Investigating sterility in the clonal shrub *Hakea pulvinifera*: comparative studies of reproductive biology, floral development and genetic variation

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Introduction

There are several clonal taxa in the family Proteaceae in which seed set is rare or absent (e.g. *Hakea aenigma* (Haegi and Barker, 1985), *Banksia elegans* (Lamont and Barrett, 1988), *Lomatia tasmanica* (Lynch *et al.*, 1998) *Grevillea infecunda* (Kimpton *et al.*, 2002)). While clonal reproduction allows established plants to persist, sometimes indefinitely (Gardner and Mangel, 1997; Drechsler *et al.*, 1999), recruitment of sexually derived offspring is crucial for the long-term survival of all plant species. Sexual reproduction provides an opportunity for recombination and the creation of new genotypes that may be better adapted to a changing environment than their parents. Natural selection and therefore evolution cannot occur in the absence of sexual reproduction. In addition, seed dispersal allows species to colonise new areas and establish new populations (Harper, 1977). Determining whether sexual reproduction is limited by internal (developmental and genetic) or external (environmental) factors is essential to ensure that appropriate management and recovery programmes are formulated for rare and endangered species.

Hakea pulvinifera is an endangered clonal shrub known from only one population near Lake Keepit in northern NSW. Originally discovered in 1949, it was thought extinct by 1971 until its rediscovery in 1988 (Barker and Morrison, 1989). Despite repeated inspection of the population since the original discovery, fruit set has never been observed in this species. In addition, Barker and Morrison (1989) suggested that *H. pulvinifera* was probably sterile after failing to observe pollen on fresh, dried and fixed flowers. *Hakea pulvinifera* is one of nine species in the Lorea or 'corkwood' group, characterised by thick, sometimes deeply fissured bark (Barker *et al.*, 1999). Species in the Lorea group have not been formally studied, and little is known of their biology or ecology. A detailed study reproductive biology of *H. pulvinifera* was recommended by Benson (1988) and Barker and Morrison (1989) to determine the most appropriate method of conservation. In addition, establishing of the level of genetic variation within the population was considered necessary to secure a genetically representative *ex situ* population (Anon., 2000).

The reproductive biology, floral development and genetic variability of *H. pulvinifera* were examined in conjunction with *H. ednieana*, a common congener within the Lorea

group. Parallel studies conducted on rare and common congeners are particularly useful when ascertaining the reasons for rarity, as both similarities and differences can yield important information, a benefit often overlooked in two-species comparisons (Scott and Gross, 2004). Factors that are similar in both species can be excluded as contributing to rarity, provided they do not limit reproduction in the common species. Differences between species can be useful to identify areas requiring further investigation.

The study

The primary objectives of this study were to verify sterility and identify the cause(s) of reproductive failure in *H. pulvinifera*, and to better understand the ecology of the Lorea group. To achieve these aims, the reproductive biology, floral development and genetic variation in *H. pulvinifera* and *H. ednieana* were investigated.

Reproductive ecology

Several aspects of reproductive ecology were examined to determine whether fruit production in *H. pulvinifera* is limited by factors in the field. A series of bagging experiments were conducted to determine whether *H. pulvinifera* and *H. ednieana* are self-compatible or obligately outcrossing, and whether pollinators are required to effect seed set. Four treatments were applied to each of five *H. pulvinifera* plants and 23 *H. ednieana* plants in two populations using the methods described in Smith and Gross (2002). One inflorescence per experimental plant was tagged and left open to pollinators to assess natural fruit set. Pollen longevity (duration of viability) and the timing of stigma receptivity were examined using 2,3,5–triphenyl tetrazolium chloride (TTC) in 12% sucrose (Cook and Stanley, 1960) and scanning electron microscopy (Smith and Gross, 2002) respectively to determine whether the study species are protandrous. Clumps of two to six flowering *H. pulvinifera* and *H. ednieana* plants were placed under observation and the behaviour of floral visitors recorded to determine the pattern of pollen movement within and among plants. Floral resources, including the number of inflorescences per plant and the number of flowers per inflorescence, pollen abundance and nectar volume and concentration, were quantified to assess the attractiveness of *H. pulvinifera* and *H. ednieana* populations to pollinators.

Floral development

To confirm or exclude ploidy as a causal factor in the sterility of *H. pulvinifera*, chromosomes were counted in root tip cells of both species using the root tip squash method in Prakash (2000). To establish the site and nature of sterility in *H. pulvinifera*, flowers at all stages of development were sectioned and stained (Prakash, 2000) then compared with the corresponding stages in *H. ednieana* flowers. Differences in pollen characteristics were quantified by measuring the size, wall thickness and number of germ pores in 20 mature microspores per flower in each of five flowers per species.

Genetic variation

Intersimple sequence repeats (ISSR), a technique that uses random primers to generate DNA fragments via the polymerase chain reaction (PCR), was used to determine the extent of clonality and genetic variation in *H. pulvinifera*. Five of the 20 primers tested generated reliable, repeatable polymorphic bands. Identical assays were conducted on three additional 'corkwood' species, *H. ednieana*, *H. fraseri* and *H. ivoryi*, to assess the relatedness between *H. pulvinifera* and other species in the Lorea group.

Outcomes

Reproductive ecology

This component of the study identified a number of factors that could potentially limit sexual reproduction in *H. pulvinifera*. Compared to *H. ednieana*, floral production within the *H. pulvinifera* population was low; fewer plants flower and fewer inflorescences were produced per plant. In addition, nectar volume and concentration were lower in *H. pulvinifera* plants, reducing the attractiveness of the population to pollinators. This is confirmed by a considerably lower incidence of bird visitation to *H. pulvinifera* plants compared to *H. ednieana* plants. Although the above factors may contribute to reproductive failure in *H. pulvinifera*, extremely low pollen viability was identified as the principal factor associated with lack of fruit set in the species. The mean percentage viability of *H. pulvinifera* pollen at anthesis was 1.0 ± 0.58 compared to 88.5 ± 1.86 (mean $\pm$ standard error) for *H. ednieana* pollen. A weak colour change (dark pink instead of red) in response to the TTC solution indicates that pollen counted as viable was not robust.
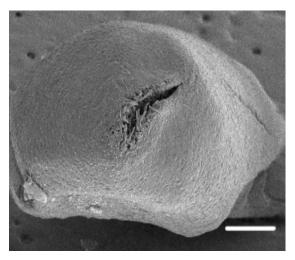
Figure 1. A deformed *H. pulvinifera* stigma at 6 h post-anthesis. Magnification x 50; bar = 100 μm.
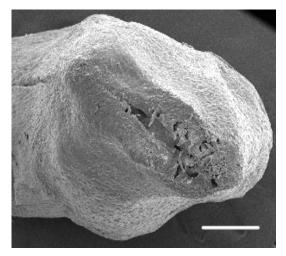

Figure 2. A large total stigmatic area and deformed pollen presenter characterise this *H. pulvinifera* flower harvested at 12 h post-anthesis. Magnification x 75; bar = 345 μm.

Moreover, *H. pulvinifera* stigmas were often deformed and did not exhibit any recognisable maturation following anthesis (Figures 1 and 2). Deformities were absent in progressively ripening *H. ednieana* stigmas. Figures 3 and 4 show *H. ednieana* stigmas at stages corresponding to those of *H. pulvinifera* presented in Figures 1 and 2.
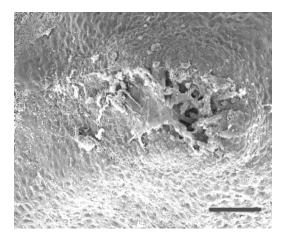

Figure 3. An unripe *H. ednieana* stigma at 6 h post-anthesis. Magnification x 60; bar = 430 μm.
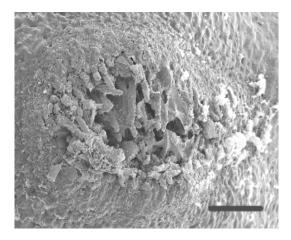

Figure 4. Some enlargement of the stigmatic cavity and growth of papillae can be seen at 12 h post-anthesis in unwiped flowers. Magnification x 220; bar = 120 μm.

As no fruit set occurred following the application of self and cross pollen to *H. pulvinifera* flowers, self-incompatibility could not be ascertained. Anthers seldom dehisced and pollen was present on only a few of the pollen presenters examined. In contrast, fruit set resulted from all treatments of *H. ednieana* flowers, providing strong evidence of self-

compatibility and the ability to set fruit both with and without assistance from pollinators, although more fruit were initiated than were matured and natural fruit set was low (Figure 5). High pollen viability at anthesis and a progressively ripening stigma confirms protandry in *H. ednieana*.
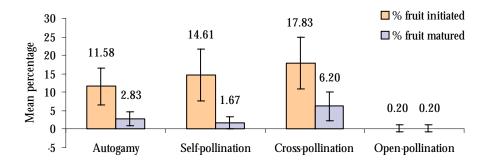

Figure 5. Mean percentage fruit initiation and mean percentage fruit maturation in *H. ednieana* following four breeding system treatments.

Floral development

The diploid chromosome number was 2n = 20 in *H. pulvinifera* and *H. ednieana*, a number in accordance with previous assessments of ploidy in the genus (Ramsay, 1963), therefore ploidy was excluded as a causal factor for sterility. Examination of anther and ovule development in the two study species confirmed male sterility in *H. pulvinifera*. While *H. pulvinifera* ovule development proceeded in concert with that observed for *H. ednieana*, pollen formation in *H. pulvinifera* was asynchronous and incomplete.
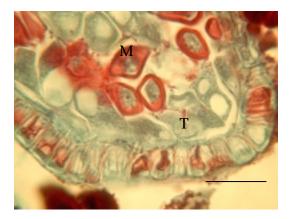

Figure 6. Recently separated young *H. ednieana* microspores (M) surrounded by a healthy tapetum (T). Bar = 40 μm
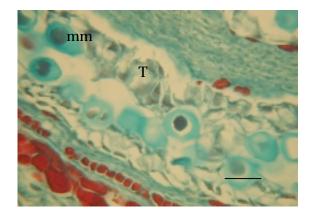

Figure 7. A *H. pulvinifera* anther showing early tapetal (T) degeneration. The microspore mother cell (mm) at top left has not yet begun meiosis. Bar = 60 μm

Early degeneration of the tapetum, a tissue integral in enzyme production, sporopollenin and nutrient supply in the anthers, was identified as the primary cause of pollen sterility in *H. pulvinifera*. Tapetal degeneration normally occurs when pollen tetrads are separating (Figure 6). In most *H. pulvinifera* anthers, this process began even before meiosis was complete (Figure 7). Precocious tapetal activity also accompanied early tapetal degeneration, causing increased thickness in *H. pulvinifera* pollen walls.

Genetic variation

The genetic composition of the 35 individual *H. pulvinifera* stems assayed were identical, providing strong evidence that the species is composed of a single plant. Analysis of data from *H. ednieana*, *H. fraseri* and *H. ivoryi* suggests that *H. pulvinifera* may be a sterile hybrid.

Summary

This study confirmed sterility and identified the causal factors associated with sterility in *H. pulvinifera*. Early tapetal degeneration resulting in incomplete pollen formation and very low anther dehiscence prevents fertilisation of viable ovules. In addition, extremely low pollinator visitation resulting from poor floral resources limits the potential for pollen transfer, despite low pollen viability. The confirmation that the only known population of *H. pulvinifera* is composed a single plant has implications for its endangered status and management; *H. pulvinifera* may be one of the rarest species in the world. It is likely that the species is a sterile hybrid, whose progenitors are either extinct or now geographically distant due to extensive destruction of the intervening habitat between *H. pulvinifera* and other 'corkwood' species. Information compiled on comparison species, *H. ednieana*, provides knowledge of the previously little studied Lorea group of Hakeas.

Literature cited

Anon. (2000). *Hakea pulvinifera* recovery plan, NSW National Parks and Wildlife Service.

Barker, R. M., Haegi, L. and Barker, W. R. (1999). Hakea. In, *Flora of Australia Proteaceae 3: Hakea to Dryandra*. Volume 17b. Australian Biological Resources Study, Department of the Environment and Heritage, CSIRO Publishing, Canberra.

Benson, J. (1988). Survey report on Hakea pulvinifera (Proteaceae) L. Johnson, Keepit State Recreation Area, New South Wales National Parks and Wildlife Service.

Barker, W. R. and Morrison, S. P. (1989). *Hakea pulvinifera* L. Johnson (Proteaceae): A rediscovered species under threat. *Journal of Adelaide Botanical Gardens*, **11**: 175-177.

Cook, S. A. and Stanley, R. G. (1960). Tetrazolium chloride as an indicator of pine pollen germinability. *Silvae Genetica*, **9**: 134-136.

Drechsler, M., Lamont, B. B., Burgman, M. A., Akcakaya, H. R., Witkowski, E. T. F. and Supriyadi (1999). Modelling persistence of an apparently immortal *Banksia* species after fire and land clearing. *Biological Conservation*, **88**: 249-259.

Gardner, S. N. and Mangel, M. (1997). When can a clonal organism escape senescence? *The American Naturalist*, **150**: 462-490.

Haegi, L. and Barker, W. R. (1985). Taxonomy of the South Australian species allied to *Hakea ulicina* R. Br. (Proteaceae). *Journal of the Adelaide Botanical Gardens*, **7**: 249-271.

Harper, J. L. (1977). *Population biology of plants*, Academic Press, Harcourt Brace, Jovanovich Publishers, London.

Kimpton, S. K., James, E. A. and Drinnan, A. N. (2002). Reproductive biology and genetic marker diversity in *Grevillea infecunda* (Proteaceae), a rare plant with no known seed production. *Australian Systematic Botany*, **15**: 485-492.

Lamont, B. B. and Barrett, G. J. (1988). Constraints on seed production and storage in a root suckering Banksia. *Journal of Ecology*, **76**: 1069-1082.

Lynch, A. J. J., Barnes, R. W., Cambecedes, J. and Vaillancourt, R. E. (1998). Genetic evidence that *Lomatia tasmanica* (Proteaceae) is an ancient clone. *Australian Journal of Botany*, **46**: 25-33.

Prakash, N. (2000). *Methods in plant microtechnique: Laboratory manual*, Botany Department, University of New England, Armidale.

Ramsay, H. P. (1963). Chromosome numbers in the Proteaceae. *Australian Journal of Botany*, **11**: 1-20.

Scott, B. and Gross, C. L. (2004). Recovery directions for monoecious and endangered *Bertya ingramii* using autecology and comparisons with common *B. rosmarinifolia* (Euphorbiaceae). *Biological Conservation*, **13**: 885-899.

Smith, J. A. (in preparation). Investigating sterility in the clonal shrub *Hakea pulvinifera*: comparative studies of reproductive biology, floral development and genetic variation. PhD thesis, University of New England, Armidale NSW, Australia.

Smith, J. A. and Gross, C. L. (2002). The pollination ecology of *Grevillea beadleana* McGillivray, an endangered shrub from northern New South Wales, Australia. *Annals of Botany*, **89**: 97-108.