

Chapter 1 Introduction

Since the nineteenth century, Darwin (1877; 1878) and many subsequent authors have been keen to know how successful mating and reproduction occur, and what aspects of reproduction, including floral morphology, development, physiology, ecology and genetics, govern sexual diversity among flowering plants (Fisher 1941; Baker 1955; Jain 1976; Lloyd 1982; Charlesworth and Charlesworth 1987; Barrett and Eckert 1990a; Barrett and Eckert 1990b; Lloyd 1992; Lloyd and Schoen 1992; Dellaporta and Calderon-Urrea 1993; Seger and Eckhart 1996; Richards 1997; Barrett 1998; Holsinger 2000). To find the selective forces responsible for the origin and maintenance of sexual systems, many of these authors have studied the relationships between floral design, breeding systems and pollinators as well as population genetics.

In flowering plants, variations in floral design and the arrangement of male and female sex organs provide an outstanding opportunity to study reproductive adaptation by natural selection (Darwin 1877; Darwin 1878; Baker 1955; Faegri and van der Pijl 1979; Lloyd 1982; Endress 1994; Barrett *et al.* 1996; Richards 1997; Barrett 1998; Holsinger 2000; Barrett 2002a; Cheptou and Schoen 2007). Floral diversity has been largely attributed to the pollination system and its influence on reproductive success and fertility. Most flowers are hermaphroditic, in which male and female organs are present within a flower (Yampolsky and Yampolsky 1922; Charnov and Bull 1976; Sjöström and Gross 2006), therefore, considerable variation in reproductive systems, e.g. cross- vs. self-fertilisation, can occur within- or between different plant species. In each reproductive mode, there are costs and benefits; evolutionary biologists use these costs and benefits to predict conditions that favour the maintenance and evolutionary transition between reproductive modes (Holsinger 2000 & refs. there in). Self-compatible species, for example, benefit from an increase in seed production from self-pollination under unfavourable pollination conditions, which helps to explain the frequent evolutionary pathway to self-compatibility from self-incompatibility. In self-incompatible species, however, the union of gametes from unrelated individuals can improve the quantity and quality of offspring, and thereby allow species to respond to environmental changes.

‘...This extraordinary diversity in the means for favouring or preventing cross- and self-fertilisation in closely allied forms, probably depends on the results of both processes being highly beneficial to the species, but in a directly opposed manner and dependent on variable conditions... with two somewhat opposed ends to be gained, namely, the safe propagation of the species and the production of cross fertilised, vigorous offspring ...’(Darwin 1878, p. 446).

This thesis is an exploration of one facet of floral diversity, the phenomena of distyly and loss of sex in *Nymphoides montana* Aston and homostyly in *N. geminata* (R. Br.) Kuntze.

1.1 The evolution and maintenance of heterostyly

In hermaphrodite flowers that require pollinators to set seed, the sex organs are often presented at the same place and at the same time, which is likely to interfere with the receipt and donation of outcross pollen (Darwin 1877; Baker 1964; Ganders 1979; Lloyd and Yates 1982; Lloyd and Webb 1986; Webb and Lloyd 1986; Lloyd and Barrett 1996; Barrett 2002b). In such floral structures, however, successful cross-pollination occurs because the pollen and stigmatic surface contact the same part of the pollinator (Lloyd and Barrett 1996). This creates a conflict between the benefit from precise pollen dispersal and the cost associated with interference of pollen deposition and removal (Lloyd and Webb 1986; Webb and Lloyd 1986; Barrett *et al.* 2000; Barrett 2002a). The benefits and costs of this floral arrangement are thought to have contributed to the great floral diversity in spatial (herkogamy) and temporal (dichogamy) separation of male and female function in outcrossing plants (Lloyd and Yates 1982; Lloyd and Webb 1986; Webb and Lloyd 1986; Harder and Barrett 1995).

Heterostyly is a floral polymorphism that has been shaped by the evolution of floral traits adapted for precision in both pollen transfer and avoidance of pollen–stigma interference (Lloyd and Webb 1986; Lloyd and Webb 1992a; Barrett *et al.* 2000). Heterostylous populations contain two (distyly) or three (tristyly) floral morphs that differ from one another in the reciprocal arrangement of stigmas (pollen receipt) and anthers (pollen removal) in a single population (Darwin 1877; Ganders 1979; Richards and Barrett 1992). Reciprocal sex-organ position in the floral morphs is thought to have evolved as an

adaptive feature for efficient cross-pollination between floral morphs because pollen is deposited onto different parts of a pollinator's body (Darwin 1877; Lloyd and Webb 1992a; Lloyd and Webb 1992b; Barrett *et al.* 2000). Moreover, heterostylous species are protected from detrimental effects of self-fertilisation through self-incompatibility systems (Charlesworth and Charlesworth 1979b; Ganders 1979; Lloyd and Webb 1992b; Barrett 2002a). Heterostyly is also associated with a suite of ancillary polymorphisms in which the shape and size of stigma papillae and pollen grains differ among stigma and anther levels (Barrett 1990; Dulberger 1992). This sexual polymorphism, therefore, provides a unique solution to the sexual conflicts that hermaphrodites encounter by achieving effective pollination but at the same time limiting interference between reproductive organs (Webb and Lloyd 1986; Lloyd and Webb 1992a; Lloyd and Webb 1992b; Nishihira and Washitani 1998; Barrett *et al.* 2000).

In 1877, Darwin, in his *The Different Forms of Flowers on Plants of the Same Species*, described the adaptive significance of heterostyly as a mechanism to promote cross-pollination. Since then several hypotheses have explained the mechanisms involved in the evolution and maintenance of heterostyly, including efficient cross-pollination, inbreeding depression, pollen–stigma interference and genetic linkage between stigma and anther heights (Mather and DeWinton 1941; Vuilleumier 1967; Charlesworth and Charlesworth 1979b; Ganders 1979; Richards 1986; Barrett 1990; Lloyd and Webb 1992a; Lloyd and Webb 1992b; Sakai and Toquenaga 2004). The Lloyd-Webb's pollen-transfer model and Charlesworth-Charlesworth's selfing-avoidance model are the two most detailed hypotheses on the evolution of heterostyly. These models differ in the selective mechanisms favouring heterostyly, the ancestral phenotypes and the sequence of establishment of reciprocal herkogamy and incompatibility systems (reviewed in Barrett 1990; Barrett 1992c). To understand the evolution of heterostyly it is necessary to study the component parts of the syndrome (Ganders 1979).

Typical features of the heterostylous syndrome include: a) strict reciprocity between stigma and anther heights in floral morphs, b) strong self- and intramorph incompatibility, c) equilibrium morph ratio within populations, and d) stigma and pollen polymorphism (Ganders 1979; Barrett 1990; Barrett 1992c; Dulberger 1992; Lloyd and Webb 1992a; Barrett *et al.* 2000; Castro *et al.* 2004; Massinga *et al.* 2005). There are, however, several taxa that do not show the typical features of heterostyly, e.g. imperfect sex-organ

reciprocity and partial self- and/or intramorph incompatibility; these include *Nymphoides* (Ornduff 1966), *Villarsia* (Ornduff 1988), *Menyanthes* (Thompson *et al.* 1998), *Oxalis* (Ornduff 1972), *Pontederia* (Glover and Barrett 1983), *Guettarda* (Richards and Koptur 1993), and *Bouvardia* and *Psychotria* (Faivre and McDade 2001). These atypical heterostylous species provide excellent examples to study the maintenance or breakdown of the heterostylous sexual system (Faivre and McDade 2001). Studies of floral morphologies, compatibility relationships and morph frequencies within populations can, either separately or in combination, provide insight in whether populations are in an evolutionary transition to, or from, heterostyly (Ornduff 1972; Charlesworth and Charlesworth 1979a; Barrett 1985; Barrett 1989a; O'Brien and Calder 1989; Richards and Koptur 1993; Eckert and Barrett 1994b; Faivre and McDade 2001).

1.2 What factors disturb heterostyly and sexual reproduction in clonal species?

In evolutionary stable heterostylous populations, equal frequencies of floral morphs (or mating partners) indicate disassortative mating due to the higher proficiency of intermorph rather than intramorph pollen transfer and/or the presence of self- and intramorph incompatibility (Barrett *et al.* 2004; Barrett and Harder 2005). Reduction in disassortative mating through extensive clonal growth may result in strongly biased populations or populations that contain a single floral morph, i.e. monomorphic populations (Ornduff 1970a; Barrett 1977; Barrett 1980a; Barrett *et al.* 1993; Uesugi *et al.* 2004; Wang *et al.* 2005; Brys *et al.* 2007). Within dense clonal patches of such populations, a considerable amount of self- and intramorph fertilisation can occur, leading to reduced fecundity or sexual sterility.

Whether the factors reducing seed production have a genetic and/or ecological component can be inferred by comparing sexual fertility between glasshouse and natural environments, as well as experimental cross-pollinations between sterile and conspecific fertile populations (Eckert *et al.* 1999; Dorken and Eckert 2001). A genetic basis to population sterility is revealed when there is no difference in sexual reproduction between the two environments. While limited compatible mates and/or pollen impair seed production, genetic factors, such as sterile polyploids (Lui *et al.* 2005), nuclear sterility mutations at sex loci (Klekowski 1988; Eckert *et al.* 1999) and the presence of different ploidy levels between floral morphs (Castro *et al.* 2007) also play a significant role.

Ecological factors that impair seed production and sexual recruitment include, for example, altitudinal variation (Eckert and Barrett 1993), repeated disturbance (Schaal and Leverich 1996), reduced light (Kudoh *et al.* 1999) and frequent fire events (Gross and Caddy 2006).

Populations that abandon sexual reproduction for clonal reproduction are likely to accumulate sterility mutations, such as poor pollen and ovule viability (Gross and Caddy 2006), reduced pollen tube growth and ovule penetration (Eckert *et al.* 1999) and reduction in flower number (Lui *et al.* 2005), disabling sexual traits. To examine whether sexual traits become degraded in sterile populations, it is necessary to compare within-flower traits associated with sexual reproduction in sterile vs. fertile populations (Eckert 2002; Gross and Caddy 2006).

Once a sterility mutation(s) has occurred, the next phase for the evolutionary degeneration of sex in clonal populations involves the spread of that mutation into the population (Eckert *et al.* 1999; Eckert 2002). Sterile plants can use the resources saved from not producing dysfunctional sexual traits to promote traits that increase clonal or vegetative fitness, i.e. the resource allocation theory. The clonal traits can also be favoured, at the expense of non-functional sexual traits, when they have a pleiotropic link with reduced sexual fertility, i.e. the antagonistic pleiotropy hypothesis (Eckert *et al.* 1999; Dorken *et al.* 2004). An assessment of whether sterile plants exhibit enhanced performance in terms of survival and clonal growth becomes necessary to distinguish between the hypotheses (Eckert 2002).

Generally, it is known that in self-incompatible species, extensive clonality reduces the opportunities for outcrossing due to a deficiency in compatible pollen, resulting in extremely low seed production (Eckert and Barrett 1993; Charpentier *et al.* 2000; Honnay *et al.* 2006). In such situations where sexual reproduction is totally suppressed, clonal growth is expected to affect negatively the level of genetic diversity when populations consist of one or only a few genetically distinct individuals (Eckert and Barrett 1993; Dorken and Eckert 2001; Rossetto *et al.* 2004). However, considerable genetic variation has been reported in a few clonal populations with reduced or no sexual reproduction and recruitment (Cheliak and Dancik 1982; Pleasants and Wendel 1989; Rottenberg and Parker 2004; Nelson *et al.* manuscript). Since reduced sexual reproduction can have various effects on the genetic structure of populations, genetic investigation becomes necessary to

understand the extent of clonality and distribution of genetic variation in sterile populations (Ellstrand and Roose 1987; Eckert *et al.* 1999; Rossetto *et al.* 2004).

1.3 Does heterostyly break down to promote self-fertilisation?

In ecological situations where opportunities for pollinator activity and outcross-pollination are limited, the ability to produce seed through self-fertilisation can be beneficial to provide reproductive assurance (Baker 1955; Jain 1976; Barrett *et al.* 1989; Ramsey and Vaughton 1996; Fausto *et al.* 2001; Goodwillie 2001; Mazer *et al.* 2004; Busch 2005; Herlihy and Eckert 2005; Jacquemyn and Honnay 2007). Heterostylous taxa that receive irregular pollinator service may break down in the direction of increased self-fertilisation in which homostyles evolve (Ornduff 1972; Piper *et al.* 1986; Barrett 1989a; Barrett *et al.* 1989; Kelso 1992; Washitani *et al.* 1994; Guggisberg *et al.* 2006; Carlson *et al.* 2007). Homostylous species possess stigmas and anthers at the same relative height within a flower, and usually have the ability to self-pollinate without pollinators (or autonomously) and self-fertilise (self-compatibility).

Since homostylous taxa are usually found in ecologically or geographically marginal habitats, where opportunities for outcrossing are limited, it raises the possibility that selection for reproductive assurance favours mutations promoting autonomous self-fertilisation (Baker 1955; Barrett and Shore 1987; Barrett *et al.* 1989; Guggisberg *et al.* 2006). Selection for autonomous self-fertilisation as a result of the breakdown of heterostyly to homostyly is associated with evolution of several characters. Modifications to the incompatibility systems of heterostylous plants, via recombination in the heterostyly supergene or changes at modifier loci, make fertilisation by self-pollen possible (Barrett and Cruzan 1994). Reduction in flower size and spatial separation of sex organs that influence self-pollen deposition is usually associated with the evolutionary transition to homostyly (Ornduff 1969; Ganders 1979; Li and Johnston 2001). The possible association between polyploidy and a reduction in detrimental effects of inbreeding by having extra gene copies is also associated with the evolution of homostyly (Barrett and Shore 1987; Kelso 1992; Shore *et al.* 2006). The establishment of polyploid homostyles is favoured in colonising new or temporary environments with limited mates or pollinators (Stebbins 1985; Kelso 1992; Petit and Thompson 1999; Guggisberg *et al.* 2006). A comparative study between homostylous and heterostylous congeneric species (or conspecific

populations) is necessary to understand the extent of variation in floral morphology and the ability to self-pollinate.

1.4 Menyanthaceae

The Menyanthaceae consist of five genera and about 60 species in freshwater aquatic and marshland environments (Chuang and Ornduff 1992; Jacobs 1992). The plants often have spreading stolons and the floating or submerged leaves arise alternately. The five-part flowers are either white or yellow, insect-pollinated, frequently hermaphroditic and heterostylous, and usually only open for a day. The fruit is a capsule. Species of Menyanthaceae have almost worldwide distribution. Four out of the five genera are distylous, including *Nymphoides* Ség., *Villarsia* Vent., *Menyanthes* L., and *Nephrrophyllidium* Gilg (= *Fauria* Franch.) (Ganders 1979). *Menyanthes* (*M. trifoliata*) and *Nephrrophyllidium* (*N. crista-galli*) are monotypic (having only one species each) and distributed only in the Northern hemisphere. *Liparophyllum* Hook.f. is the only taxon lacking distylous species, and is monotypic (*L. gunnii*), endemic to New Zealand and Tasmania, and self-compatible (Ornduff 1982).

In the two major genera of Menyanthaceae, at least 15 out of c. 50 species of *Nymphoides* (Tippery 2007) and 10 out of c. 16 species of *Villarsia* (Dulberger and Ornduff 2000) are distylous. *Nymphoides* is cosmopolitan, whereas *Villarsia* occurs in the southern hemisphere. In *Nymphoides*, the distylous species are *N. beaglensis* Aston, *N. bosseri* A.Raynal, *N. crenata* (F.Muell.) Kuntze, *N. elliptica* Aston, *N. fallax* Ornduff, *N. humboldtiana* (Kunth) Kuntze, *N. indica* (L.) Kuntze, *N. montana* Aston, *N. moratiana* A.Raynal, *N. peltata* (S.G.Gmel.) Kuntze, *N. planosperma* Aston, *N. quadriloba* Aston, *N. spongiosa* Aston, *N. subacuta* Aston, and *N. triangularis* Aston.

Seven out of c. 50 species of *Nymphoides* and four out of c. 16 species of *Villarsia* (Dulberger and Ornduff 2000) are homostylous. In *Nymphoides*, the homostylous species are *N. aurantiacum* (Dalz.) O. Kuntze, *N. disperma* Aston, *N. geminata* (R. Br.) Kuntze, *N. parvifolium* (Griseb.) O. Kuntze, *N. simulans* Aston, *N. sivarajanii* K.T.Joseph, and *N. tenuissima* A.Raynal. Other reproductive modes have also been recorded in *Nymphoides*, including the dioecious *N. macrospermum* Vasud and *N. krishnakesara* Sivar & Joseph, and the monoecious (or gynodioecious) *N. hydrophylla* (Lour.) O. Kuntze (Sivarajan and Joseph 1993).

No phylogenetic analysis of Menyanthaceae is currently available to describe the relationships among different lineages and the evolutionary history of breeding systems. A phylogenetic study of the family that incorporates a variety of breeding systems and floral morphologies is already underway by Tippery (2007).

1.5 Aims of the project

Distylous and monomorphic styler populations of *Nymphoides montana* as well as homostylous populations of a congener *N. geminata* occur in the Northern Tablelands, New South Wales, Australia. This provides a valuable opportunity to study the patterns of floral variation and breeding system in the study species. The overall aim of this thesis is;

- i. To understand the factors that may have been important in the maintenance and disruption of distyly, and
- ii. To study the possible benefits of homostyly in unfavourable pollination conditions.

1.6 Outline of the thesis

Chapter 1 gives a general introduction to the concepts of heterostyly and the features one must look for in understanding the origin and maintenance of this breeding system. It also discusses the steps involved in the loss of sexual reproduction in the absence of a mating partner. Additionally, it gives a brief review on the evolutionary transition to homostyly.

In Chapter 2, the reproductive biology of distylous *N. montana* is studied to explore variation in floral traits and incompatibility systems of the two floral morphs. The following questions are addressed:

- 1) What are the frequencies of floral morphs in the natural populations?
- 2) Does *N. montana* reproduce successfully in its natural habitat?
- 3) Does *N. montana* show clear sex-organ reciprocity; if not, what floral traits reduce reciprocity?

- 4) Does *N. montana* show between-morph variation in ancillary dimorphic traits?
- 5) Does *N. montana* exhibit a diallelic incompatibility system; if so, to what extent?

The loss of one floral morph or mating partner from a heteromorphic incompatible population is often associated with reduced or an absence of sexual reproduction. In Chapter 3, the consequences of loss of the mating partner on sexual and clonal reproduction of a monomorphic population of the distylous *Nymphoides montana* are studied, and the following questions are addressed:

- 1) Is the population sterile; if so, what is the basis of sterility?
- 2) To what extent have the pollen and ovules lost their function?
- 3) What is the ploidy level of the monomorphic plants?
- 4) Is sexual sterility associated with a change in clonal growth?
- 5) What is the impact of sexual sterility on genetic and/or clonal diversity of the population? Is the population maintained by clonal propagation?

In general, homostylous forms are considered to be a derived condition from their related heterostylous taxa (Ganders 1979). A breakdown of heterostyly to homostyly is usually associated with a shift toward temporary or marginal habitats to provide reproductive assurance. This transformation involves a breakdown of self-incompatibility and floral barriers that prevent self-pollination. Although homostylous *Nymphoides geminata* occurs within the geographical distribution of distylous *N. montana*, its populations usually occupy temporary habitats with unreliable water regimes. In Chapter 4, the reproductive biology of the homostylous *N. geminata* is studied to explore to what degree its floral biology and breeding system are modified compared with that of distylous *N. montana*. The following questions are addressed:

- 1) What floral features define *N. geminata* as a homostylous species? What floral features promote autonomous self-pollination?

- 2) Is *N. geminata* a self-compatible species? What is the capacity for autonomous self-fertilisation?
- 3) Are floral visitors so few that reproductive assurance could be a major factor favouring autonomous self-fertilisation?
- 4) Does *N. geminata* reproduce successfully in its natural habitat?
- 5) Is *N. geminata* associated with a higher level of ploidy?

The closing chapter, Chapter 5, presents the discussions and conclusions of this thesis, and recommendations for future work.