

Apomixis could also maintain the morphological diversity of mulga by enabling the reproduction of morphotypes that may otherwise be sterile (Martonfi *et al.* 1996; Bonilla and Quarin 1997), such as the triploid, narrow-leaved form of *A. minyura*. Such odd-ploid morphotypes are often the product of hybridisation events and may be lost from future generations in the absence of apomixis. Putative hybrid forms, reported in many parts of the group's range (Pedley 2001), could also result from recent or ancient hybridisation events and subsequent persistence of the hybrid as a clone.

Many of the features of mulga are also found in the genus *Senna* (Caesalpinioideae), at present the only legume in

which apomixis has been clearly demonstrated (Randell 1970; Holman and Playford 2000). The series *Subverrucosae* is widespread in the Australian arid zone and facultative apomixis may represent an adaptation to the irregular fluctuations in conditions in arid areas, by providing a balance between 'fitness and flexibility' (Randell 1970). Adaptive gene complexes could be maintained by asexual seed production, facilitating niche differentiation, which has been observed in other apomictic groups (Vavrek 1998) and may occur in mulga (Fox 1980). On the other hand, new morphologically different clones may be produced from fertilised seeds, as mulga appears to show some degree of sexuality. Fluctuating aridity in the Quaternary could have provided ideal conditions for the evolution and spread of apomicts (Hopper and Maslin 1978; Maslin and Hopper 1982). Ploidy level variation is found in a number of Australian arid-zone taxa and is thought to have facilitated invasion of the arid zone by these groups (Barlow 1982). Polyploids tend to be more physiologically plastic than diploids and are therefore more successful in colonising new environments (Kirschner and Stepanek 1996; Petit *et al.* 1996). This phenomenon has been noted particularly in weeds, but is likely also to have occurred in mulga and other groups that have rapidly expanded in the Australian arid zone (Barlow 1982). Several characteristics of facultative apomicts are likely to be favourable in harsh environments and habitats subject to irregular fluctuations in edaphic conditions. The persistence of genets across generations and the resulting dispersal advantage increase the probability of finding a favourable environment.

The likelihood of apomixis has important consequences for the taxonomy of mulga. The results presented here show that it is possible to recognise discrete morphological entities, or morphotypes, in the study population, despite variation within individuals. Furthermore, the majority of these morphotypes correspond to taxa contained in Pedley's (2001) treatment and appear to be reproductively isolated at this site. However, the taxonomic level at which apomictic lines should be recognised is difficult to define and would require an investigation of mulga genetics over a geographical range. This would help to determine whether mating is more likely to occur between individuals of different morphotypes or whether mulga morphotypes share a common evolutionary fate, being temporarily segregated into discrete morphotypes.

A more-detailed investigation of the distributions and genetic relationships within and among morphotypes is required before the taxonomy of the mulga species complex can be confidently described. Perhaps the most crucial avenue of future research is the cytology and embryology of the mulga complex. A detailed cytological survey of mulga, coupled with observations of embryo growth, would shed light on the reproductive strategies of this enigmatic group. Confirmation of the hypothesis of apomixis would have far-reaching implications for our understanding of Australian arid-zone evolution and the directions to be taken in future mulga research.

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