

Paternal bottlenecks in fragmented populations of the grassland daisy *Rutidosia leptorrhynchoides*

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Summary

Allozyme markers were used to estimate mating system parameters in nine fragmented populations of the grassland daisy *Rutidosia leptorrhynchoides* that differed in size and spatial isolation. Multilocus estimates of outcrossing rate did not differ significantly among populations, all indicating a high level of outcrossing ($t_m = 0.84\text{--}1.0$). Small populations showed greater divergence than large populations between the allele frequencies in the population and those in the pollen pool, indicating paternal bottlenecks. Isolated populations of fewer than 200 individuals also exhibited higher correlations of outcrossed paternity (r_p) than larger populations, indicating the production of more full-sibs within families. The combination of paternal bottlenecks and correlated paternity increases the genetic identity of progeny across families and predisposes populations to biparental inbreeding in subsequent generations. As over half the remaining populations of *R. leptorrhynchoides* contain fewer than 200 plants, such second-order inbreeding may threaten the viability of the species if it is associated with significant inbreeding depression.

1. Introduction

Habitat fragmentation has emerged as a dominant landscape process over the last century, and is now widely regarded as a major threat to species diversity. The destruction of large areas of vegetation leaving only isolated, or partially connected, remnants greatly depletes the overall numbers of species, and reduces the average size of their populations. A major consequence is the loss of genetic diversity within species, due initially to bottleneck effects, and subsequently to genetic drift if populations remain isolated for a number of generations – as a growing body of evidence highlights (e.g. van Treuren *et al.*, 1991; Prober & Brown, 1994; Sun, 1996; Young *et al.* (in press). Such losses limit a species' ability to respond to environmental change through selection (Frankel *et al.*, 1995) and may increase the probability of population extinction (Newman & Pilson, 1997).

A second consequence is the effect fragmentation may have on mating events within populations. The main concern here is increased inbreeding and accompanying inbreeding depression in small or isolated remnant populations that might reduce their

long-term viability and conservation value, subject to the mitigating effects of purging of genetic load if selection is strong (Barrett & Charlesworth, 1991). The few studies that have quantified outcrossing rates in fragmented plant populations have so far found little evidence of an increase in the frequency of self-fertilization (van Treuren *et al.*, 1993, 1994), though Rajimann *et al.* (1994) found some increase in maximum selfing rates in small populations of *Gentiana pneumonanthe*.

However, the frequency of self-pollination versus cross-pollination is only one component of mating events. The mating system of plant populations can conceivably respond to fragmentation in at least three distinct yet interacting ways:

1. In species that permit self-fertilization, the proportion of outcrossing events may decline.
2. The number of plants in the population providing pollen may become low, leading to a bottleneck in the pollen pool and a biased sample of population allele frequencies in the next generation.
3. The number of plants contributing pollen to each single maternal plant may decline, increasing the proportion of full- to half-sibs

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within progeny arrays. This may occur even without a bottleneck in the overall number of paternal plants in a population.

Changes in mate availability may trigger any or all of these responses. Such changes may be due directly to reduced size of remnant populations, or decreased immigration due to isolation. They may also come about indirectly owing to, for example, associated changes in flowering phenology or altered abundance or behaviour of pollinator species following fragmentation, for which there is now strong evidence (e.g. Powell & Powell, 1987; Didham *et al.*, 1996).

Polymorphic marker genes offer the opportunity to detect which one or more of these responses is operative in a fragmented system. They enable estimation of outcrossing rates (Fyfe & Baily, 1951; Brown, 1989), of paternal bottlenecks (Waples, 1989; Young & Brown, 1998) and of the correlation of outcrossed paternity (Ritland, 1989). Here we report such estimates for nine fragmented populations, that vary in size and degree of spatial isolation, of the endangered daisy *Rutidosia leptorrhynchoides* F. Muell.

2. Materials and methods

(i) Plant species studied

Rutidosia leptorrhynchoides (Asteraceae) is a multi-stemmed herbaceous perennial daisy 20–40 cm in height endemic to the grassland and grassy woodland communities of southeastern Australia. These communities have been reduced to about 0.5% of their original 2 million ha extent since the mid-1800s, due mainly to conversion of land for sheep grazing (Kirkpatrick *et al.*, 1995). Herbarium records indicate a marked reduction in both the number and size of *R. leptorrhynchoides* populations since 1874 (Fig. 1). More recently, reduction in fire frequency and invasion

of grassland communities by exotic weeds have also reduced the size of several populations (Scarlett & Parsons, 1989). The species is currently listed as nationally endangered (Briggs & Leigh, 1995) and is known from only 24 populations occupying remnant grassland vegetation mainly along roadsides, railway easements and in cemeteries. Populations range in size from five to about 100 000 flowering plants, with half of these consisting of fewer than 200 individuals.

Rutidosia leptorrhynchoides is cytologically complex, with northern populations being diploid ($2n = 22$) while southern ones are either all diploid or primarily tetraploid ($2n = 44$), with low frequencies of triploids ($2n = 33$), hexaploids ($2n = 66$) and a range of aneuploids ($2n = 42–46$) (A. G. Young & B. G. Murray, unpublished data). Flowering is protracted, lasting from mid-spring until the end of summer, and the species is insect pollinated. Although seeds are wind dispersed, dispersal distances are commonly less than 0.5 m and there is no long-term storage of seed in the soil (Morgan, 1995*a, b*). Limited seed production and low recruitment appear to limit regeneration in very small populations. Assessment of allozyme variation in 18 diploid populations has shown small populations to be genetically depauperate relative to large ones (Young *et al.*, in press).

(ii) Controlled pollinations

To examine the potential for self-fertilization and agamospermy, controlled pollinations were conducted on 10 diploid plants raised from open-pollinated families collected from two large populations (Stirling Ridge and Queanbeyan; Table 1). Plants were grown, and all pollinations conducted, in a greenhouse under natural light. Flower heads contain about 90–100 florets which are hermaphroditic and partially protandrous, maturing from the outermost whorl inwards over a period of 3–4 days.

Three pollination treatments were imposed on each plant: (1) unmanipulated – flower heads were bagged as buds to exclude pollinators and left bagged until seed recovery; (2) selfed – two flower heads on the same plant were rubbed together to transfer pollen from the inner florets of one to the outer florets of the other; (3) outcrossed – one flower head from each of two plants originating from different families within the same population were rubbed together to transfer pollen from the inner florets of one to the outer florets of the other. Selfed and outcrossed pollinations were initiated on the day on which the first florets on a head opened and were repeated for three consecutive days to ensure pollination of the majority of florets in the head. After pollination on the first day heads were kept bagged to exclude pollinators. After the last cross on the third day heads remained bagged until seeds were harvested 3–4 weeks later. All crosses were

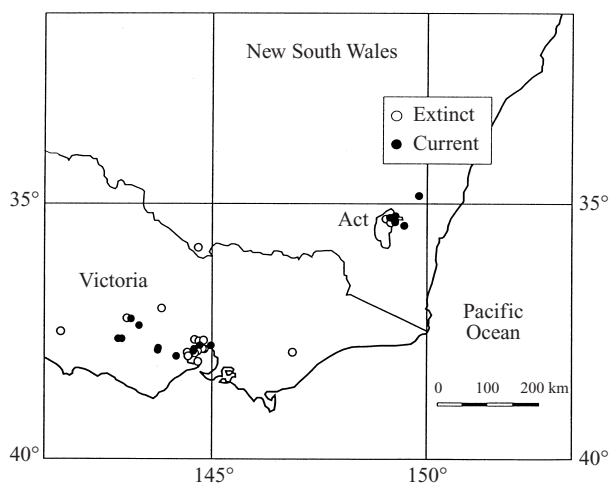


Fig. 1. Distribution of *Rutidosia leptorrhynchoides* 1874–1998.

Table 1. Size and isolation status of *Rutidosia leptorrhynchoides* populations

Population size (no. of flowering plants)	Isolation distance to nearest population	
	< 2 km	> 5 km
Large	Stirling Ridge (70 000) Queanbeyan (10 000)	Goulburn (95 200) Majura (27 626)
Medium	Capital Circle (220) Barton (133)	Captains Flat (161) St Albans (137)
Small	West Block (5)	

reciprocal, giving a total of 20 flower heads per treatment. Differences in the number of filled seed set per head among treatments were assessed using ANOVA.

(iii) Populations, seed sampling and starch gel electrophoresis

Nine diploid populations were examined, covering three size and two isolation classes that represent the range of current conditions of *R. leptorrhynchoides* populations. For size these were: large, 1000s; medium, 100s; and small, five plants. For isolation the classes were: < 2 km or > 5 km from the nearest other population (Table 1). There are no small populations isolated by > 5 km. Two populations, Capital Circle ($n = 220$) and West Block ($n = 5$), are known to have undergone reductions in size over the last 15 years.

Within each population samples of between 15 and 20 open-pollinated seed, from a total of at least three heads, were collected from all five remaining plants at West Block and from 15 randomly chosen plants in other populations. Seeds were vernalized for 3 days at 4 °C and then germinated at room temperature for 2 weeks, at which time percentage germination was assessed for each family. Allozyme analysis of seedlings was conducted as previously described by Young *et al.* (in press) for four enzyme systems encoded by eight putative loci. These were: glucose-6-phosphate isomerase (GPI) EC 5.3.1.9 (1 locus), phosphoglucosmutase (PGM) EC 5.4.2.2 (3 loci), menadione reductase (MNR) EC 1.6.99 (1 locus) and aspartate aminotransferase (AAT) EC 2.6.1.1 (3 loci).

(iv) Estimation of mating system parameters

Maximum likelihood estimates of single-locus (t_s) and multilocus (t_m) outcrossing rates were made following the mixed-mating model with maternal genotypes being inferred from progeny arrays (Brown & Allard, 1970). Correlation of outcrossed paternity (r_p) was estimated following Ritland's (1989) sibling-pair

model, in which r_p is the probability that two individuals drawn at random from the same progeny array are full-sibs. These parameters, and maternal fixation index (F_{is}), were calculated using the computer program MLTR version 0.9 (Ritland, 1994). Standard errors for estimates of t_s , t_m and r_p were based on 500 bootstraps with resampling among maternal plants.

The extent of divergence between the estimates of allele frequencies in the pollen pool $\{y_{ij}: i = 1, \dots, k_j; j = 1, \dots, l\}$ (where l denotes the number of polymorphic marker loci in a population and k_j is the number of alleles at the j th locus) and their corresponding estimates in the population $\{x_{ij}\}$ was measured following Waples (1989) using the Pollak estimator (F_k):

$$F_k = [\sum_{ji} 2(x_{ij} - y_{ij})^2 / (x_{ij} + y_{ij})] / \sum_{ji} (k_j - 1).$$

The values for $\{x_{ij}\}$ were those from a previous sample from these same populations (Young *et al.*, in press) and were based on a larger sample of up to 35 maternal plants.

Two-way ANOVA was used to examine relationships between population size and isolation as independent variables (fixed effects) and outcrossing rate (t_m), correlation of outcrossed paternity (r_p) and F_k as dependent variables. Populations within size and isolation classes were treated as random effects.

3. Results

(i) Self-incompatibility and outcrossing rate

Seed set differed significantly among the three pollination treatments (among-treatment $F_{2,54} = 26.9$, $P > 0.0001$). Outcrossed flower heads produced far greater numbers of filled seed (mean = 28.5 seeds per head) than either selfed (mean = 1.83 seeds per head) or unmanipulated treatments (mean = 0.23 seeds per head), which usually produced no seed at all. A single plant from Stirling Ridge was found to be fully self-compatible.

Multilocus estimates of outcrossing rates for all populations were high, with only Stirling Ridge ($t_m = 0.84$) and Capital Circle ($t_m = 0.86$) being significantly

Table 2. Mating system parameters and percentage seed germination for *Rutidosia leptorrhynchoides* populations

Population	No. of flowering plants	Isolation distance (km)	Maternal F_{IS}^a	t_m^b	$t_m - t_s^c$	r_p^d	Percentage seed germination
<i>Large</i>							
Stirling Ridge (SR)	70000	< 2	0.007	0.84 (0.06)*	0.02 (0.03)	0.33 (0.08)	91 (2)
Queanbeyan (QB)	10000	< 2	0.013	0.92 (0.06)	0.01 (0.04)	0.37 (0.08)	80 (5)
Mean				0.88		0.35	86
Goulburn (GB)	95200	> 5	0.011	0.96 (0.04)	0.05 (0.01)	0.11 (0.04)	86 (3)
Majura (MJ)	27626	> 5	0.009	0.93 (0.04)	0.09 (0.02)	0.34 (0.07)	75 (1)
Mean				0.95		0.23	81
<i>Medium</i>							
Capital Circle (CC)	220	< 2	0.001	0.86 (0.06)*	0.01 (0.03)	0.32 (0.09)	60 (7)
Barton (BR)	133	< 2	0.003	0.93 (0.06)	0.04 (0.04)	0.27 (0.06)	81 (3.6)
Mean				0.90		0.30	71
Captains Flat (CF)	161	> 5	0.005	0.92 (0.07)	0.04 (0.05)	0.53 (0.10)	73 (4)
St Albans (SA)	137	> 5	0.003	0.94 (0.06)	0.03 (0.02)	0.47 (0.07)	73 (3)
Mean				0.93		0.50	73
<i>Small</i>							
West Block (WB)		< 2	0	1.0 (0.01)	0.04 (0.01)	0.96 (0.05)	79 (9)

^a Maternal fixation index.

^b Multilocus outcrossing rate.

^c Multilocus outcrossing rate minus the mean single locus outcrossing rate.

^d Correlation of outcrossed paternity.

^e Based on $n = 400$ seed apart from West Block $n = 100$.

* $P < 0.05$ that $t_m = 1$.

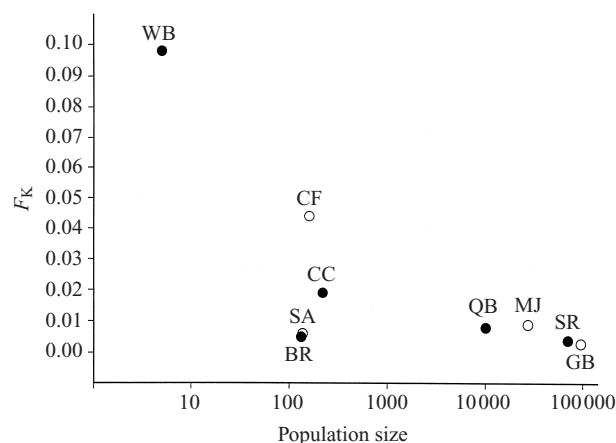


Fig. 2. Divergence between population and pollen pool allele frequencies, as measured by the Pollak estimator (F_k) (Waples, 1989), in relation to population size. Filled circles, population isolated by < 2 km; open circles, population isolated by > 5 km. For full names of populations see Table 2.

less than one (Table 2). There was no significant difference in mean population outcrossing rate among the five size and isolation classes. Differences between multilocus and single locus outcrossing rates were negligible for all populations except Majura, for which the mean of single locus values was 9% lower than the multilocus value of $t_m = 0.93$. Percentage seed germination was also the same for all levels of population size and isolation (Table 2).

Table 3. Two-way ANOVA table for correlated paternity (r_p)

Source	d.f.	Mean square	F	P
Size	2	0.182	24.0	0.006
Isolation	1	0.017	2.3	0.20
Size \times isolation	1	0.041	5.4	0.08
Residual	4	0.008		

(ii) Paternal bottlenecks

In contrast to results for outcrossing rate, the relative divergence between allele frequencies in the pollen pool and the population as assessed by the Pollak estimator (F_k) was negatively related to the log of population size ($F_{2,4} = 16.2$, $P = 0.012$) (Fig. 2). There was no obvious effect of isolation on F_k , nor was there a significant interaction between isolation and size.

(iii) Intraplant correlation of paternity

Correlations of outcrossed paternity ranged from $r_p = 0.11$ in the largest population at Goulburn to $r_p = 0.96$ in the smallest at West Block (Table 2). Two-way ANOVA showed a significant effect of population size on r_p and a marginally significant size by isolation interaction term (Table 3). There were no effects of isolation on r_p independent of size.

The four large populations had low r_p , regardless of isolation status. These low correlations probably mean that the populations have been sufficiently large and stable that available mates and pollinators are not limiting, regardless of their isolation status. Correlations between $r_p = 0.11$ and $r_p = 0.37$ may reflect the composite nature of the inflorescence, which predisposes the plant to pollination of multiple florets in a head by a single pollinator.

The medium-sized populations at Capital Circle and Barton, which were within 2 km of the large Stirling Ridge site, had values of r_p in line with large populations. In contrast, the two populations at Captains Flat and St Albans, isolated by > 5 km, exhibited correlations some 20% higher, indicating reduced paternal diversity in open-pollinated families.

The small West Block population of five plants had the highest correlation ($r_p = 0.96$), despite its proximity to a large population, with families being almost exclusively full-sibs. This very high paternal correlation, despite proximity to the large Stirling Ridge population, suggests that populations can become so small that gene flow from their large neighbours is no longer sufficient to preclude restricted mating patterns.

4. Discussion

(i) Self-incompatibility

In the crossing experiment unmanipulated flower heads yielded virtually no filled seed and selfed heads yielded very few. This suggests that *Rutidosis leptorrhynchoides* produces no apomictic seed, and has a strong self-incompatibility mechanism. Self-incompatibility is characteristic of the Asteraceae, with many species possessing multiallelic sporophytic systems (Frankel & Galun, 1977). However, our finding of one individual that set numerous seed after deliberate selfing shows that in *R. leptorrhynchoides* such self-incompatibility can be lost.

The low maternal fixation indices and uniformly high outcrossing rates for the nine populations show that cross-fertilization prevails, despite the variation between them in spatial isolation and differences in size of 5 orders of magnitude. The possibility of an increase in abortive self-pollination in small or isolated populations cannot be ruled out as seed set was not quantified. However, high seed germination in all populations suggests that late-acting post-zygotic selection against selfs may be of limited importance.

The maintenance of full outcrossing in depleted populations of *R. leptorrhynchoides* is perhaps surprising given that rare fully self-compatible individuals occur, and that these should be at a significant selective advantage when populations become small and mates are limited. There are several possible explanations. First, the lack of seed-set from the

unmanipulated pollination treatment suggests that self-fertilization requires a pollinator to visit the flower. This constrains the evolution of self-compatibility as the pollinators will generally carry some outcross pollen. Secondly, the high levels of genetic load maintained in self-incompatible species, and expressed when they produce selfed seed, may offset the reproductive advantage of autogamy (Les *et al.*, 1991). Thirdly, self-compatible mutants appear to be rare ($q \sim 0.1$ in our sample taken from two large populations). It is likely that bottleneck effects at the time of fragmentation eliminate them from the surviving small remnant *R. leptorrhynchoides* populations. Finally, reductions in population size may have been too recent for selection to have raised the frequency of self-compatible types, so as to lower the overall outcrossing rate, in current *R. leptorrhynchoides* populations.

(ii) Paternal bottlenecks

The second possible effect of fragmentation on mating patterns is the reduction in paternal effective population size. Estimates of divergence of allele frequencies between the total population and the pollen pool (Fig. 2) show that smaller populations are increasingly prone to paternal bottlenecks, where relatively few males contribute most of the gametes to the next generation. This is especially true in the small West Block population, for which the indicator of divergence in pollen allele frequencies (F_k) was 10 times greater than in the large populations. Such paternal bottlenecks could reflect the erosion of alleles at self-incompatibility loci, as DeMauro (1993) demonstrated for small populations of the daisy *Hymenoxys acaulis* var. *glabra*. This may increase the paternal fitness of plants with rare compatibility types. This result is analogous to observations of inter-plant variation in maternal fitness (seed-set) in small populations of self-incompatible species (e.g. Byers & Meager 1992; Godt & Hamrick, 1995) and potentially has the same consequences, as a form of fecundity selection (Vekemans *et al.*, 1998). Loss of allelic richness at allozyme loci in small populations of *R. leptorrhynchoides* suggests that this is a real possibility (Young *et al.*, in the Press). Other causes may be changes in pollinator abundance or behaviour, or changes in population age structure such that a few older and larger individuals dominate.

(iii) Intraplant correlation of paternity

The third effect of fragmentation on mating system is evident from the variation in correlated outcrossed paternity, with the proportion of full-sibs in progeny

		F_k	
		Low	High
r_p	Low	Random mating N_e high Inbreeding low	Immigration N_e high Outbreeding?
	High	Biparental mating N_e high Inbreeding low	Male polygamy N_e low Inbreeding high

Fig. 3. Interaction between F_k and r_p and the relationship with mating pattern, effective population size (N_e) and the potential for biparental inbreeding in subsequent generations.

arrays being highest in the smallest population, and elevated in isolated medium-sized populations. The maintenance of low r_p in the two medium-sized populations at Barton and Capital Circle is presumably because gene flow from the nearby large population at Stirling Ridge keeps the effective population size high. Thus, it seems that potential exists at this scale of fragmentation (isolation < 2 km) for remnant populations to form a network of interacting units linked by gene flow.

Increases in paternal correlation will not reduce progeny fitness directly. However, if seed dispersal is limited, and mating restricted, they predispose populations to biparental inbreeding through mating among full-sibs in subsequent generations. Reductions in fitness due to such biparental inbreeding have been shown to be significant in several other plant species, including *Gaillardia pulchella* (Heywood, 1993) and *Raphanus sativus* (Nason & Ellstrand, 1995).

If seed dispersal is extensive, the potential for biparental inbreeding depends on the relationship between r_p and F_k (Fig. 3). If r_p and F_k are both high, a few plants account for the paternity of most of the progeny across the population. In this case, the effective paternal population size will be low, progeny identity among families will be high, and subsequent biparental inbreeding may be great. This is the situation in Captains Flat and West Block. In contrast, when r_p is high but F_k remains low, many males still contribute relatively evenly to overall mating events, and progeny relatedness at the population level may be little different from that in large populations (though still higher within families) (Fig. 3). In this case r_p reflects a shift towards biparental mating, and the risk of full-sib mating in the next generation is not as great. This appears to be the situation in the St Albans population. The situation in which F_k is high but r_p is low obtains when a small population is subject to extensive pollen immigration from a genetically differentiated and diverse source. This

situation opens the possibility of outbreeding depression.

(iv) Implications for conservation

In terms of management of existing *R. leptorhynchoides* populations for conservation, these results show that populations of less than a few hundred individuals surviving in remnant grassland habitats may be experiencing shifts towards increased genetic identity of progeny due to elevated correlations of outcrossed paternity, especially if they are isolated from other populations by more than 5 km. When this is associated with paternal bottlenecks, as is the case for two of the populations examined, there is the possibility of biparental inbreeding through mating among full-sibs and the potential for inbreeding depression to reduce population viability.

It must be emphasized that these results should be treated with caution, as they are based on limited replication of populations within size and isolation classes. Nevertheless, as over half of the 24 currently known populations of the species are smaller than 200 plants, and many are isolated by distances greater than 5 km, this is a significant finding. Identifying the effects of biparental inbreeding on individual fitness, and the implications for long-term survival of remnant populations, are research priorities for effective conservation management.

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