

## Somatic mutations contribute to genotypic diversity in sterile and fertile populations of the threatened shrub, *Grevillea rhizomatosa* (Proteaceae)

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- **Background and Aims** *Grevillea rhizomatosa* is a spreading shrub which exhibits multiple breeding strategies within a narrow area in the fire-prone heathlands of eastern Australia. Reproductive strategies include self-compatibility, self-incompatibility and clonality (with and without sterility). The close proximity of contrasting breeding systems provides an opportunity to explore the evolution of sterility and to compare and contrast the origins of genotypic diversity (recombinant or somatic) against degrees of sexual expression.
- **Methods** ISSR markers for 120 band positions (putative loci) were used to compare genetic diversity among five populations at a macro-scale of 5 m between samples ( $n = 244$  shrubs), and at a micro-scale of nearest neighbours for all plants in five 25-m<sup>2</sup> quadrats with contrasting fertilities ( $n = 162$  shrubs). Nearest-neighbour sampling included several clusters of connected ramets. Matrix incompatibility (MIC) analyses were used to evaluate the relative contribution of recombination and somatic mutation to genotype diversity.
- **Key Results** High levels of genotypic diversity were found in all populations regardless of fertilities (fertile populations,  $G/N \geq 0.94$ ; sterile populations,  $G/N \geq 0.97$ ) and most sterile populations had a unique genetic profile. Somatic mutations were detected along connected ramets in ten out of 42 ramet clusters. MIC analyses showed that somatic mutations have contributed to diversity in all populations and particularly so in sterile populations.
- **Conclusions** Somatic mutations contribute significantly to gene diversity in sterile populations of *Grevillea rhizomatosa*, the accumulation of which is the likely cause of male and female sterility. High levels of genetic diversity therefore may not always be synonymous with sexual fitness and genetic health. We hypothesize that frequent fires drive selection for clonal reproduction, at the cost of flowering such that sexual functions are not maintained through selection, and the build-up of somatic mutations in meristems results in high genotype diversity at the cost of pollen and ovule fertilities.

**Key words:** Clone diversity, ISSR, matrix incompatibility, multiple breeding strategies, sterility, *Grevillea rhizomatosa*, somatic mutations.

### INTRODUCTION

The world is replete with examples of rare and endangered plant species on the brink of extinction (wild macadamias, for example; Pisanu *et al.*, 2009; Neal *et al.*, 2010); however, in the face of human-induced adversity there are also many rare plant species that persist in the landscape (e.g. cliff-dwelling *Bertya ingramii*; Scott and Gross, 2004). Long-livedness is one life-history trait that may promote the persistence of populations through individuals living long and having many opportunities to reproduce thus compensating for times where seed production or establishment are not favourable. In support of this, Sjöström and Gross (2006), suggest that a lack of trees on the recently extinct plant database for Australia is a direct result of the long-livedness of the tree habit imparting resilience to populations of such species. Indeterminate growth through clonal reproduction may also afford longevity and there are several remarkable examples of seemingly 'immortal' individuals (see table 1 in de Witte and Stöcklin, 2010). However, the costs of indeterminate growth, where meiosis is avoided and, as the clone spreads, the number of mitotic cell

divisions increases, may include an accumulation of mutations. The accumulation of mutations is likely to buoy the level of genetic diversity in clonal individuals as deleterious mutations are less likely to be purged in the absence of sexual recombination. Indeed there are many examples where clonal organisms have high levels of gene diversity (Ellstrand and Roose, 1987), although the benefits, if any, of this are rarely tested and require assessments of asexual fitness (e.g. Ally *et al.*, 2010).

Since Muller's work (Muller's Ratchet; Felsenstein, 1974) in 1964 and Klekowski's somatic mutation theory of clonality in 1997 (Muller, 1964; Klekowski, 1997), there has been a growing interest in the study of sterility in clonal plants (e.g. Eckert, 2002). One model pathway for the advent of sterility is that if the sexual machinery is not under active selection (i.e. due to a lack of fertilization) and vegetative reproduction is possible and dominates, background somatic mutations are not purged but build up to have the effect of disrupting function including processes critical to sexual fertility (e.g. Eckert *et al.*, 1999). The insertion of T-DNA through mutagenesis in the MAP3Kε1 and MAP3Kε2 protein kinase genes of *Arabidopsis*, for example, has been shown to cause plasma

membrane irregularities following pollen mitosis I which causes pollen lethality (Chaiwongsar *et al.*, 2006).

Less work has been undertaken on the environmental mechanisms that may promote clonal growth. In landscapes where frequent wildfire prevents individuals from flowering and fruiting, and clonal growth is promoted, the scene is set for somatic mutations to build up in the absence of purging. Such mutations may disable sexual recombination such that when flowering is permitted during fire-free periods, seed cannot be produced. The types of species prone to this could include those that inhabit fire-prone ecosystems, which take several years to reach sexual maturity, require several years of a fire-free period to re-initiate flowering and resprout after fires. Many species in the Proteaceae exhibit these life history traits and exist in fire-prone habitats. The growing membership of proteads with infertile, obligate-clonal species in *Grevillea* (Kimpton *et al.*, 2002; Holmes *et al.*, 2008, 2009), *Lomatia* (Lynch *et al.*, 1998), *Banksia* (Millar *et al.*, 2010) and *Hakea* (Smith, 2004), for example, is thus of great interest. Where entire populations are infertile and obligately clonal, somatic mutations may accrue, resulting in a very different genetic profile than sexually reproducing populations of the same species. However, studies incorporating both sterile and fertile populations are few for woody species with bisexual flowers, and lacking in the large southern hemisphere family, Proteaceae. We set out to compare genetic profiles across different sexual strategies using the threatened species *Grevillea rhizomatosa*, a long-lived shrub that has self-incompatible, self-compatible and fully sterile populations, all of which occur in a fire-prone habitat and have individuals that reproduce vegetatively (Gross and Caddy, 2006). Our first objective was to evaluate and compare levels of divergence in gene diversity in sterile populations compared with that found in nearby sexually reproducing populations. We expected overall genotypic diversity to be highest in self-incompatible populations, followed by the self-compatible population and lowest in sterile populations. Information on levels of divergence can inform estimations of clone age (Ally *et al.*, 2008) and speciation trajectories (Vanormelingen *et al.*, 2008) and, when multiple breeding systems are present, it can provide information on life-history strategies and population dynamics.

Our second objective was to determine if somatic mutations are frequent events in *Grevillea rhizomatosa*. The unequivocal detection of somatic mutations for wild species can be an elusive task. It is dependent upon a variable molecular marker and a suitable target organism (ideally one with rapid vegetative reproduction) with ramets that can be distinguished (e.g. with extant connections between ramets). This is the problem of 'being at the right place at the right time with the right tools'. As it can be difficult to satisfy all of these requirements, the likelihood of making incorrect conclusions (i.e. incorrectly excluding somatic mutagenesis as a contributor to gene diversity) is an issue. Several approaches were employed to try and minimize the risk of these Type I errors including the excavation of ramets. We aimed to determine if somatic mutations are more common in sterile compared with fertile populations where purging through recombination and natural selection would be more frequent.

## MATERIALS AND METHODS

### *Species and study sites*

*Grevillea rhizomatosa* Olde & Marriott (Proteaceae) is a spreading shrub growing to 1.5 m in height that flowers sporadically throughout the year but mainly in August–November. It is restricted to a 27-km<sup>2</sup> area on the Gibraltar plateau (see fig. 1 in Caddy and Gross, 2006) where it often grows adjacent to creeks on granitic soil. Reproduction occurs through the proliferation of rhizomatous suckers and less often by seed production (Gross and Caddy, 2006). Ramets are produced from subterranean stems or thickened roots that can extend below ground for several metres before a stem sucker emerges above ground. Often lines of smaller plants can be seen emerging from a central plant (a guerilla strategy). Excavations revealed that these are stem ramets and not root fusions between genets. The suckering habit of *G. rhizomatosa* enables rapid regeneration after fire (i.e. resprouter). As such, populations are comprised of individuals that are of an indeterminate age and plants in small patches could be derived from the same genet.

Six populations within a 9 km × 3 km area were studied within the contiguous national parks of Gibraltar Range and Washpool in north-eastern NSW, Australia (29°28'03"S to 29°32'37"S, 152°18'18"E to 152°21'39"E; see fig.1 in Caddy and Gross, 2006). Populations are small, each with fewer than 300 plants: Washpool (approx. 225 individuals), Dandahra Trail (approx. 250 individuals), Cascade (41 individuals), Swamp (165 individuals), Mulligan's Hut (approx. 250 individuals) and Cascade Walk (approx. 80 plants). The Mulligan's Hut and Swamp populations are sterile (Caddy and Gross, 2006) as are the plants in the Cascade Walk population (C. L. Gross, unpubl. res.). The Cascade and Dandahra Trail populations have a self-incompatibility mechanism that is absent from the Washpool population (Gross and Caddy, 2006). We do not know if the incompatibility mechanism is the result of an S-allele system or the result of early-acting inbreeding depression. In the Washpool population seed set can occur from assisted self-pollination, autogamous pollination as well as from outcrossed pollen (Gross and Caddy, 2006). The species is diploid in both sterile and fertile populations ( $2n = 20$ ; Nelson, 2006).

### *Genetic diversity at macro- and micro-scales*

Genotypic diversity was investigated at two scales. At a macro-scale we aimed to quantify the number of genotypes in the greater study area by sampling extensively from five populations. Using a micro-scale survey we looked for genetic variation in small neighbourhoods (NH = all plants within a 25-m<sup>2</sup> quadrat) where groups of plants were likely to contain ramets. Here our aim was to detect the extent of clonality in small patches and whether connected plants were of the same genotype. Field sampling was undertaken between 2006 and 2010.

### *Macro-scale genetic variation*

Leaf material was collected from at least 35 plants in each of five populations (Washpool,  $n = 54$  plants; Dandahra trail,

$n = 50$  plants; Cascade,  $n = 50$  plants; Swamp,  $n = 35$  plants; Mulligan's Hut,  $n = 55$  plants). To minimize sampling the same genet, leaf tips were taken from individuals that were separated by at least 5 m. Samples were labelled and dried in silica gel in preparation for DNA extraction.

#### Micro-scale genetic variation

Neighbourhood quadrats (NH = all plants in a 25-m<sup>2</sup> quadrat were sampled) were set out in three populations. Populations that had a high risk of erosion from excavation work were not used. Within quadrats, nearest neighbour distances were measured so that neighbourhood maps could be constructed. Sampled plants were checked for connections to other plants through careful excavations. Two neighbourhoods were sampled in fertile and self-incompatible Cascade (Cas–NH1 = 25 plants, Cas–NH2 = 23 plants), one in sterile Cascade Walk (CasW–NH3 = 56 plants) and two in sterile Mulligan's Hut (Mull–NH4 = 32 plants, Mull–NH5 = 26 plants). At Cascade the two quadrats were separated by a creek. At Mulligan's Hut the quadrats were approx. 30 m apart and separated by boulders. Leaf tips (10 cm) were taken from every shrub in the quadrat (25 m<sup>2</sup>). Samples were labelled and dried in silica gel in preparation for DNA extraction.

#### DNA extraction

Apical shoots (20 mg) were placed in 2-mL centrifuge tubes with a 3-mm tungsten carbide bead and pulverized in a mixer mill for 30 min, followed by a digestion with 400  $\mu$ L AP1 buffer (DNeasy plant mini kit; Qiagen) for 30 min at 65 °C. Then 130  $\mu$ L AP2 buffer was added and the mixture incubated on ice for 5 min and centrifuged at high speed for 8 min. From each sample, 200  $\mu$ L was placed in a reaction plate and total genomic DNA was extracted by the X-tractor Gene robotic system (Corbett) using a standard protocol (Pusterla *et al.*, 2008). The quality of DNA in samples was tested on a 1% agarose gel with standard concentrations of Lambda DNA (1, 20, 50 and 100 ng $\mu$ L<sup>-1</sup>) followed by visual assessment of each sample. DNA was quantified using a spectrophotometer (Nanodrop ND-1000; Nanodrop Technologies) and diluted to a concentration of 20 ng $\mu$ L<sup>-1</sup> in PCR-grade water. Twenty-six ISSR primers (Godwin *et al.*, 1997; Wolfe *et al.*, 1998) based on the UBC Primer set 9 (obtained from GeneWorks, Melbourne, Australia) were tested on four individuals from each of six populations. Four primers that generated polymorphic and reproducible bands were selected. Primer 809 [(AG)<sub>8</sub>G] yielded 30 bands, primer 840 [(GA)<sub>8</sub>YT] yielded 38 bands, primer 841 [(GA)<sub>8</sub>YC] yielded 31 bands and primer 888 [BDB(CA)<sub>7</sub>] yielded 21 bands. These primers were then used for amplification of all samples taken across all populations. The reaction mixture comprised 10  $\mu$ L of GoTaq<sup>®</sup> Green Master Mix (Promega), 1  $\mu$ L of primer (10  $\mu$ M), 1  $\mu$ L of template DNA (20 ng) and 8.0  $\mu$ L of PCR-grade water for each sample to give a total volume of 20  $\mu$ L. The PCR programme consisted of an initial denaturation at 94 °C for 10 min and each cycle then comprised a 45-s denaturation phase at 94 °C, a 45-s annealing phase at 41 °C, and a 2-min extension phase at 72 °C. After 40 cycles there

was a final extension phase of 10 min at 72 °C. The PCR products were then analysed using a multicapillary electrophoresis system with a modified 0M500 method file (QIAxcel DNA High Resolution Kit). To check for DNA extraction repeatability and to set the thresholds of similarity, two samples were extracted separately from the same leaf for six leaves, one leaf from each population. ISSR band outputs were then scored automatically using the QIAxcel Bio Calculator (with thresholds for similarity set at baseline filter = 100 rfu, threshold = 15%, minimum distance = 2.00 bp). Then each sample profile was checked visually for miscalled or poorly identified peaks which were eliminated. The QIAxcel Bio Calculator was used to produce a presence/absence binary score for each sample. Due to a large number of samples, a second stage of data quality-control was undertaken by searching the data matrix for miscalled data. This was done by checking the data for (a) any two bins separated by <5 bp; (b) paired bins (size brackets), which were then merged if they were separated with <5 bp or separated if differences were >5bp; (c) bins with <20 fragments; and (d) all bins which were fully populated across a population were double checked against their respective run files to verify them. Miscalled data occurred in <2 % of the data matrix. In addition the alignments between samples from different analyses were calibrated by shuffling samples and repeating the analyses. This process was repeated for each primer across the entire dataset. In addition and where somatic mutations were detected, samples were re-run and visualized on agarose gels using the methods in Fatemi and Gross (2009).

#### Statistical analysis

Using the protocols of Fatemi and Gross (2009), bands were scored as either present (1) or absent (0) for all individuals. Analyses were conducted using the binary option for diploid organisms in GenAlEx 6.1 (Peakall and Smouse, 2006). The number of private bands (bands unique to a single population), percentage polymorphism and Shannon's Information Index were calculated at both the macro- and micro-population levels. The distribution of genetic variability was assessed by conducting an analysis of molecular variance (AMOVA). The relationship between individuals and populations was investigated by principal co-ordinate analysis (PCoA).

Clonal or genotypic diversity was estimated by calculating the following two parameters in the macro- and micro-scale populations: (1) the proportion of distinguishable genotypes was measured as  $G/N$ , where  $G$  is the number of genotypes detected and  $N$  is the total number of individuals (ramets) sampled; (2) Simpson's Diversity Index ( $D$ ) corrected for finite sample sizes by Pielou (1969) was calculated as:

$$D = 1 - \sum \{[n_i(n_i - 1)] / (N(N - 1))\}$$

where  $n_i$  is the number of ramets of  $i$ th genotype and  $N$  is the sample size. The index  $D$  is 0 in a population with only one genet and 1 in a population comprised of genets with only one member. The evenness measure of Fager (1972) was calculated as:

$$E = D_{\text{obs}} - D_{\text{min}} / D_{\text{max}} - D_{\text{min}}$$



where  $D_{\min} = [(G - 1)(2N - G)]/[N(N - 1)]$  and  $D_{\max} = [N(G - 1)]/[G(N - 1)]$ .  $E$  is 0 in a population where all samples represent different genets or where all samples belong to the same genet.  $E = 1$  when there is an even distribution of genotypic diversity (i.e. where all genets are replicated an equal number of times in a population). To estimate the number of genotypes in populations, similarity thresholds were also used for both the macro- and micro-sampling scales by calculating average genetic distances among ramets to provide percentage identity. Scoring errors and mutations may cause individuals from the same clonal lineage (clone-mates) to have a pairwise distance larger than zero. Using the program GenoDive Version 2.0b20 (software for analysis of population genetic data, 2011; see Meirmans and Van Tienderen, 2004) ramets were assigned to genotypes at the 100 % identical (threshold 0) and thresholds 1, 2, 3 and 4 which are based on genetic distances calculated from band profiles. The most conserved limit for allocating clones is threshold 0 (i.e. to be considered a clone-mate, finger-print profiles have to be 100 % identical between pairs) and commonly used limits range from 2 % to 4 % (i.e. any pair of individuals having <2–4 % differences in the profiles are classified as clone-mates; see also Lo *et al.*, 2010, and references therein).

Finally, matrix incompatibility analyses (Mes, 1998) was used to evaluate if genotype profiles were generated via somatic mutations or from recombination events. With binary ISSR data the presence or absence of bands at two loci presents four possible combinations (0/0, 1/0, 0/1, 1/1). The presence of all four combinations is more parsimoniously explained by sexual recombination than by three mutation events, assuming the infinite allele model. The level of incongruence among profiles is referred to as 'incompatibility'. The incompatibility value is used as a measure of recombination when summed over all pairwise comparisons. The jackknifing JACTAX option in PICA (Wilkinson, 2001) was used to determine the contribution of a particular genotype to matrix incompatibility. The genotypes with the highest contribution

were sequentially removed from the dataset until the matrix incompatibility count (MIC) became zero or only four multilocus genotypes were left in the analyses. The number of steps (genotypes) required to reduce the MIC to zero is an indication of the origins of the genetic diversity. If the deletion of most genotypes is required to remove matrix incompatibility, indicative of recent recombination events, there should be a gradual decrease in MIC until only four multilocus genotypes are left in the analyses. If only a few genotypes contribute to overall matrix incompatibility, there should be a sharp decrease in MIC upon deletion of these genotypes, indicating that the majority of genotypes were derived from a pathway involving somatic mutation. These analyses were conducted at a population level for all macro- and micro-scales. In the micro-scale analysis the Cascade Walk neighbourhood (CasW–NH3,  $n = 56$  plants) the quadrat was split into two, giving two batches of 28 samples so that the MIC analysis with the other neighbourhoods was comparable in terms of sample size.

## RESULTS

### Macro-scale genetic variation

At the macro-scale, 244 shrubs over five populations were sampled. ISSR markers provided 120 band positions of which all were polymorphic across populations, although within populations per cent polymorphisms ranged from 48 % to 65 % (Table 1). At the conservative level of 100 % similarity (where identical genotypes have to share exact matches at each band position) there were 243 genotypes present in the 244 sampled individuals (Table 1). No genotypes were shared among populations and private bands were detected in all populations (Table 1). Genotypic diversity ( $G/N$ ) was high within populations (0.97–1.00) regardless of fertility levels, and Simpson's Diversity Index showed that all genets were made up of one member except at Swamp where 90 % of the population was made up of one member (Table 1). An evenness value of 0 was found in all

TABLE 1. Summary of genetic/genotypic data collected in populations of *G. rhizomatosa* at the macro-scale of sampling with at least 5 m between plants within populations

Population	<i>n</i>	No. of genotypes (no. of plants in each clone network) at threshold levels 0–4					At threshold 0					
		0	1	2	3	4	Private bands	<i>P</i> %	<i>G/N</i>	<i>I</i>	<i>D</i>	<i>E</i>
Cas–SI	50	50 (1)	49 (1–2)	47 (1–2)	46 (1–3)	39 (1–4)	3	50	1.00	0.19	1.0	0.0
Dand–SI	50	50 (1)	50 (1)	50 (1)	49 (1–2)	49 (1)	6	65	1.00	0.24	1.0	0.0
Mull–sterile	55	55 (1)	53 (1–2)	48 (1–3)	40 (1–11)	29 (1–15)	3	48	1.00	0.16	1.0	0.0
Wash–SC	54	54 (1)	54 (1)	54 (1)	53 (1–2)	53 (1–2)	2	62	1.00	0.21	1.0	0.0
Swamp–sterile	35	34 (1–2)	32 (1–4)	32 (1–4)	31 (1–4)	29 (1–4)	1	57	0.97	0.21	0.9	0.0
Total	244	243	238	231	219	199	15					

*n* = number of plants sampled.

No. of genotypes = number of different genotypes [based on thresholds of 0 (100 % matching bands) and thresholds 1–4 which indicate the maximum genetic distance that is allowed between two individuals to still be clone-mates with the same multilocus genotype]. The size of the clone network is given in parenthesis as the number of clone-mate plants.

Private bands = the number of bands unique to the population; *P*% = percentage of bands that were variable within populations; *G/N* = proportion of distinguishable genotypes; *I* = Shannon's Information Index; *D* = Simpson's Diversity Index; *E* = Genotypic evenness measure of Fager.

Populations: Cascade (Cas), Dandahra (Dand), Mulligan's Hut (Mull), Washpool (Wash) and Swamp.

SI = plants are self-incompatible; SC = plants are self-compatible; sterile = flowers male and female sterile.

macro-populations (Swamp was 0.000000330), indicating that the plants represent different genets. In fact, clones (plants with identical genotypes) were only detected in Swamp (Table 1). The self-incompatible populations (Cascade and Dandahra Trail) exhibited levels of polymorphisms and Shannon's Information Indices comparable to the range found within the sterile population at Swamp. The sterile Mulligan's Hut plants had the lowest detected levels of polymorphic band positions and a low Shannon's Information Index (Table 1).

The overall area occupied by a population on the PCoA reflects the amount of genetic variability contained within that population. A large area of occupancy on the figure indicates high variability and overlapping individuals indicate shared genotypic profiles. The population groupings from the PCoA (Fig. 1) showed that populations form aggregated entities with some overlap. Plants from the Mulligan's Hut population formed a discrete group. The self-incompatible

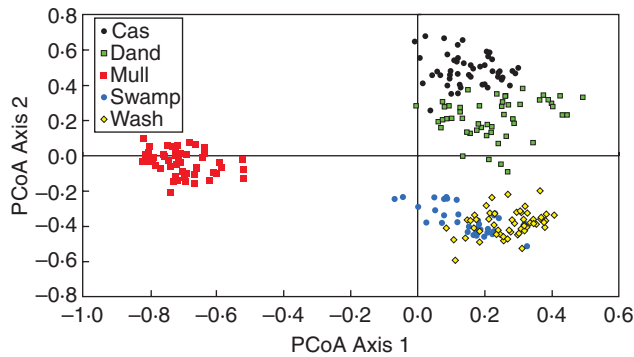


FIG. 1. Principal co-ordinate analyses of ISSR data collected from individuals sampled from five populations of *G. rhizomatosa* in the Washpool and Gibraltar Range National Parks, NSW (PC1 = 31.62 % variation, PC2 = 24.18 % variation, PC3 = 16.68 % variation). Populations are Cascade (Cas), Dandahra (Dand), Mulligan's Hut (Mull), Swamp and Washpool (Wash).

populations were grouped together loosely and had a pairwise Nei's Genetic Identity of 0.928. The self-compatible plants from Washpool overlapped in gene diversity with sterile Swamp plants (Fig. 1) and had a pairwise Nei's Genetic Identity of 0.945. The PCoA showed that the Mulligan's Hut population is the most dissimilar to the other populations (Fig. 1) and it had the lowest pairwise Nei's Genetic Identities with the other populations (range 0.884–0.889). The clumped array of genotypes from the Swamp and Washpool populations indicates that there is a comparatively high level of similarity among individuals from those populations (Fig. 1). AMOVA indicated that the majority of genetic variability (54 %) was located within populations ( $\Phi_{PT} = 0.458$ ,  $P = 0.010$ ).

#### Micro-scale genetic variation

At the micro-scale, 162 shrubs over three populations and five neighbourhoods were sampled and 95 band positions resolved. We detected 146 genotypes and none was shared among neighbourhoods. All neighbourhoods had private bands with sterile Cascade Walk containing the highest number of private bands (Table 2). Genotypic diversity ( $G/N$ ) in the micro-scale population was high and ranged from 0.77 to 1.00 in sterile neighbourhoods (Table 2) and was 1.00 in both fertile neighbourhoods at Cascade (Table 2). Simpson's Diversity  $D$  and Fager's Evenness  $E$  where 1.0 and 0, respectively, in the fertile neighbourhoods, indicating that every plant was a unique genotype. In the sterile neighbourhoods, less diversity and less evenness in genotypes was found in  $D$  and  $E$ , indicating the presence of clones (Table 2). In Mulligan's Hut NH4, plant pairs 2a and 2b and 31a and 31b were each joined below ground by a root-bearing stem (joined ramets) and within pairs the same genotype was found. No plants were joined in the Mulligan's Hut NH5 quadrat and this neighbourhood had the lowest levels of polymorphic bands (Table 2). In the Cascade Walk neighbourhood, where 56 shrubs were sampled, 43 genotypes were detected

TABLE 2. Summary of genetic/genotypic data collected in neighbourhoods (NH) within three populations of *G. rhizomatosa* at the micro-scale of sampling every plant within a 25-m<sup>2</sup> area

Population	<i>n</i>	No. of genotypes (no. of plants in each clone network) at threshold levels 0–4						Threshold 0				
		0	1	2	3	4	Private bands	<i>P%</i>	<i>G/N</i>	<i>I</i>	<i>D</i>	<i>E</i>
Cas–NH1-SI	25	25 (1)	22 (1–3)	20 (1–4)	17 (1–7)	15 (1–7)	3	54	1.00	0.207	1.00	0.0
Cas–NH2-SI	23	23 (1)	22 (1–2)	20 (1–3)	16 (1–4)	11 (1–8)	6	50	1.00	0.205	1.00	0.0
CasW–NH3-sterile	56	43 (1–5)	41 (1–5)	36 (1–6)	31 (1–11)	15 (1–35)	16	49	0.77	0.175	0.986	0.84
Mull–NH4-sterile	32	30 (1–2)	29 (1–3)	20 (1–10)	12 (1–21)	10 (1–22)	1	34	0.94	0.149	0.996	0.52
Mull–NH5-sterile	26	25 (1–2)	24 (1–2)	21 (1–3)	16 (1–9)	8 (1–18)	2	29	0.96	0.126	0.997	0.052
Total	162	146	138	117	92	59	28					

*n* = number of plants sampled.

No. of genotypes = number of different genotypes [based on thresholds of 0 (100 % matching bands) and thresholds 1–4 which indicate the maximum genetic distance that is allowed between two individuals to still be clone-mates with the same multilocus genotype]. The size of the clone network is given in parenthesis as the number of clone-mate plants.

Private bands = the number of bands unique to the population; *P%* = percentage of bands that were variable within populations; *G/N* = proportion of distinguishable genotypes; *I* = Shannon's Information Index; *D* = Simpson's Diversity Index; *E* = Genotypic evenness measure of Fager.

Populations: Cascade (Cas) neighbourhood 1 and 2, Cascade Walk (CasW) neighbourhood 3, Mulligan's Hut (Mull) neighbourhoods 4 and 5.

SI = plants are self-incompatible; sterile = flowers male and female sterile.

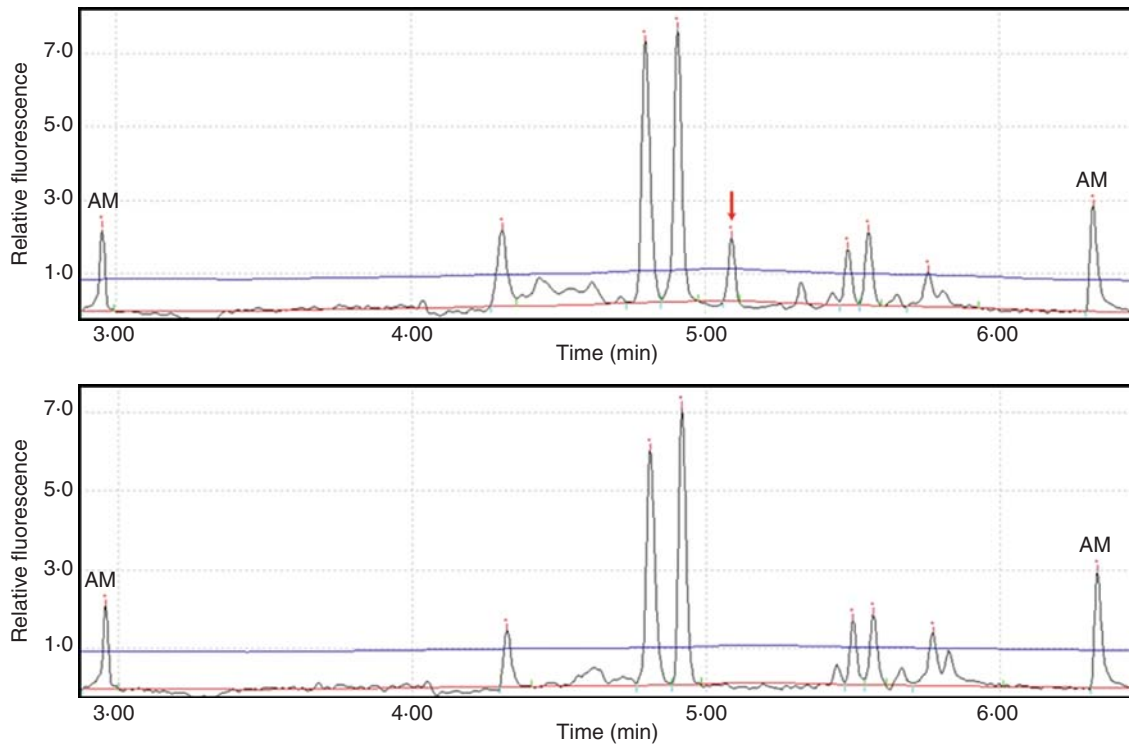


FIG. 2. ISSR profiles of two connected individuals using primer 888. The arrow indicates a band in individual CasWk-5-1 (top) which is not present in an otherwise identical individual CasWk-5-2 (bottom).

(Table 2) with five individuals sharing one genotype. In this neighbourhood our sampling included 36 plants that were connected below ground to one or more plants [15 plant clusters of 2 plants ( $n = 11$ ) or 3 plants ( $n = 3$ ) or 5 plants ( $n = 1$ )]. Six of the 15 ramet clusters had at least one band difference (Figs 2 and 3) while the remaining nine clusters did not express different genotypes on connected pairs of plants. At the Cascade site the two neighbourhoods were separated by a creek. Cascade NH1 and Cascade NH2 had similar levels of gene diversity (Table 2) and were weakly differentiated (Fig. 4), although there were no shared genotypes between these two neighbourhoods. On two occasions in Cascade NH1 we found joined ramets, each involving two plants joined below ground. In one pair, the ramets expressed different genotypes at one band position and in the second joined pair two genotypes were detected, differing at two band positions. At Cascade NH2 we found two pairs of plants that were connected below ground. One pair expressed a different genotype by one band difference and the other pair was of the same genotype. Across all neighbourhoods, ten pairs of connected plants were found where each pair member held a different genotype. Threshold analyses (Tables 1 and 2) provided scenarios of decreased genotypic diversity when the threshold limits from 0 were relaxed to thresholds 1–4. These models suggest that we may have overestimated the number of genotypes in our populations at threshold 0 if, for example, the marker was prone to reading errors, and consequently we may have overestimated the incidence of connected ramets having different genotypes. However, many of the ten plant pairs that expressed different genotypes were still distinguished as separate genotypes at the higher threshold limits of threshold 1 (7/10

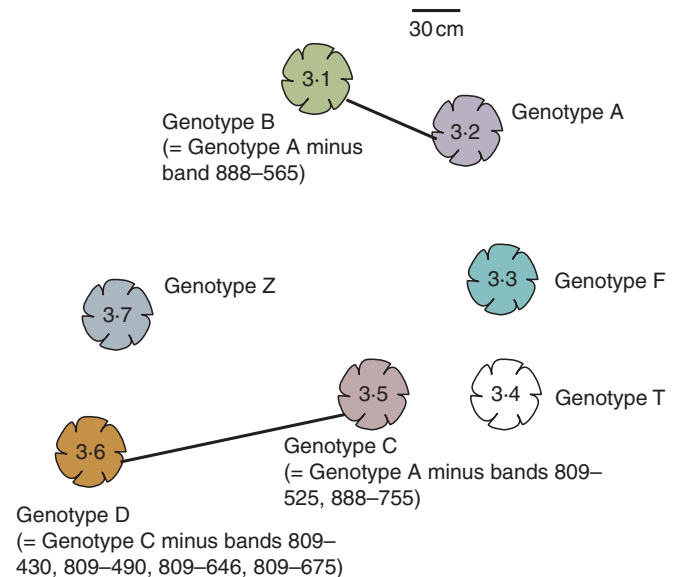


FIG. 3. An example of a cluster of seven connected or unconnected plants and their genotypes at CascadeWalk. Plants 3-1 and 3-2 were connected below ground as were plants 3-6 and 3-5. Genotypes F–Z differed from genotype A by five to seven band differences.

pairs), threshold 2 (5/10 pairs), threshold 3 (4/10 pairs) and threshold 4 (2/10 pairs). Furthermore, repeat assays of suspected somats consistently revealed band differences as shown in Fig. 2 and thus we consider threshold 0 to be a good indication of genotype membership.

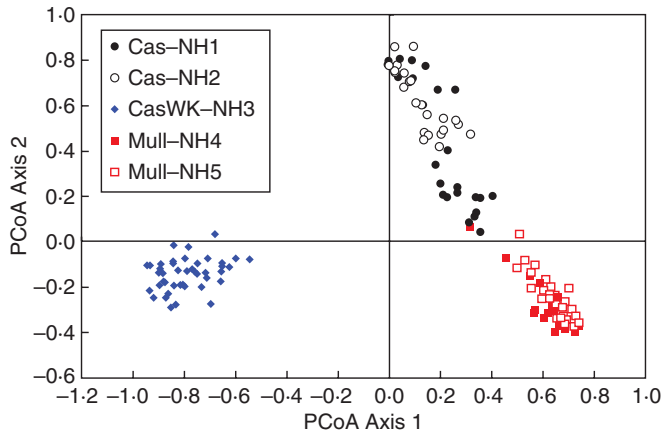


FIG. 4. Principal co-ordinate analyses of ISSR data collected from individuals sampled from five neighbourhood populations of *G. rhizomatosa* in Gibraltar Range National Park, NSW (PC1 = 56.44 % variation, PC2 = 19.71 % variation, PC3 = 9.18 % variation). Populations are Cascade (Cas) neighbourhoods NH1 and NH2, Cascade Walk (CasWk) NH 3 and Mulligan's Hut (Mull) NH4 and NH5.

In summary, across neighbourhoods we found 42 instances of connected plant clusters (one or more connected ramets) and, of these, ten connected plant pairs exhibited different genotypes at the 0 threshold limit, which is unambiguous evidence of somatic mutations.

The PCoA of the neighbourhood data (Fig. 4) shows that the genetic structure within and among the five neighbourhood populations clusters strongly at the within-population level. There is minimal sharing of variation among populations with variation discretely aligned within populations. Cascade NH1 and NH2, the only populations capable of sexual reproduction in the micro-scale study, and in close proximity to each other, overlap in the distribution of several samples indicating a shared genetic history. This finding is supported by the highest values of Nei's Genetic Similarity (Table 3). The Mulligan's Hut neighbourhoods overlap on the PCoA (Fig. 3) whereas Cascade Walk is a discrete group and dissimilar to the other neighbourhoods.

#### Genotype variation: sexual recombination versus somatic mutations

The analysis of character incompatibility (Table 4 and Fig. 5A) showed that at the macro-scale, more genotypes from the fertile than the sterile populations had to be removed to produce compatibility (Fig. 5A). The self-incompatible populations contained the highest percentage of genotypes that were sexually derived (92–94 %; Table 4). The sterile populations at Mulligan's Hut and Swamp had a high proportion of sexually derived genotypes as well (79–82 %) but a greater proportion of their genotypes were derived from somatic mutations compared with the fertile populations (Table 4). In the micro-scale assessments where we sampled intensively in a 25-m<sup>2</sup> quadrat, all of the sterile neighbourhoods had the highest percentages of genotypes derived from somatic mutagenesis (34–48 % compared with 17–20 % in self-incompatible neighbourhoods). The sterile

TABLE 3. Nei's pairwise population matrix of genetic identity for the micro-scale investigation of genetic similarity among five neighbourhoods

	Mull-NH4	Mull-NH5	Cas-NH1	Cas-NH2	CasWk-NH3
Mull-NH4	1.000	–	–	–	–
Mull-NH5	0.985	1.000	–	–	–
Cas-NH1	0.899	0.897	–1.000	–	–
Cas-NH2	0.866	0.859	0.947	1.000	–
CasWk-NH3	0.780	0.771	0.850	0.838	1.000

1.00 indicates complete similarity.

Mull = Mulligan's Hut, Cas = Cascade, CasWk = Cascade Walk. NH = neighbourhoods 1–5.

TABLE 4. The percentage of genotypes derived from sexual recombination in five populations sampled at the macro-scale of plants no closer than 5 m and at the neighbourhood micro-scale of every plant within 25 m<sup>2</sup>

Population	<i>n</i>	No. of genotypes	% of genotypes sexually derived
Macro-scale			
Cascade-SI	50	50	92
Dandahra Trail-SI	50	50	94
Mulligan's Hut-sterile	55	55	82
Washpool-SC	54	54	90
Swamp-sterile	35	34	79
Micro-scale			
Cascade-NH1-SI	25	25	80
Cascade-NH2-SI	23	23	83
Cascade Walk-NH3-sterile	56	43	42
Mulligan's Hut-NH4-sterile	32	30	66
Mulligan's Hut-NH5-sterile	26	25	80

*n* = number of plants sampled.

No. of genotypes = the number of distinct ISSR profiles detected.

The percentage of genotypes sexually derived was determined from matrix incompatibility analyses as the number of genotypes that had to be removed for compatibility, divided by the total number of genotypes.

SI = plants are self-incompatible, SC = plants are self-compatible and sterile = flowers male and female sterile. NH = neighbourhoods 1–5.

neighbourhoods took fewer steps to resolve incompatibilities (Fig. 5B), indicating that somatic mutagenesis is a significant contributor to the origins of the genotypes in these neighbourhoods.

## DISCUSSION

### Detection of somatic mutations in wild populations

The detection of somatic mutations requires variation to be revealed as unambiguous within a whole organism – whether that be a genet (*Thuja plicata*; O'Connell and Ritland, 2004), an agamospermous array (*Taraxacum officinale*; King and Schaal, 1990) or among connected ramets (*Olea europaea* subsp. *laperrinei*; Baali-Cherif and Besnard, 2005). The variable breeding systems and the semi-persistent ramets in *Grevillea rhizomatosa* have provided a rare opportunity to compare genetic diversity across three types of



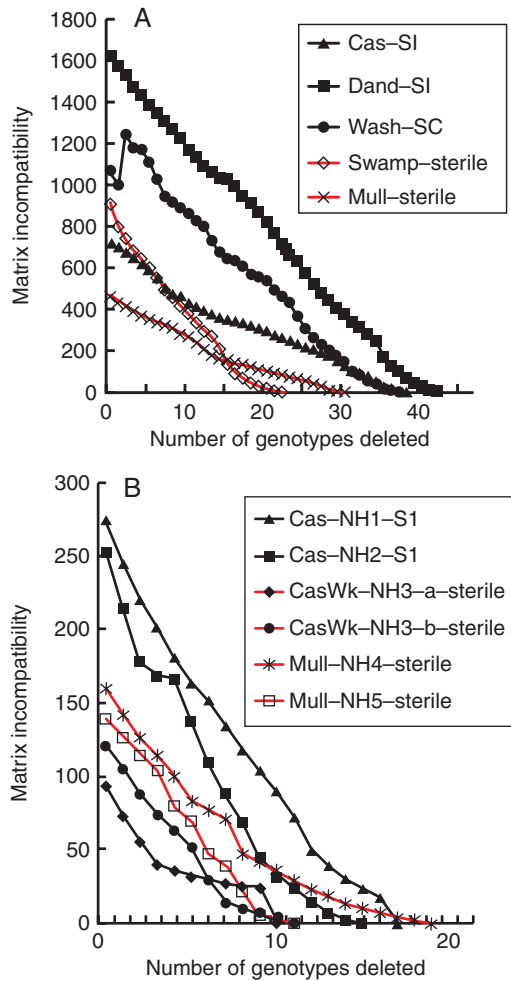


FIG. 5. The matrix incompatibility count (MIC, the number of incompatible character states) after sequential removal of genotypes that contribute most to overall MIC at (A) for the macro-sampled populations and (B) for the micro-sampled populations. Populations are (A) Cascade, Dandahra, Mulligan's Hut (Mull), Washpool (Wash) and Swamp; (B) Cascade (Cas) neighbourhoods NH1 and NH2, Cascade Walk (CasW) NH 3, Mulligan's Hut (Mull) NH4 and NH5. For the Cascade Walk NH3 analyses, the sample was split in two to give  $2 \times n = 28$  plants to even the sampling among neighbourhoods. SI = plants are self-incompatible; SC = plants are self-compatible; sterile = flowers male and female sterile.

breeding systems, to investigate the origins of sterility in *G. rhizomatosa* and to test the hypothesis that somatic mutations contribute significantly to genetic diversity. Moderate levels of genetic diversity in *G. rhizomatosa* were found in both fertile (50–65 % polymorphic bands) and sterile populations (48–57 % polymorphic bands). Somatic mutations were detected in most ramet clusters that were investigated. Mutations (single- or multiple-band differences) were found on pairs of connected ramets growing in both sterile and fertile populations. In sterile Mulligan's Hut NH4, and considering all connected and unconnected plants, there were more genotypes than within the fertile populations at Cascade, although the Cascade NH1 held genotypes with 20 % more polymorphic bands than Mulligan's Hut NH4. This suggests that at Cascade sexual recombination is an important contributor to genotype diversity at this site. In the Cascade Walk

neighbourhood, a sterile population with many ramet-clusters, a clone of four plants with identical genotypes was found, none of which was joined. Within this clone, members were connected to plants in seven other clusters and ramets connected in this super network mostly did not share genotypes. There were eight separate ramet clusters, suggesting that ramet connections eventually disintegrate or are disrupted giving rise to isolated plants or satellite ramet-clusters.

#### Genotypic diversity and breeding strategies

Our expectation that genotypic diversity would be highest in the self-incompatible populations was not supported – gene diversity was uniformly high across all populations. SC Washpool and SI Dandahra had the highest percentage of polymorphisms but genotypic diversity was equally high across all breeding strategies. The genotypic diversity found in all sterile populations was considerably higher than values reported in a meta-analysis by Honnay and Jacquemyn (2008;  $G/N = 0.44 \pm 0.04$ ). The same patterns of genotypic diversity were observed at the micro-scale level in which the sterile populations showed a relatively high level of variation. We suggest that somatic mutations have contributed to the high levels of observed genotypic diversity detected in this study and particularly so in SI Cascade and the sterile populations. Matrix incompatibilities showed that in all populations there are genotypes that are derived from sexual recombination. This is of particular interest in the sterile populations. In the Mull–NH5 neighbourhood for example, 80 % of genotypes had band patterns congruous with a sexual recombination event. We interpret this as persistence of initial diversity, a similar finding to that made by Kimpton *et al.* (2002) for *Grevillea infecunda*. The mutations we discovered in *G. rhizomatosa* using ISSR markers (i.e. neutral allele sites) are unlikely to be the mutations that induce floral sterility. Further work is required in *G. rhizomatosa* to investigate mutation levels in functional alleles. We hypothesize, however, that the sterility in plants is due to an accumulation of genetic damage at functional alleles within meristems (see Thomas, 2002) that has deactivated the sexual machinery, a process which can be accentuated in old and senescing plants (Ally *et al.*, 2010). Alternative hypotheses are that rare sexual events occur in the population or that the majority of plants at this site are sterile  $F_1$  progeny from hybridization events. However, we consider it unlikely that multiple migration events involving sterile  $F_1$  progeny [of at least 25 seeds (80 % of 32 genotypes)] have occurred at Mull–NH 5, especially as nearby Mull–NH4 does not show this pattern of recombination events.

#### Disturbance, resprouters, sterility and somatic mutation: cause and effect?

Sterile individuals of *G. rhizomatosa* exhibit a collection of faulty sexual characters and processes; pollen is not distributed onto the pollen presenter prior to anthesis, pollen is mostly unviable, and viable pollen from fecund populations does not instigate seed set in the sterile Mulligan's Hut population (Gross and Caddy, 2006); i.e. sterile populations of *G. rhizomatosa* are both male and female sterile. Ecological factors that reduce the frequency of sexual events (e.g.



flowering) and thus reduce the opportunities for successful sexual reproduction are likely to prevent the selection of sexual characters, resulting in an accumulation of mutations in sexual traits (Klekowski, 1997). This is the ‘Use it or lose it’ hypothesis discussed in Eckert (2002). The primary ecological factor in the study area that could retard flowering, alter germination success and promote clonal reproduction is a disrupted fire regime (more frequent and or severe fires). Fire is a known stimulator of clonal reproduction in some Australian ecosystems (e.g. Lacey, 1974), and fire is a significant ecological factor in the heath habitat of *G. rhizomatosa* (Croft *et al.*, 2006). In 2006 much of the habitat of *G. rhizomatosa* had experienced two fires in the previous 10 years (Clarke *et al.*, 2006). Croft *et al.* (2006) note that a fire-free interval of 10 years would be needed to ensure the persistence of *G. rhizomatosa*. Frequent fire episodes may prevent sexual reproduction by killing juveniles (and seed reserves) before sexual maturity (Keith, 1996), by depleting starch reserves in standing plants (Keith, 1996) or by preventing resprouting adults from flowering every year. At Washpool our field records for *G. rhizomatosa* show that fire destroys the above-ground biomass but plants quickly resprout from underground suckers. After the 2002 fires it took 8 years for re-sprouting plants at Washpool to recommence flowering (C. L. Gross and P. A. Nelson, unpub. res.). Frequent fires may select for the resprouting habit which allows individuals to vegetatively proliferate, thereby excluding opportunities for seedling recruitment. As summarized by Lloret *et al.* (1999), however, vegetative reproduction is common in many phylogenetic lineages and biogeographical contexts not necessarily associated with fire (e.g. hurricanes). In grasslands, for example, vegetative reproduction can be promoted through management regimes (e.g. Schaal and Leverich, 1996) and this has been linked to the accumulation of somatic mutations in meadows (Warren, 2009). Thus our results may be relevant to other biogeographical contexts. In this study we have shown that the mutations occur in ramets that are resprouting. Lamont and Wiens (2003) predicted that frequent axillary branching (induced by trauma) via abundant stored buds within resprouters might favour expression among their genets and ramets of beneficial somatic mutations present in their meristematic tissues. We have no evidence that the mutations we detected are those directly responsible for the observed declines in sexual fitness in *G. rhizomatosa* – only that somatic mutations are present. Moreover, the effects of the somatic mutations may be largely masked in the diploid adult phase but be deleterious in the haploid pollen stage, causing pollen unviability. We also do not know whether the mutations we detected are beneficial to *G. rhizomatosa*; i.e. at the expense of sexual reproduction, vegetative reproduction may be enhanced (antagonistic pleiotropy; see Dorken *et al.*, 2004). As the production of somatic mutations is likely to be amplified in *G. rhizomatosa*, a long-lived species, a high mutation rate may also result in the loss of functional traits (Muller, 1964). Selection for optimization of mutation rates has been proposed for asexual organisms and, as pointed out by King and Schaal (1990), the processes that generate genetic variation may themselves be under selection. As sexual functionality has been lost in several populations of *G. rhizomatosa*, it would be extremely informative to

investigate the functional relationship between sterility and genetic diversity in this species. Sterility in populations of *G. rhizomatosa* was not commensurate with a complete lack of genotypic diversity. The Mulligan’s Hut and Swamp populations had relatively high levels of diversity when compared with fertile populations.

The detection of high genotype variability in sterile populations suggests several scenarios: (a) sterile populations were founded by fertile genotypes and sterility has subsequently developed; (b) sterile populations originated from genetically variable but sterile individuals, e.g. F<sub>1</sub> hybrids; or (c) that populations consist of large clones in which somatic mutation has generated genetic variability but this variability has resulted in a loss of traits. These hypotheses are not mutually exclusive. Interestingly, the two sterile populations are genetically distinct from one another. Swamp is more similar to SC Washpool than sterile Mulligan’s Hut. This suggests that each population may have been founded independently and that different pathways for sterility may be involved.

#### *Multiple breeding systems in Grevillea rhizomatosa*

Smith and Gross (2002, table 1) collated data for 21 species of *Grevillea* and 19 of these were self-compatible. Further assessments of breeding systems in *Grevillea* have revealed additional self-compatible species (*G. repens*; Holmes *et al.*, 2008), although some weakly so [*G. iaspicula*; fig. 2 in Hoebee and Young, 2001 (however, illegal access to pollinator exclusion bags by honeybees bringing in outcross pollen may have been involved here; S. Hoebee, pers. comm., 10 June 2010)] and fully sterile species (*G. infecunda*; Kimpton *et al.*, 2002). Interestingly, for two species (*G. robusta* and *G. sphacelata*) both self-compatible and self-incompatible populations have been detected [*G. robusta* (Brough, 1933; Kalinganire *et al.*, 2000); *G. sphacelata* (Hermanutz *et al.*, 1998; Richardson *et al.*, 2000)]. For *G. robusta* the incompatibility mechanism has been determined to be gametophytic self-incompatibility (Kalinganire *et al.*, 2000). In *G. rhizomatosa*, plants at Washpool are self-compatible but at Cascade and Dandahra plants are self-incompatible. Here, we hypothesize that selfing is a synapomorphic character for *Grevillea* and that self-incompatibility is imposed through inbreeding depression on *G. rhizomatosa* at Cascade and Dandahra where selfing has brought together a high load of lethal mutations. Inbreeding depression is elevated in perennials with high mitotic mutation rates (Morgan, 2001) and Barrett *et al.* (1996) suggest that the evolution of selfing would be constrained by high genetic loads. We hypothesize that the populations at Cascade and Dandahra have high genetic loads resulting in strong inbreeding depression. The self-incompatible populations (Cascade and Dandahra) had the highest occurrence of polymorphic bands, and somatic mutations were also detected in Cascade (Dandahra not tested). We suggest that a high level of polymorphism in these populations is evidence of a high mutational load rather than evidence of superior genetic health. At Mulligan’s Hut, Swamp and Cascade Walk, we propose that the mutational load has passed a threshold resulting in dysfunctional reproductive characters (faulty anthesis, inviable pollen, female sterility). Support for this hypothesis can be found in Gross and Caddy (2006, fig. 4) where high levels of unviable

pollen were detected at Cascade and Dandahra compared with Washpool, suggesting that populations at Cascade and Dandahra may be moving towards a mutational meltdown. Finally, we propose that the mutational meltdown that induces sterility in *G. rhizomatosa* is unable to be rectified through selection against the deleterious mutations at the cellular level because the proliferation of the clones is actively selected in the fire-prone landscape where flowering can be retarded after fire.

#### Clonality in context and the Australian flora

Clonal growth can afford persistence in harsh environments (Song *et al.*, 2002). However, while the importance of clonal behaviour in plants is appreciated for many floras (e.g. central Europe, Klimeš and Klimešová, 1999; north-east China, Song *et al.*, 2002), the rich Australian flora (approx. 20 000 angiosperms) has not yet been evaluated from this perspective at a meta-analysis level (e.g. Sjöström and Gross, 2006; Gross, 2005). In a continent that is subjected to extreme environmental disturbances and conditions (e.g. drought, floods, fire, cyclones, infertile soils) the clonal strategy in Australian ecosystems is potentially prevalent and very important for population persistence during such times where flowering and fruiting is arrested or prevented. There are some well-recognized and significant occurrences of facultative clonal growth patterns among the Australian flora that occupy ecosystems dependent upon disturbance (e.g. *Triodia*; Lazarides, 1997) and within the alien flora there are many that flourish in disturbed habitats [e.g. facultatively clonal *Phyla canescens* (Xu *et al.*, 2010; Price *et al.*, 2011); obligately clonal *Arundo donax* (A. Haddadchi *et al.*, unpubl. res.)]. Increasingly, covert clonality is being discovered in the Australian endangered tree flora [e.g. *Elaeocarpus williamsonianus* (Rossetto *et al.*, 2004); *Wollemia nobilis* (Peakall *et al.*, 2003)] that is most certainly associated with persistence under adversity [see also *Erythroxylum pusillum* (van der Merwe *et al.*, 2010)]. Within certain shrubby and herbaceous flora clonality appears to be important too and may provide assurance during other adverse situations such as a shortage of pollinators [*Canavalia rosea* (Gross, 1993); *Tetratheca juncea* (Gross *et al.*, 2003)] or in marginal environments (*Banksia ionthocarpa* ssp. *chrysophoenix*; Millar *et al.*, 2010). There is thus much to be learnt about the benefits and extent of clonal reproduction in the Australian flora. Our work has shown that genotypic diversity in a clonal species that is endemic to a fire-prone landscape is derived from both recombination and somatic mutagenesis and the latter is particular prevalent in sterile populations. The compensation that somatic mutagenesis may provide for sterile (and fertile) populations in lieu of the benefits from recombination is generally unknown. We suggest that, as a starting point, partitioning the origins of genetic diversity alongside the elucidation of clonality will contribute very important information on the evolution, speciation and persistence ecology of the Australian flora.

#### Conclusions

Asexual reproduction allows *Grevillea rhizomatosa* to persist in a landscape where fecundity is likely to be retarded

by frequent fires. However, a lack of sexual recombination in some populations has not diminished genotype diversity and we have shown here that diversity is generated through the advent of somatic mutations. Tuskan *et al.* (1996, and references therein) suggest that somatic mutations provide clonal organisms with a mechanism for enhanced survival and fitness, particularly for long-lived organisms that occupy variable environments. We concur with this view but add that the mutational increase in somatic mutations may have been at the expense of successful sexual reproduction. Further work is required to determine the ultimate causes of sterility in *G. rhizomatosa*, and whether a build-up of somatic mutations in apical meristems handicaps the sexual machinery. From a conservation perspective, management regimes should aim to minimize disruption to sexual processes, a theme emphasized by Honnay and Bossuyt (2005).

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