Chapter 4 Breakdown of Distyly

4.1 Introduction

The evolution of self-pollinating taxa from an obligate outcrossing ancestor is one of the most common evolutionary pathways in flowering plants (Baker 1955; Stebbins 1957; Stebbins 1970; Ornduff 1972; Jain 1976; Lloyd 1979; Barrett 1988b; Lloyd 1992; Fausto *et al.* 2001; Herlihy and Eckert 2002). The ability of plants to produce seed following self-pollination evolves via the breakdown of self-incompatibility (Barrett 1988b) and floral barriers preventing self-pollination, e.g. herkogamy (Lloyd 1992; Takebayashi and Delph 2000) or dichogamy (Totland and Schulte-Herbrüggen 2003). This evolutionary shift is often associated with the migration of plants to ecologically or geographically marginal habitats and occurs in response to a lack of mates and/or poor pollinator visitation and outcross-pollination (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). In such habitats, selection may favour mutations that increase the ability of plants to self-pollinate and set seed autonomously, i.e. without pollinators, ensuring reproductive success (Darwin 1878; Jain 1976; Schemske 1978; Lloyd 1979; Piper *et al.* 1986; Lloyd 1992; Schoen *et al.* 1996; Holsinger 2000; Mazer *et al.* 2004; Carlson *et al.* 2007). Selection for reproductive assurance is thought to be one of the principle factors involved in the evolutionary transition from outcrossing to selfing (Schoen *et al.* 1996; Barrett 2002a).

Population biologists have long been interested in heterostylous families to study the evolution and modification of mating systems (Mather 1950; Baker 1966; Ornduff 1966; Barrett 1979; Charlesworth and Charlesworth 1979a; Ganders 1979; Barrett 1985; Shore and Barrett 1985; Barrett 1989a; Fenster and Barrett 1994; Schoen *et al.* 1997). The most frequent modification of heterostyly involves the evolutionary breakdown of heterostyly in the direction of increased self-fertilisation by the formation of homostyly (Ornduff 1972; Piper *et al.* 1984; Barrett and Shore 1987; Barrett 1988a; Barrett *et al.* 1989; Fenster and Barrett 1994; Kohn *et al.* 1996; Schoen *et al.* 1997; Guggisberg *et al.* 2006; Carlson *et al.* 2007; Nakamura *et al.* 2007; Sakai and Wright 2008). Most genera/species with

heterostylous members contain other species/populations that lack floral polymorphism (distyly or tristyly) and possess floral monomorphisms known as homostyly. Homostylous species self-pollinate autonomously because of the close proximity of the stigmas and anthers within a flower, and are usually highly self-compatible (Ornduff 1969; Ganders 1979; Barrett 1989a; Schoen *et al.* 1997; Nakamura *et al.* 2006; Sakai and Wright 2008). In heterostylous species, however, the sex-organ reciprocity and incompatibility systems promote outcrossing while limiting stigma–anther interference, and hence self-pollination (Ganders 1979; Webb and Lloyd 1986; Barrett 1990; Lloyd and Webb 1992a; Lloyd and Webb 1992b; Barrett 2002a).

A breakdown of heterostyly is often accompanied by a shift to temporary habitats within the species range (Schoen *et al.* 1997) or to the geographical margins of the range of related heterostylous species (Ornduff 1972; Shore and Barrett 1985; Barrett and Shore 1987; Guggisberg *et al.* 2006). In such situations, homostyly is usually favoured over outcrossing morphs that suffer from mate deficiency and reduced pollinator activity (Baker 1955). Homostyly is associated with the loss of self-incompatibility systems, so that fertilisation by self-pollen becomes possible, floral traits are modified to promote self-pollen deposition (Ganders 1979; Barrett and Shore 1987; Kohn *et al.* 1996; Schoen *et al.* 1997), and there is a reductions in size and attractiveness of floral organs (Ornduff 1969). A breakdown of heterostyly has been reported in closely related homostylous species, including several species of *Primula* (Kelso 1992; Wedderburn and Richards 1992; Guggisberg *et al.* 2006), *Turnera* (Barrett and Shore 1987; Tamari *et al.* 2001) and *Amsinckia* (Schoen *et al.* 1997). This suggests that the evolutionary shift favouring selfing is associated with speciation events (Barrett 1988b). Alternatively, homostyly can be found within heterostylous populations as a result of a recent breakdown of heterostyly, e.g. *Oxalis dillenii* subsp. *filipes* (Ornduff 1972), *Eichhornia crassipes* (Barrett 1979), *E. paniculata* (Barrett *et al.* 1989), *Amsinckia lunaris* and *A. spectabilis* (Schoen *et al.* 1997).

Dissolution of the genetically controlled self-incompatibility systems of heterostyly to promote self-fertilisation takes several distinct forms. The most well-known is evolution of homostyly through a crossover within a supergene that controls floral morphology and incompatibility components of the heterostylous syndrome (Dowrick 1956; Lewis and Jones 1992). The recombinant origin of homostyly has been tested in *Primula vulgaris*

(Crosby 1949) *P. halleri* and *P. japonica* (Dowrick 1956; Wedderburn and Richards 1992), *Turnera ulmifolia, T. orientalis, T. velutina* (Shore and Barrett 1985; Barrett and Shore 1987) and *T. aurelii,* and *Piriqueta cistoides* (Tamari *et al.* 2001) when crossed to distylous morphs. The derived homostyles combine stamen length and male compatibility features of one morph with stylar length and female compatibility features of the alternative morph. Such homostyles are generally self-compatible, and sometimes, largely self-pollinate autonomously.

A second way in which the relaxation or complete loss of self-incompatibility can occur is when a series of modifier genes, which have a non-allelic association with the heterostylous supergene change morphological features of the syndrome (Shore and Barrett 1986; Ornduff 1988; Fenster and Barrett 1994). In 'quasi-homostylous' races of tristylous *Oxalis dillenii* and *O. corniculata,* a severe reduction in overall flower size, stigma height and anther height contributed to autonomous seed production (Ornduff 1972). Elsewhere, in 'semi-homostylous' populations of tristylous *Eichhornia paniculata,* a single low-level anther positioned adjacent to the stigma of mid-styles led to autonomous autogamy and self-fertilisation (Barrett 1985; Fenster and Barrett 1994). In tristylous *Pontederia cordata,* however, the incompatibility modification was not associated with changes in morphological features; this was suggested to result from the pleiotropic effects of the supergene rather than to linked modifiers (Barrett and Anderson 1985).

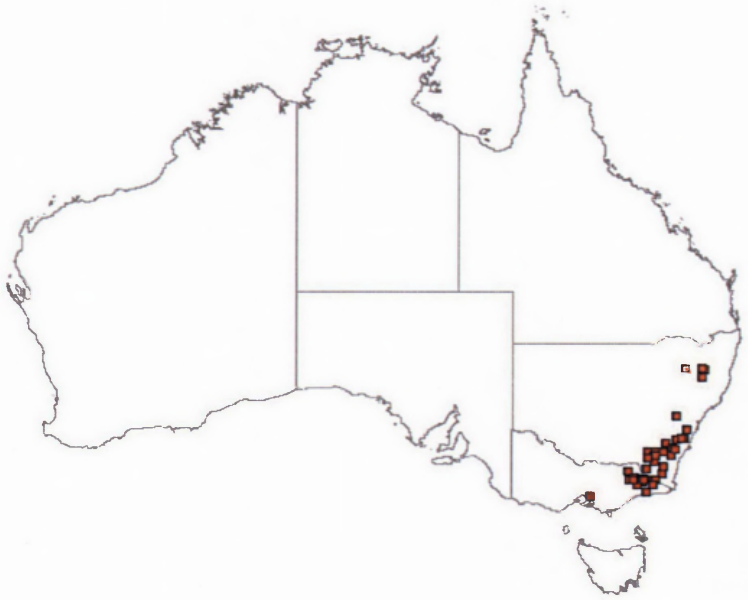
A breakdown of heterostyly to homostyly frequently accompanies polyploidisation, as described in *Turnera* (Barrett and Shore 1987; Truyens *et al.* 2005), *Damnacanthus* (Naiki and Nagamasu 2004), *Primula* (Guggisberg *et al.* 2006) and *Ophiorrhiza* (Nakamura *et al.* 2007). Because most homostyles are self-compatible, they may suffer from increased inbreeding depression (Lloyd 1979; Lande and Schemske 1985). Polyploidy, therefore, creates a buffer against the fixation of homozygosity and accumulation of deleterious mutations by having multiple gene copies (Lande and Schemske 1985; Barrett and Shore 1987; Barrett 1989b; Kelso 1992). Because the newly arrived homostylous polyploids are capable of producing fertile offspring, polyploidy is more likely to become established in homostyles than self-incompatible heterostyles as a result of selection for reproductive assurance (Kelso 1992; Schoen *et al.* 1997; Guggisberg *et al.* 2006). There are, however, several studies that report a higher level of ploidy may not necessarily disrupt the

maintenance of self-incompatibility (Mable 2004) and heterostyly (Ornduff 1970a; Ornduff 1974; Barrett and Shore 1987).

The Menyanthaceae possess distylous floral polymorphisms and members of the family have been studies for mating system change (Ornduff 1966; Ornduff 1969; Ornduff 1974; Thompson *et al.* 1998). In this family, distylous taxa, including *Nymphoides*, *Villarsia* and *Menyanthes*, have been reported to contain members that are homostylous. In *Nymphoides* and *Villarsia*, the derivative nature of homostyly from heterostylous ancestors was first mentioned by Ornduff (1969). In *V. albiflora*, Ornduff (1988) proposed the recombination in the distyly locus caused the breakdown of distyly to homostyly. The monomorphic species appeared to be a long-homostyle with the styles and incompatibility behaviour of long-styles but with the anthers of short-styles. Ornduff (1988), however, favoured his second hypothesis for the origin of homostyly that is, populations of *V. albiflora* represented a situation where the flowers were short-styles, and the long-styles of a presumed distylous ancestor had been lost following periodic severe reductions in population size.

Nymphoides geminata R. Br. Kuntze (Menyanthaceae) is a homostylous short-lived perennial (or annual) plant growing in either permanent or temporary pools (Jacobs 1992). This species is native to Australia, and has a widespread distribution across eastern Australia compared with its congener the distylous *N. montana* (Figure 4.1). Flowers are similar to *N. montana* in the number of flower parts and the disposition of hairs surrounding the petal edges and the corolla tubes (Chapter 2: section 2.3.1), but differ in flower size and the relationship between stigma and anther heights. A study of *N. geminata* was of interest, given its widespread distribution and distinctive floral morphology, in trying to understand the interactions between habitat changes, pollinator activity, polyploidy and reproductive biology.

A) *Nymphoides montana*



B) *Nymphoides geminata*

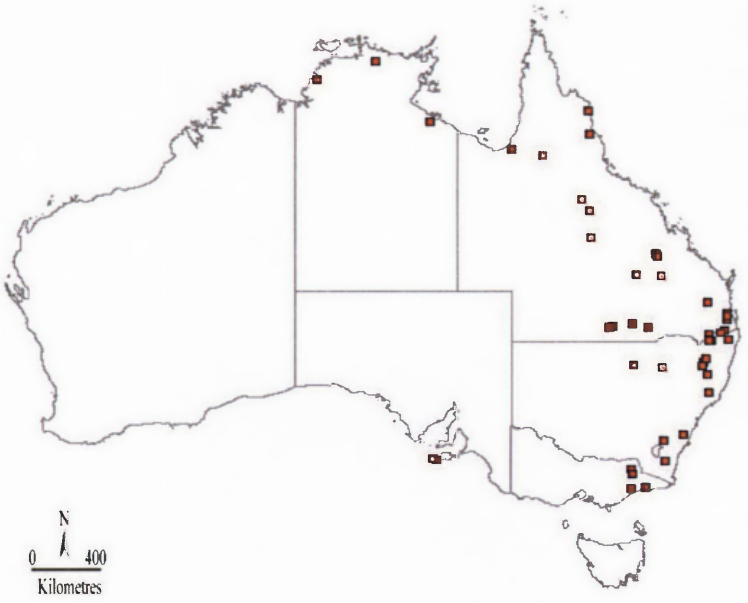


Figure 4.1. Distribution map of A) the distylous *Nymphoides montana* and B) the homostylous *N. geminata* in Australia (Australia's Virtual Herbarium, viewed at 10 September 2007).

4.2 Aims

In this chapter, the reproductive biology of homostylous *Nymphoides geminata* is studied to explore the extent to which its breeding system and floral morphology 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?

4.3 Material and Methods

4.3.1 Study species

Nymphoides geminata (R. Br.) Kuntze is an aquatic plant with floating leaves; it reproduces vegetatively by spreading stolons and sexually by seeds (Figure 4.2 and Figure 4.3). The five-part flowers are pale yellow with epipetalous stamens attached to the floral tube. The ovary is superior, 3-carpelate and fused to from a single locule; placentation is parietal. The fruit is a capsule with dark brown, spinose seeds. The seeds are almost half the size of the seeds of distylous *N. montana* (Figure 4.4; $\leq$ 0.75 mm). The seed coat ornamentation also differs between the two homostylous and distylous species (Figure 4.4).

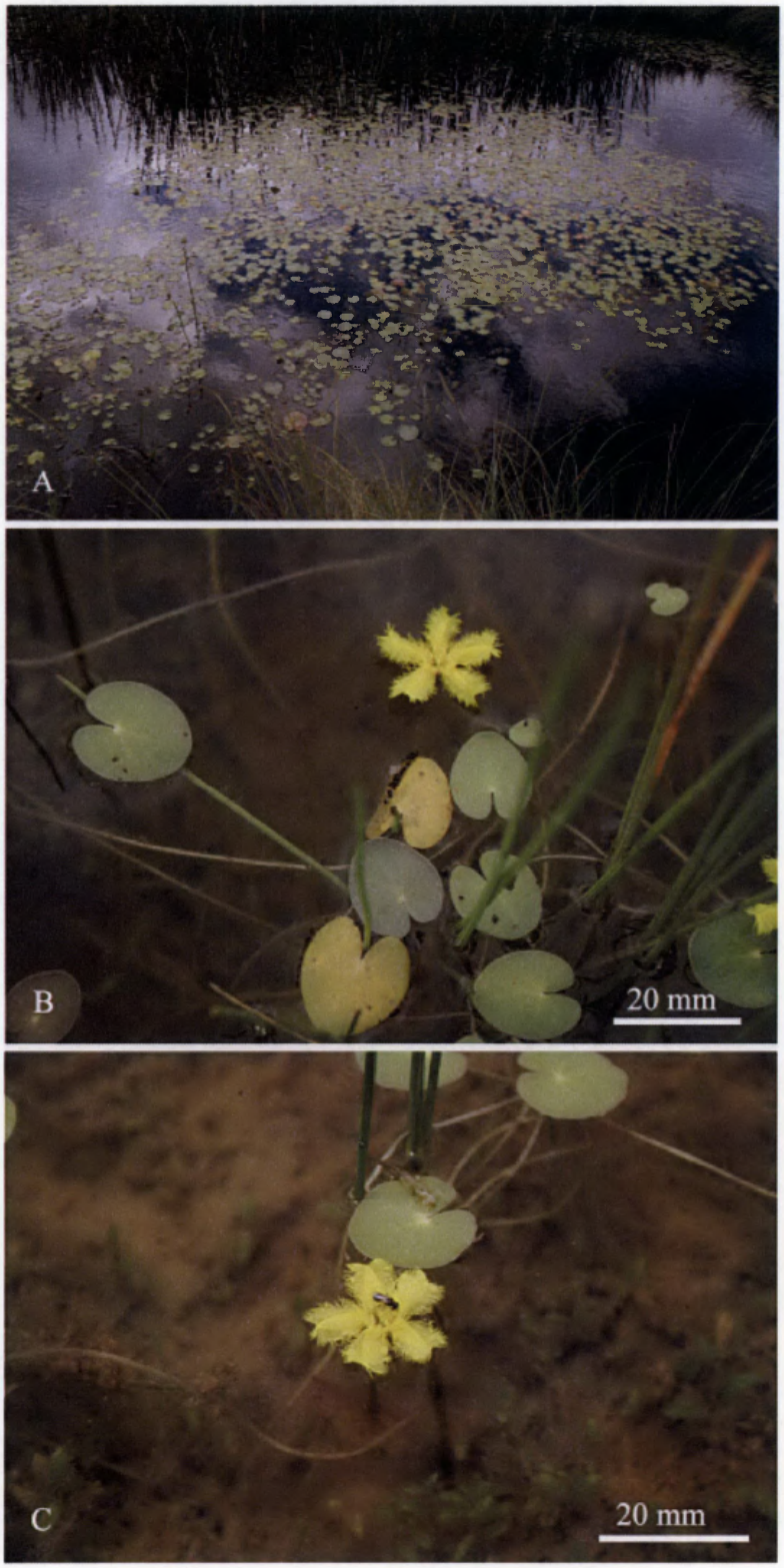


Figure 4.2. Photographs of *Nymphoides geminata* showing A) habitat at a permanent man-made lagoon located in a travelling stock route, B) floating seeds and spreading stolons and C) a floral visitor.

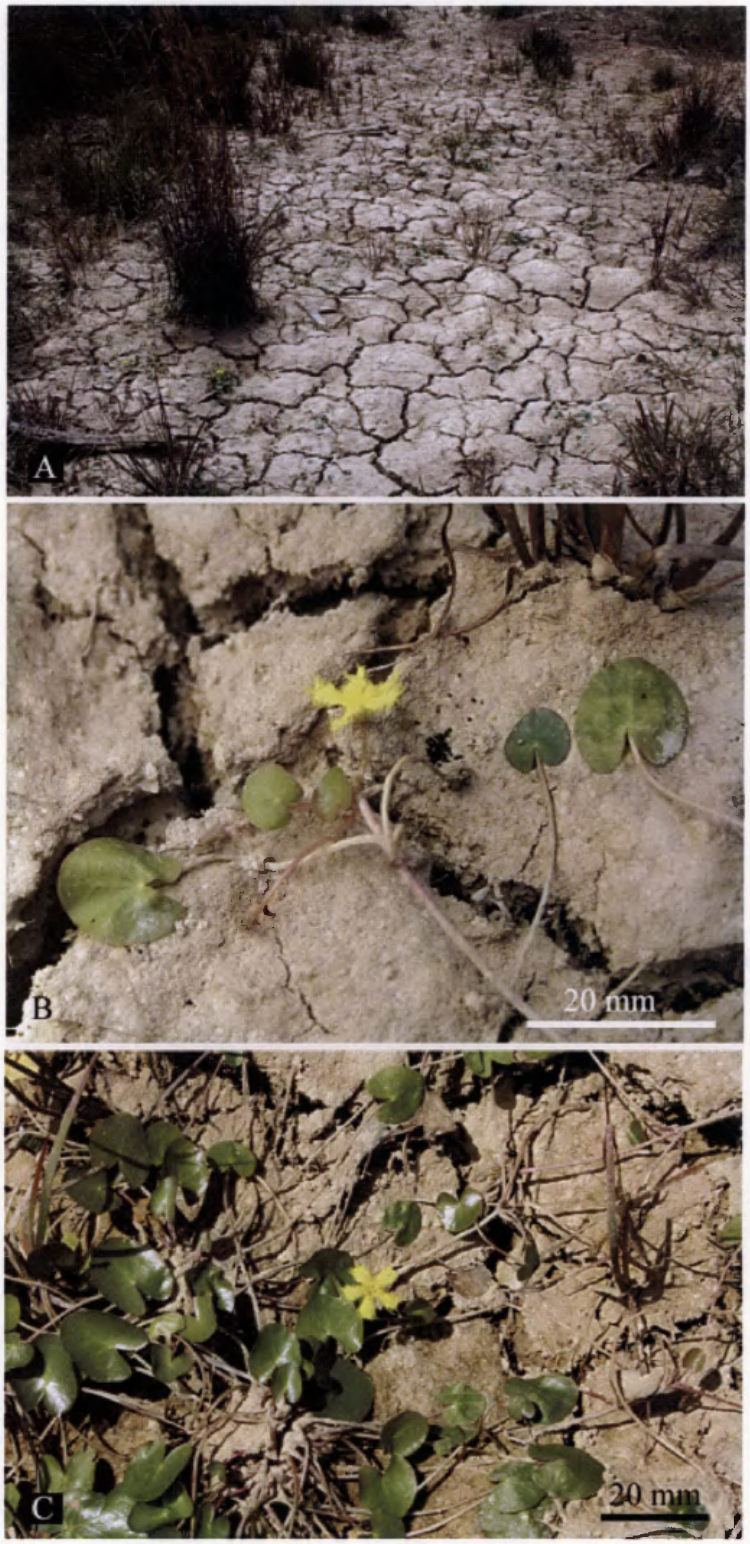


Figure 4.3. Photographs of *Nymphoides geminata* showing A) habitat at a disturbed area of dry sclerophyll forest (Yooroonah State Forest), B) seeds and a ruptured fruit and C) spreading stolons. Note the ability of the plants to persist and reproduce successfully in an ecologically marginal habitat with an unreliable water regime.

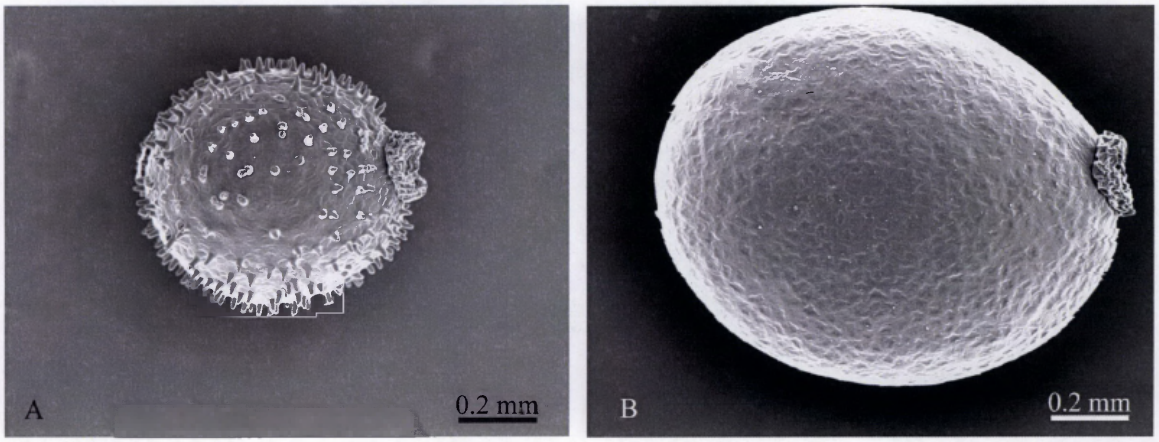


Figure 4.4. SEM micrographs of seeds of A) the homostylous *Nymphoides geminata* and B) the distylous *N. montana*. Note the differences in the seed ornamentation and size between the two species.

4.3.2 Study sites

Three populations of *Nymphoides geminata* in the Northern Tablelands of New South Wales, Australia (Figure 4.5) were used in this study. The largest population is located in a lagoon of approximately 4 ha in a travelling stock route 21.5 km East of Glen Innes (TSR, 1195 m elevation; 29° 38′ 54″ S, 151° 51′ 41″ E). Plants are rooted in water approximately 20 cm deep, along the edge of the lagoon (Figure 4.2). The second population is located in shallow depressions with a surface area of approximately 100 m^2 alongside a road, among annual grass/sedge lands (Crisps Road, CRR, 1125 m elevation; 29° 42′ 46″ S, 151° 49′ 36″ E). The third population in Yooroonah State Forest has plants restricted to a disturbed area of dry sclerophyll forest (YSF, 1170 m elevation; 30° 30′ 07″ S, 152° 13′ E). This population is very small with a surface area of approximately 20 m^2 and no standing water (Figure 4.3). The YSF and CRR populations are seasonally inundated, whereas the TSR population is in a permanent man-made water body (Figure 4.2). All the three populations are within the geographical range of distylous *N. montana*, and are at a similar elevation (Chapter 2: section 2.3.2 and Figure 2.2).

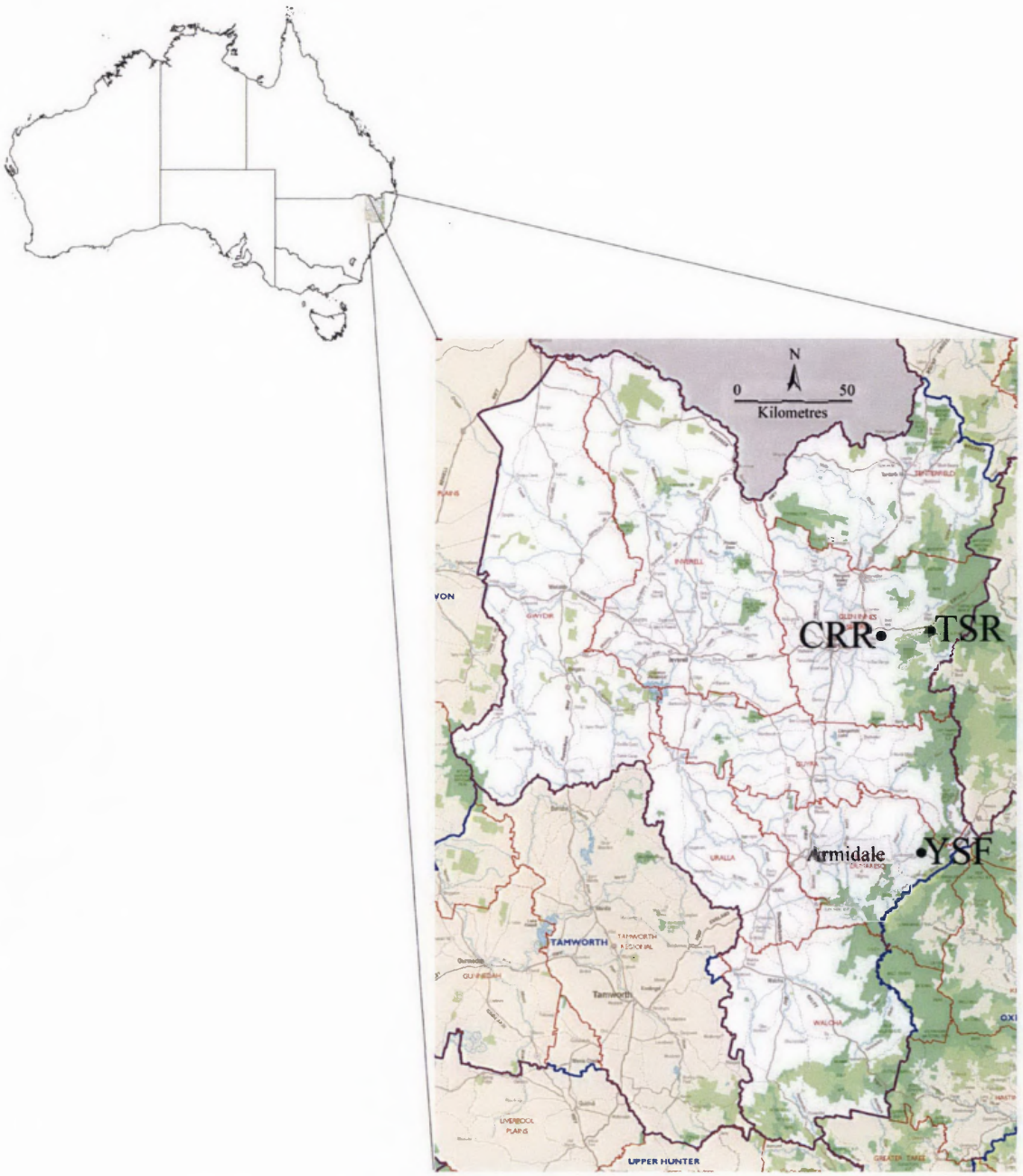


Figure 4.5. Location of the study sites of *Nymphoides geminata* in the Northern Tablelands, NSW, Australia. The homostylous populations are located within the range of the distylous *N. montana*. Two populations occupy disturbed habitats; one at the drying edge of a dry sclerophyll forest (Yooroonah State Forest, YSF) and the other alongside a road (Crisps Road, CRR). The largest population is in a man-made lagoon located in a travelling stock route (TSR). Source: www.aec.gov.au

4.3.3 Floral measurements

Differences in floral traits among the homostylous populations (YSF, TSR and CRR) were identified by randomly collecting flowers from plants located at least 3 m apart; this minimised the likelihood of selecting ramets. In each population, one flower from each of 15 different plants were collected and preserved in 70% ethanol until measured. The floral traits were measured from the base of the superior ovary using a Nikon digital sight DS-L1 attached to a dissecting microscope (Figure 4.6). Traits measured were stigma height, stigma width, stigma length, style length, anther height, anther length, filament length, stamen insertion height, stigma–anther separation, corolla tube length, and corolla diameter.

Figure 4.6. A cross-section of a homostylous flower of *Nymphoides geminata* from the Glen Innes (TSR) population. Letters correspond to the following measurements: a) corolla tube length, b) anther height, c) anther length, d) filament length, e) stigma height, f) stigma length, g) stigma width, h) corolla tube width, and i) corolla diameter.

Pollen grain size was assessed by collecting pollen from two flowers from each of 10 randomly chosen plants, each at least 3 m apart, from two populations, TSR and YSF. Pollen grains were prepared semi-permanently on microscope slides (Beattie 1971). Fifteen triangular pollen grains on each flower were measured. The area of each was calculated from its height and base measurements (Figure 2.6). In total, 15 pollen × 2 flowers × 10 plants × 2 populations = 600 pollen grains were measured.

Pollen grain number was determined by quantifying the number of pollen grains produced in all five anthers of one flower from each of 15 plants, each at least 3 m apart, in each of the two populations, TSR and YSF. Pollen grains were prepared and counted as described in Chapter 2: section 2.3.7.

The number of ovules per flower was assessed using 10 mature fruits from the YSF and TSR populations. The number of unfertilised ovules, aborted seeds and mature seeds were counted and added together to estimate ovule number. Aborted seeds are readily recognisable, being shrivelled with soft brownish seed coats and smaller than mature seeds.

The pollen:ovule ratio was calculated as the ratio of the mean number of pollen grains per population to the mean number of ovules per population in each of the homostylous populations, YSF and TSR. Standard errors were calculated as described in Chapter 2: section 2.3.9.

One-way analyses of variance (ANOVAs) were performed on the pollen and ovule data to test for differences between the TSR and YSF populations, and on the floral trait data between the YSF, TSR and CRR populations. Normality and homogeneity of variances were checked using the Ryan-Joiner test and Levene's test, respectively. Where necessary, data were $\log_{10}$ transformed to satisfy assumptions of ANOVA.

An association between the floral traits and breeding systems (homostyly *vs.* distyly) was defined using Principal Component Analysis (PCA) on all the floral traits measured from the homostylous populations of *Nymphoides geminata* and the distylous populations of *N. montana* (Chapter 2; DD, TL and GC). A covariance matrix was used to extract eigenvalues. The first principal component (PC1) scores for the two breeding systems were compared using a one-way ANOVA. Traits that contributed most in distinguishing among the three floral morphs (homostyle, S-morph and L-morph) were identified by the DFAs.

Differences in floral traits were tested separately between homostyly and each of the distylous morphs, using nested ANOVAs with morph as a fixed factor and population nested within morph as a random factor.

4.3.4 Pollen and stigma morphology

Stigma and pollen morphology were observed using two or three mature flowers from each of five plants, each at least 3 m apart, from each of the TSR and YSF populations. Fresh and air-dried samples were fixed, critical point dried, and viewed with a scanning electron microscope (SEM) as described in Chapter 2: section 2.3.10.

4.3.5 Chromosome count

To assess ploidy level, root tip meristems were prepared and fixed for chromosome observations as described in Chapter 3: section 3.3.4. A total of 250 cells (5 cell/2 root tips/25 ramets) from the TSR population and 70 cells (5 cell/2 root tips/7 ramets) from the YSF population were observed at the metaphase stage. Ten cells per population with a good spread of chromosomes were enlarged to 400x magnifications on a Nikon digital sight DS-L1 attached to the microscope and counted. The large numbers of aggregated chromosomes and the small size of the chromosomes made an accurate chromosome count difficult; therefore, mean (± SD) chromosome numbers are presented here.

4.3.6 Breeding systems

During February-March 2005, 40 plants (20 plants per population) were randomly collected from the two populations, TSR and YSF. Plants were collected at least 3 m apart, placed in 10-cm pots and transferred to a pollinator-free glasshouse, as described in Chapter 2: section 2.3.11.

To assess the capacity of *Nymphoides geminata* for autonomous self-fertilisation in the homostylous populations, a series of experiments were performed on 15 and 14 plants from the YSF and TSR populations, respectively. On each plant, 6–9 flowers were allocated to the following pollination treatments: a) autonomous self-pollination – flowers were not hand-pollinated, b) self-pollination – flowers were hand-pollinated with self-pollen, and c) cross-pollination – flowers were hand-pollinated with pollen from a different individual. Two to three replicates of each treatment were conducted on each plant. Mature fruits were harvested two weeks later. Percent fruit set was calculated by scoring

the number of mature fruits containing at least one seed. The proportion of ovules forming to mature seeds was also counted to calculate percent seed set.

Split-plot analyses of variance (ANOVAs) were used to test the effects of population, plant, and pollination treatment on the percent seed set. Population and plant nested within population were considered as random factors. Pollination treatment was considered as a fixed factor. Percent seed set was arcsine transformed to meet the assumptions of ANOVA. Because the population × pollination treatment was significant ($F_{2,54} = 9.03$, $P< 0.001$), a random block ANOVA was performed separately for each population to test whether pollination treatments differed.

A test for agamospermy, i.e. seed set without fertilisation, was also performed on two flowers from each of 10 randomly chosen plants in each of the two populations, YSF and TSR, under glasshouse conditions. On each plant, one flower was dissected at the bud stage and then emasculated. The second flower on the same plant was also dissected without emasculation to control for possible negative effects of flower manipulation on seed set.

4.3.7 Open pollinations

During November-December 2006, the reproductive success of the homostylous plants under natural pollinations was assessed by estimating open-pollinated fruit and seed set in the three populations, YSF, TSR and CRR. In each population, 10–40 mature flowers were tagged, and the number of flowers forming to mature fruits was scored two weeks later. Percent fruit set was calculated as described in Chapter 2: section 2.3.13.

To assess percent seed set, 40 mature fruits in the TSR population and 30 fruits in each of the YSF and CRR populations were dissected, and percent seed set was calculated as described in Chapter 2: section 2.3.13. A one-way ANOVA was performed to compare percent seed set between populations. The percentage data were arcsine transformed to satisfy assumptions of ANOVA.

4.3.8 Floral visitor observations

During November-December 2006, floral visitor movements were examined in the three populations, YSF, TSR and CRR; this period corresponds to peak flowering. At each site, 5–30 randomly selected patches of flowers (with a mean of eight flowers per patch)

were observed for 5 min each, per hour throughout the day (09:30–15:30). The number of visited flowers and the visitor's behaviour during each pollinator visit were recorded. Only insects that clearly contacted stigmas and anthers during their visits were considered pollinators. The type of visitors that were excluded as potential pollinators were: a) flying insects that entered the quadrats but did not forage for nectar or pollen and flew away, and b) insects that alighted onto non-reproductive parts of the flowers (e.g. petals or corona) and not inside the floral tube. This observation was carried out for 4 days in the TSR population and 2 days in each of the YSF and CRR populations in both months.

4.4 Results

4.4.1 Floral measurements

The mean values and the results of the ANOVAs on all the floral trait, pollen and ovule data are given in Table 4.1. All floral traits differed significantly among the three populations. The YSF population showed the smallest corolla tube length and corolla diameter as well as the greatest stigma–anther separation compared with the other two populations, TSR and CRR. The TSR population, however, produced significantly more and smaller pollen compared with the YSF population (Table 4.1). Ovule number did not differ significantly between the YSF and TSR populations (Table 4.1). The pollen:ovule ratios were relatively low in each of the two populations (YSF: 79.13 ± 311.94 and TSR: 142.55 ± 42.3), corresponding to a facultative selfing breeding system (Cruden 1977).

Table 4.1. Means of floral trait, pollen and ovule data (± S.E.), and *F*-values from one-way ANOVAs of the Yooroonah State Forest (YSF), Glen Innes (TSR) and Crisps Road (CRR) populations of homostylous *Nymphoides geminata*. The populations differed significantly in all traits except the ovule number. These traits were used in comparison with the distylous *N. montana*. Superscripts denote: NS > 0.05, * $P<0.05$, ** $P<0.01$, *** $P < 0.001$.

No.	Traits	Populations			*F* value
		YSF	TSR	CRR	
1	Stigma height (mm)	3.96 ± 0.07	5.01 ± 0.11	5.04 ± 0.07	50.66^{***}
2	Stigma width (mm)	1.67 ± 0.04	1.95 ± 0.05	2.02 ± 0.06	12.35^{***}
3	Stigma length (mm)	1.57 ± 0.06	2.21 ± 0.07	2.21 ± 0.06	36.35^{***}
4	Style length (mm)	2.39 ± 0.05	2.81 ± 0.07	2.84 ± 0.05	19.80^{***}
5	Anther height (mm)	4.56 ± 0.06	5.36 ± 0.11	5.05 ± 0.05	29.56^{***}
6	Filament length (mm)	1.13 ± 0.03	1.45 ± 0.04	1.37 ± 0.02	27.75^{***}
7	Anther length (mm)	1.24 ± 0.02	1.55 ± 0.05	1.37 ± 0.02	21.89^{***}
8	Stamen insertion height (mm)	3.32 ± 0.05	3.82 ± 0.10	3.68 ± 0.05	12.40^{***}
9	Stigma–anther separation (mm)	0.60 ± 0.07	0.35 ± 0.06	0.01 ± 0.04	27.17^{***}
10	Corolla tube length (mm)	2.52 ± 0.09	3.32 ± 0.08	3.40 ± 0.07	36.72^{***}
11	Corolla diameter (mm)	10.58 ± 0.43	19.37 ± 0.48	16.80 ± 0.84	30.32^{***}
12	Pollen grain number	10,000 ± 1018	17,667 ± 1233	—	23.06^{***}
13	Pollen grain size (μm)	359.03 ± 5.30	338.40 ± 6.34	—	6.24^{*}
14	Ovule number	126.00 ± 7.28	123.93 ± 5.69	—	0.07^{NS}

Degrees of freedom: 2,42 for the first 11 traits, 1,28 for pollen grain number, 1,18 for pollen grain size and ovule number.

4.4.1.1 Comparisons of floral traits (homostyly vs. *distyly)*

The loadings of the traits of the PCA are given in Table 4.2. The first three principal components explained ≥ 99% of the variation in the traits (Table 4.2). The homostyles and the distylous S-morphs and L-morphs formed three distinct, non-overlapping groups along the PC1 axis (Figure 4.7). For the PC1 and PC2 scores, stigma–anther separation and corolla tube length had strong loadings (Table 4.2). In the DFA, cross validation verified this result and 91% was correctly classified. The distylous populations had greater PC1 scores than the homostylous populations (5.18 ± 0.11 *vs.* 3.05 ± 0.03, $F_{1,133} = 155.41$, $P < 0.001$). This indicates that the floral traits of the distylous plants, more specifically herkogamy and flower size, were greater than those of the homostylous plants.

The mean values and the results of the ANOVAs on all comparisons between the homostyles and each of the distylous morphs are given in Figure 4.8 and Table 4.3, respectively. The homostylous flowers were smaller than the distylous flowers in stigma height, anther height, stigma–anther separation, corolla tube length, and corolla diameter. Also, the homostylous flowers produced less pollen and more ovules than the distylous flowers. Moreover, there was less variation in pollen size between the homostyles and the S-morphs than the L-morphs.

Table 4.2. Loadings of traits on the first three scores of the Principal Component Analysis. Eigenvalues and percent total variance are given in parentheses and brackets, respectively. Character loadings of an absolute value > 0.55 are given in bold. Flower size and herkogamy are the two most diagnostic traits that segregated homostyly and distyly.

Character	PC1 (1.88) [61.0]	PC2 (1.16) [37.6]	PC3 (0.01) [0.6]
Stigma height	0.10	−0.02	0.29
Stigma width	0.08	−0.02	0.16
Stigma length	0.08	−0.06	0.31
Style length	0.11	0.01	0.29
Anther height	0.02	0.08	−0.08
Filament length	−0.11	0.20	**−0.76**
Anther length	−0.02	−0.05	−0.16
Stamen insertion height	0.02	0.09	−0.17
Stigma–anther separation	**0.79**	**−0.55**	−0.27
Corolla tube length	**0.57**	**0.80**	0.05

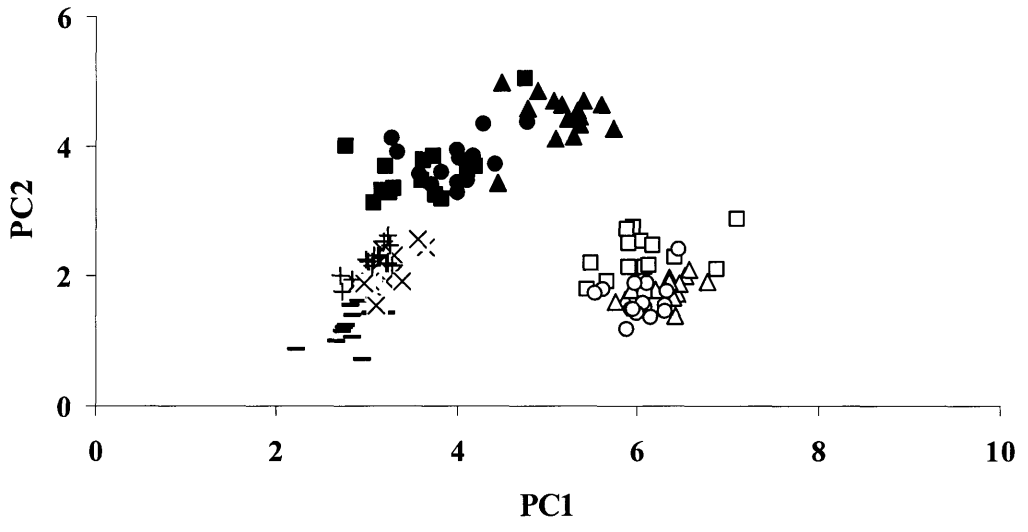


Figure 4.7. Plot of the first and second PCA scores for the homostylous *N. geminata* (bottom left) and the distylous S-morphs (top, filled symbols) and L-morphs (bottom right, open symbols) of *Nymphoides montana*. PC1 and PC2 explained 61.0% and 37.6% of the variation among the individuals, respectively. Symbols referring to the homostylous populations are: –, Yooroonah State Forest (YSF); ×, Glen Innes (TSR) and +, Crisps Road (CRR). Symbols referring to the distylous populations are: ▲Δ, Dumaresq Dam (DD); ■□, Thomas Lagoon (TL); ○●, Glencoe (GC).

Table 4.3. *F*-values from nested ANOVAs showing the effects of morph and population nested within morph on floral traits for comparisons between the homostylous *Nymphoides geminata* and each of the distylous S-morphs and L-morphs of *N. montana*. There was less variation in pollen size between the homostyles and the S-morphs than the L-morphs. Analyses refer to the mean values in Figure 4.8. Superscripts denote: NS > 0.09, † 0.05 < *P* < 0.09, * *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001.

No.	Traits	Homostyle *vs.* S-morph		Homostyle *vs.* L-morph	
		Morph	Population (morph)	Morph	Population (morph)
1	Stigma height	8.69^{*}	21.54^{***}	84.90^{***}	34.13^{***}
2	Stigma width	2.71^{NS}	11.58^{***}	69.52^{***}	9.34^{***}
3	Stigma length	1.76^{NS}	21.08^{***}	14.03^{*}	37.74^{***}
4	Style length	68.80^{***}	6.68^{***}	261.84^{***}	8.97^{***}
5	Filament length	10.58^{*}	25.78^{***}	40.82^{**}	21.75^{***}
6	Anther height	26.27^{**}	44.49^{***}	12.81^{*}	12.16^{***}
7	Anther length	10.74^{*}	17.38^{***}	2.35^{NS}	58.16^{***}
8	Stamen insertion height	25.12^{**}	30.32^{***}	$6.14^{\dagger}$	11.33^{***}
9	Stigma–anther separation	15.34^{*}	41.48^{***}	173.61^{***}	13.13^{***}
10	Corolla tube length	17.50^{*}	42.38^{***}	30.50^{**}	21.90^{***}
11	Corolla diameter	16.95^{*}	44.14^{***}	18.55^{*}	36.82^{***}
12	Pollen grain number	18.09^{*}	6.19^{**}	21.70^{*}	14.66^{***}
13	Pollen grain size	6.38^{NS}	6.44^{**}	35.24^{*}	9.90^{***}
14	Ovule number	852.51^{***}	0.10^{NS}	277.74^{**}	0.46^{NS}

Degrees of freedom for morph and population (morph), respectively, were: traits 1–10, 1,4 and 4,84; character 11, 1,3 and 3,70; character 12, 1,2 and 2,56; and traits 13 and 14, 1,2 and 2,36.

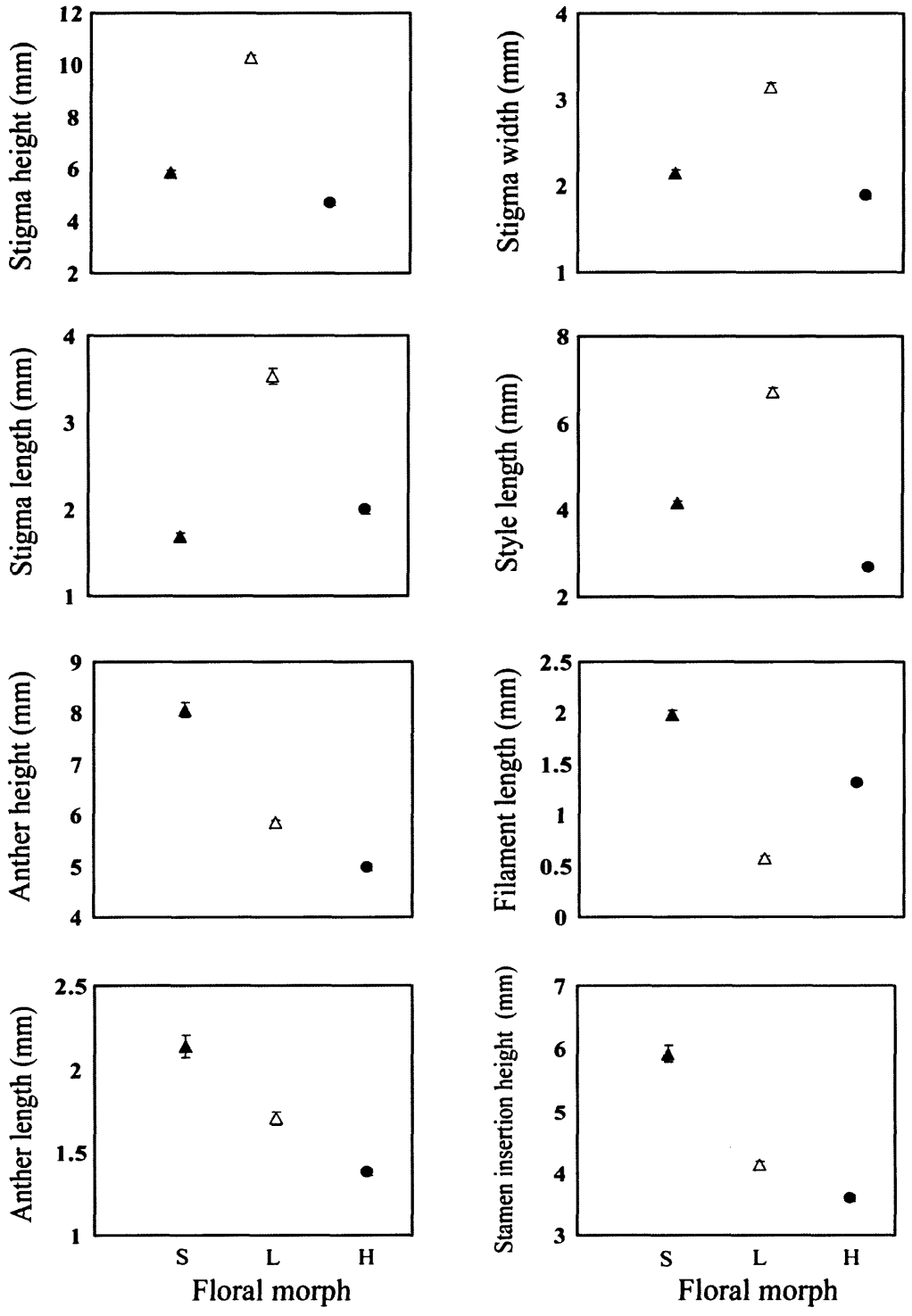


Figure 4.8. Comparisons of means of floral traits (± SE) among the three floral morphs; the homostylous (H) *N. geminata* and the S-morphs (S) and L-morphs (L) of distylous *Nymphoides montana*. The homostylous flowers were smaller with over 80% shorter stigma–anther separations, and produced more ovules and less pollen than the distylous flowers.

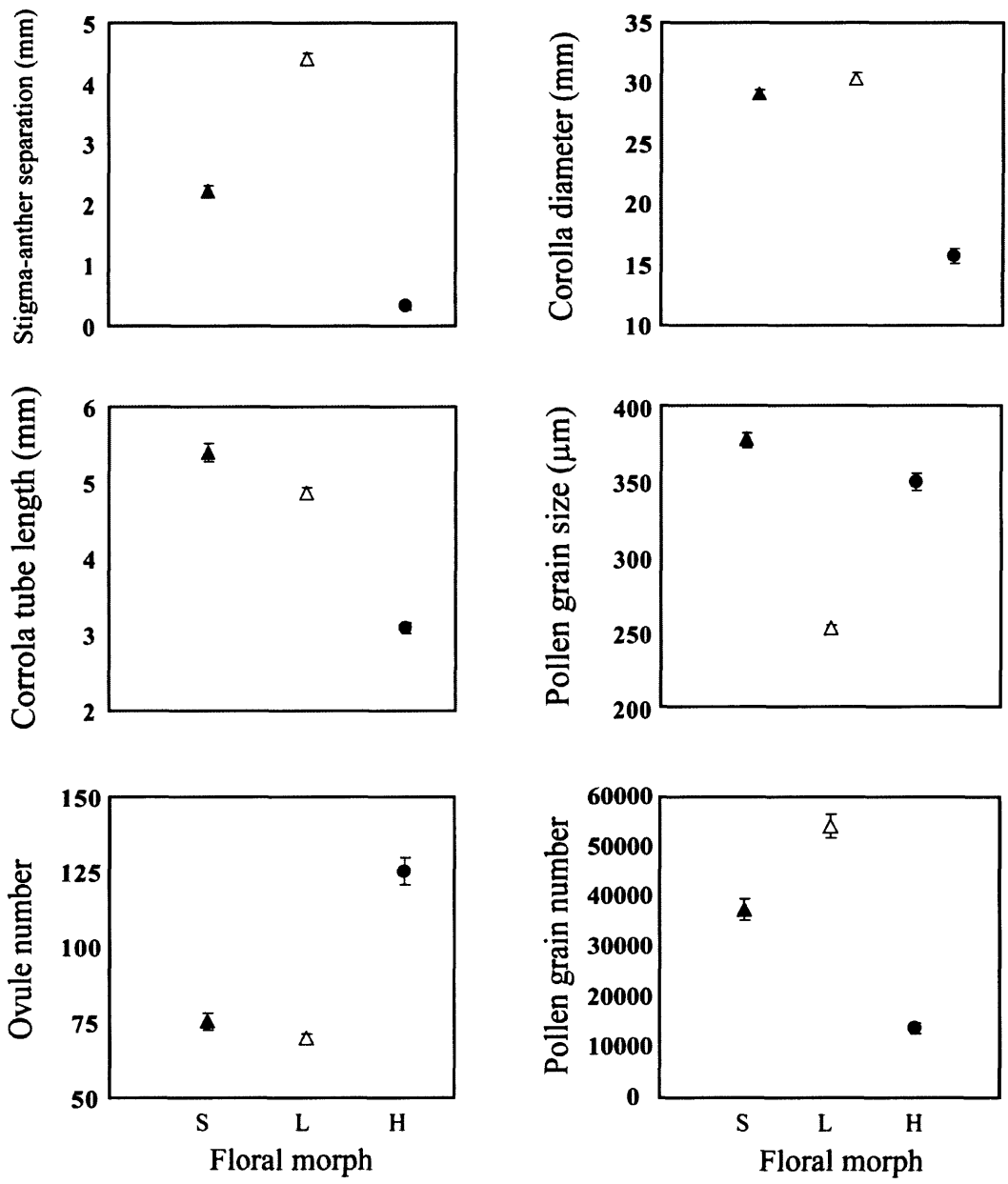


Figure 4.8. (*Continued*)

4.4.2 Pollen and stigma morphology (homostyly vs. *distyly)*

The SEM images of the pollen grains of the homostylous *N. geminata* show that the pollen grain exine sculpture resembles the S-morph pollen sculpture of the distylous *N. montana* (Figure 4.9). The muri of the homostylous pollen has minute granulae over the whole surface, similar to the muri of the S-morph pollen. The morphology of the stigma and stigmatic papillae of the homostyles are, however, similar to those of the L-morphs (Figure 4.10). In both the homostyles and the L-morphs, each stigmatic lobe is divided into two sub-lobes, and the papillae are cylindrical and longer than those of the S-morph.

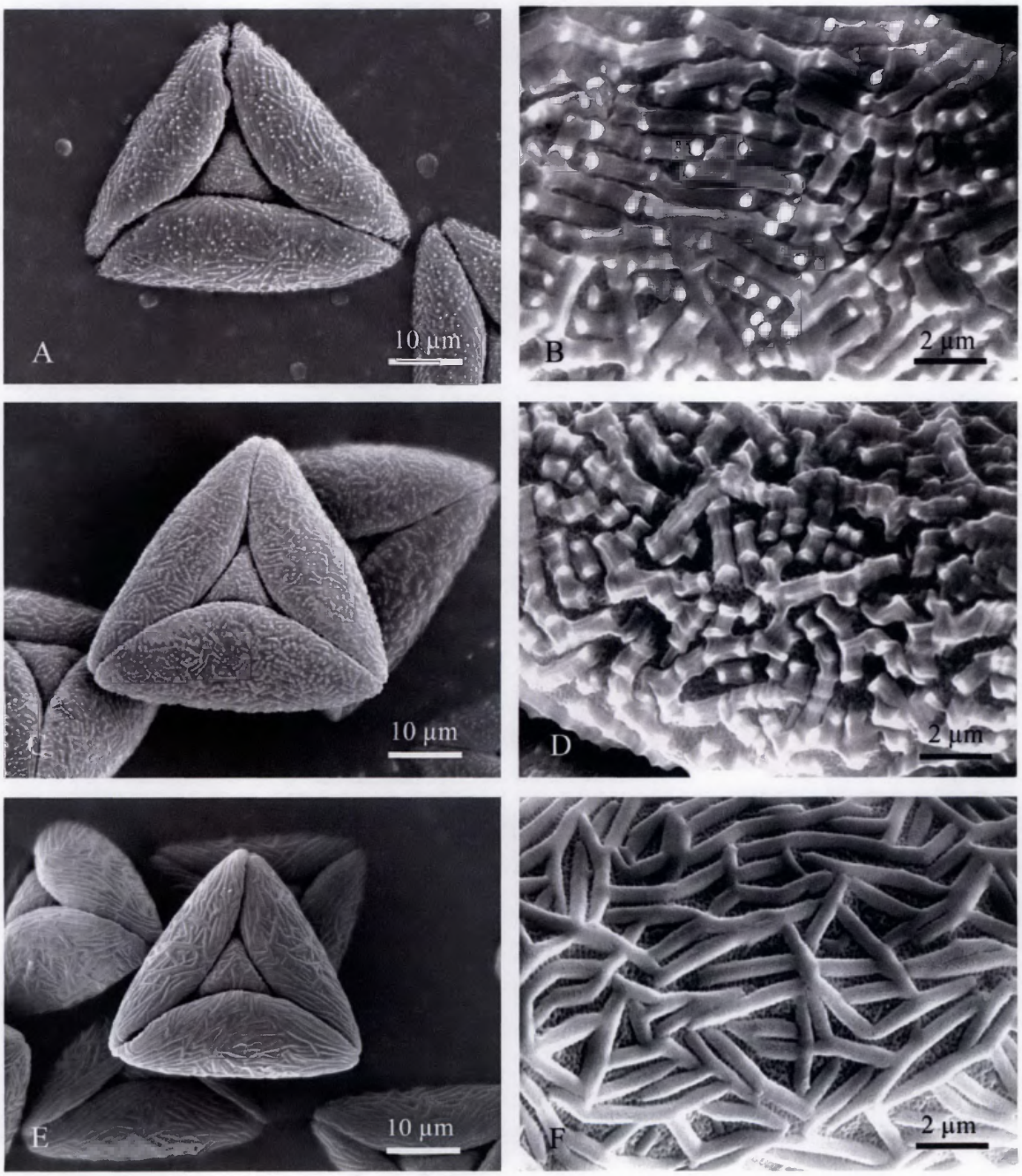


Figure 4.9. SEM micrographs of air-dried pollen grains of the homostylous *Nymphoides geminata* (A and B), and the distylous S-morph (C and D) and L-morph (E and F) of *N. montana*. The pollen grain sculpture and size of the homostyles are similar to those of the S-morphs.

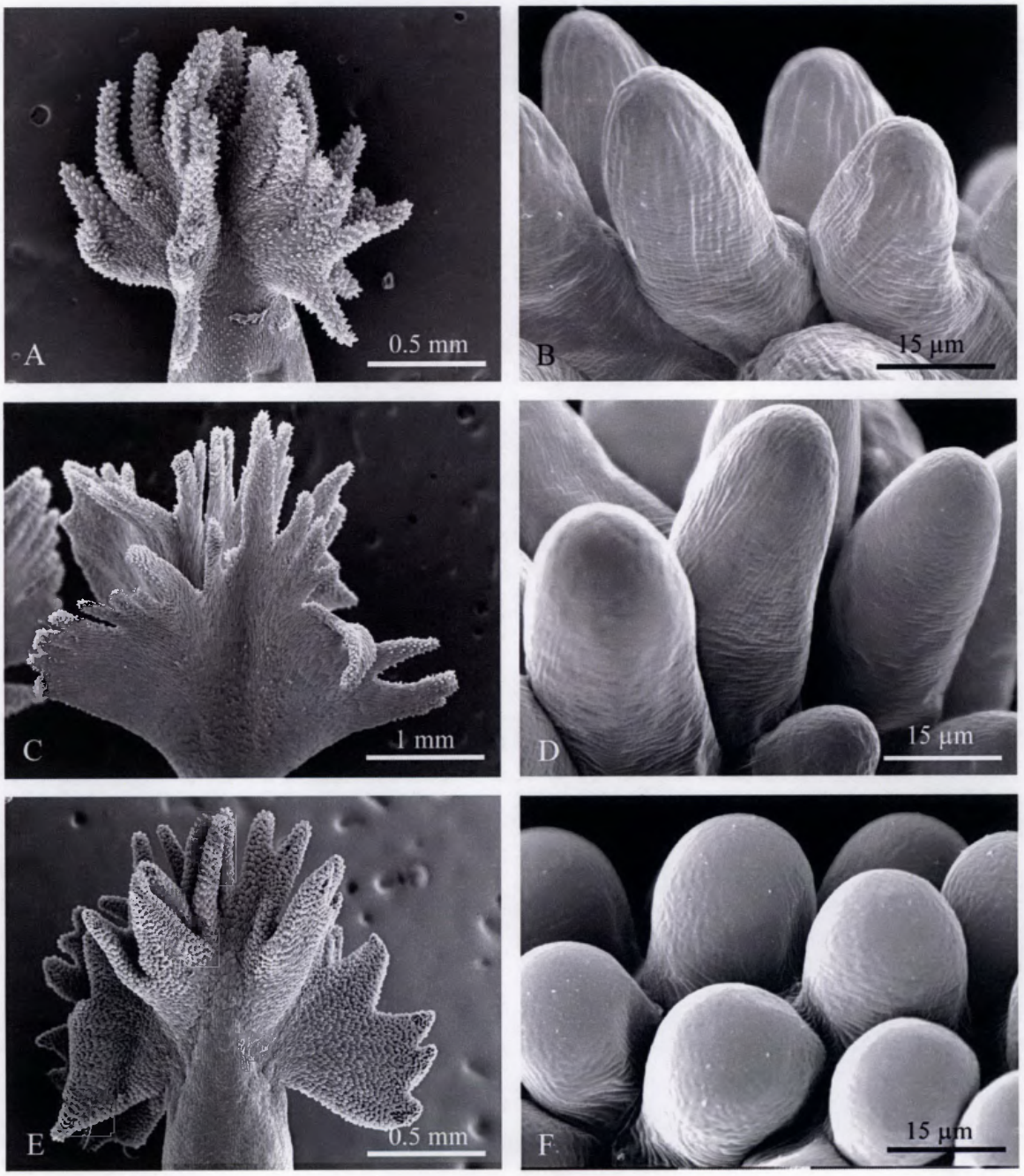


Figure 4.10. SEM micrographs of stigmas of the homostylous *Nymphoides geminata* (A and B), and the distylous L-morph (C and D) and S-morph (E and F) of *N. montana*. The shape and size of the stigmatic papillae of the homostyles are similar to those of the L-morphs.

4.4.3 Chromosome count

Somatic chromosome counts in both populations of homostylous *N. geminata* showed average counts close to the hexaploid number $6x = 54$ (YSF: mean ± SD = 52.80 ± 2.25 and TSR: mean ± SD = 50.18 ± 3.13).

4.4.4 Breeding systems

In the glasshouse environment, 100% of auto-, self- and cross-pollinated flowers set fruits. Results of the split-plot ANOVA on seed set demonstrated that the effect of pollination treatment depended on population (Table 4.4). Random block ANOVAs showed that there were no significant differences between the pollination treatments in the YSF population (Table 4.5; $F_{2,28} = 0.56$, $P = 0.579$). About 90% of ovules developed into seeds following autonomous self-pollination, hand-self-pollination and cross-pollination in this population (Figure 4.11). However, the TSR population showed significant differences between the pollination treatments ($F_{2,41} = 10.62$, $P < 0.001$). A high percentage of ovules developed into seeds following self- (88%) and cross- (87%) pollination treatments compared with 68% seed set following autonomous self-pollination (Figure 4.11). In both analyses, the plants differed significantly (Table 4.4 and Table 4.5).

Asexual reproduction through agamospermy did not occur in either YSF or TSR populations, whereas unemasculated control flowers produced mature fruits and seeds.

4.4.5 Open pollinations

During November-December 2006, 100% of open-pollinated flowers set fruits in each of the three populations studied. Although the result of the one-way ANOVA on open-pollinated seed set demonstrated significant differences between the populations, 70–90% of the ovules developed into mature seeds in each population (Figure 4.12; $F_{2,97} = 4.48$, $P = 0.014$).

Table 4.4. Results of the split-plot ANOVAs to test the effects of population, plant and pollination treatment on percent seed set in the homostylous *Nymphoides geminata*. The significant population × pollination treatment interaction was further analysed by the random block ANOVAs (see Table 4.5).

Source	Type III *SS*	df	*MS*	*F*	*P*
Between subject factors					
Population	0.20	1	0.20	0.69	0.465
Plant (population)	2.38	27	0.09	3.37	<0.0001
Within subject factors					
Pollination treatment	0.64	2	0.32	1.35	0.425
Population × pollination treatment	0.47	2	0.24	9.03	<0.0001
Error	1.41	54	0.03		

F-test for the effect of population used a synthetic denominator mean square (*MS*). The denominator was $MS_{\text{plant (population)}} + MS_{\text{population*pollination treatments}} - MS_{\text{error}}$.

Table 4.5. Results of the random block ANOVAs describing variations in percent seed set between the pollination treatments in the homostylous *Nymphoides geminata* from the Yooroonah State Forest (YSF) and the Glen Innes (TSR) populations. There was a significant difference between the pollination treatments in the TSR population.

Source	Population							
	YSF				TSR			
	df	*MS*	*F*	*P*	df	*MS*	*F*	*P*
Plant	14	0.09	4.09	0.001	13	0.08	2.82	0.012
Pollination treatment	2	0.01	0.56	0.579	2	0.52	17.05	0.000
Error	28	0.02			26	0.03		

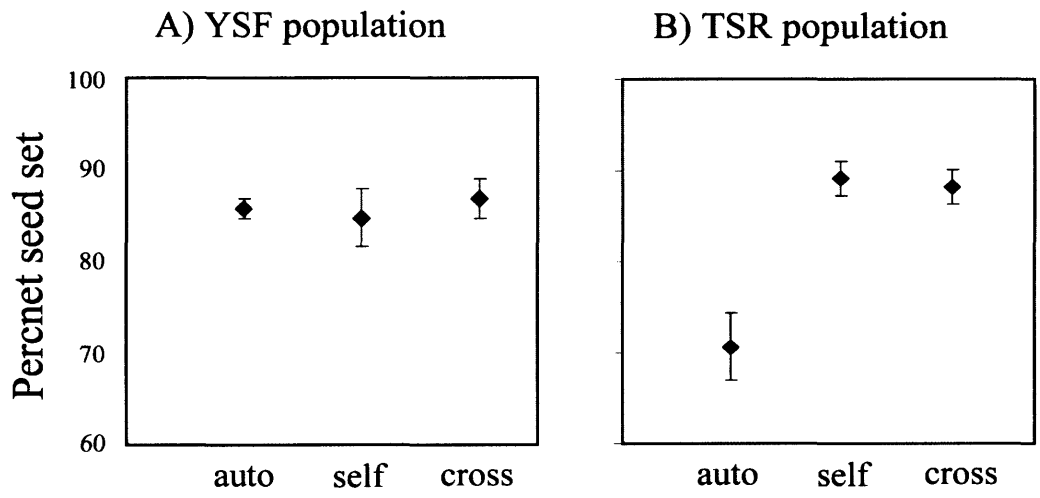


Figure 4.11. Results of the glasshouse-pollinated seed set of the homostylous *Nymphoides geminata* from A) the Yooroonah State Forest (YSF) and B) the Glen Innes (TSR) populations. Mean percent seed set following autonomously self-pollinated (auto), and hand-pollinated (self and cross) treatments is given. In both populations, a relatively high proportion of the ovules developed into mature seeds following autonomous self-pollination, and hand-self-pollination and cross-pollination. In the TSR population, however, autonomously self-pollinated flowers did not produce as many seeds as the hand-pollinated flowers.

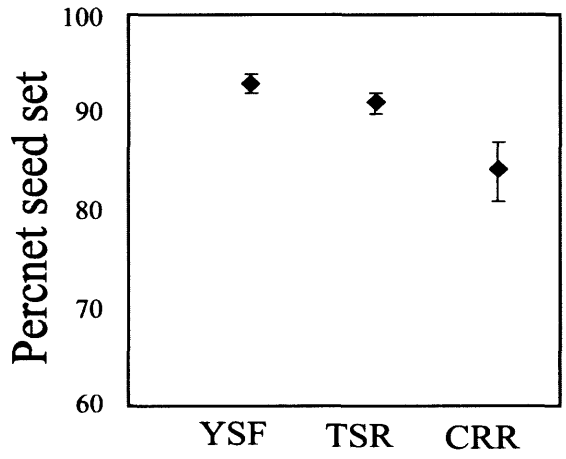


Figure 4.12. Results of the open-pollinated seed set of the homostylous *Nymphoides geminata* from the Yooroonah State Forest (YSF), the Glen Innes (TSR), and the Crisps Road (CRR) populations. In all three populations, the proportions of open-pollinated ovules forming to mature seeds were high.

4.4.6 Floral visitor observations

During a total of 40 h observation in November-December 2006, very few flower visitors were recorded visiting the 816 *N. geminata* flowers that were observed. In the TSR population, only one flower visitor was observed to contact the stigma and the anthers, and considered a potential pollinator (Figure 4.2). Most flying insects did not forge on the flowers. No insects were observed visiting the flowers in the YSF and CRR populations during this time.

4.5 Discussion

The floral morphology of *Nymphoides geminata* suggests that this species is a homostyle with the stigma and the anthers positioned at the same level within a flower. Plants have small flowers, and are self-compatible and capable of self-pollination without the service of pollinators. *Nymphoides geminata* also tolerates ecologically marginal habitats, e.g. roadsides, where pollinator activity appears to be very low. The homostylous condition should provide an opportunity for reproductive assurance in environments with unreliable pollinators.

4.5.1 Floral adaptation to auto-fertility and breakdown of self-incompatibility

This study shows that flower size and herkogamy are the two most distinctive floral traits that segregate homostyly and distyly (Table 4.2 and Figure 4.7), as reported in other studies (Ornduff 1969; Ganders 1979; Barrett and Shore 1987; Kohn *et al.* 1996; Schoen *et al.* 1997; Li and Johnston 2001). The homostylous flowers of *Nymphoides geminata* are smaller, with over 80% shorter stigma–anther separations, than the distylous flowers (Figure 4.8). The reduced herkogamy should facilitate autonomous self-pollination in the homostylous flowers because mature pollen has been brought into close contact with the stigma. Modifications of floral traits that allow self-pollination in the absence of pollinators will only be effective if a breakdown of self-incompatibility (discussed below) allows the self-pollen to fertilise the ovules. The high level of self-compatibility in the homostylous *N. geminata* contrasts with the low level of self-compatibility in the distylous *N. montana*. In Chapter 2, it was reported that the occurrence of herkogamy and self-incompatibility in the distylous flowers reduced seed set (< 7%) following autonomous and

hand-pollinated self-pollination. By contrast, when the homostylous flowers were left untouched or manually self-pollinated in a pollinator-free glasshouse, over 70% of the ovules developed into mature seeds. The ability to self-pollinate autonomously, however, differed between the two study populations (Figure 4.11); this could be associated with the observed floral variations (Table 4.1). In the homostylous flowers, anthers are positioned at the throat of the corolla tube (Figure 4.6). The physical contact between the mature pollen and stigma may, therefore, decrease in the relatively wider flowers of the TSR population than the flowers of the YSF population, leading to the less autonomous self-pollination and fertilisation.

The most well-known form of a breakdown of self-incompatibility in heterostylous plants is when homostyly evolves via recombination within the distyly *S* locus (Piper *et al.* 1986; Ornduff 1988; Kelso 1992; Lewis and Jones 1992; Washitani *et al.* 1994; Richards 1997; Schoen *et al.* 1997; Naiki and Nagamasu 2004; Shore *et al.* 2006). The *S* locus is a supergene consisting of at least three (and possibly six) tightly linked genes which control style length (*G/g*), anther height (*A/a*), pollen size (*P/p*), as well as stigmatic surface (*S/s*), pollen incompatibility (*Ip/ip*) and stylar incompatibility (*Is/is*) of distyly (Lewis and Jones 1992). In the present study, the homostylous flowers possessed similar pollen and stigma papillae characteristics (size and morphology) to those of the distylous S-morphs and L-morphs, respectively (Figure 4.9 and Figure 4.10). It is possible that *N. geminata* is an example of recombinant homostyly, combining the pollen compatibility features of the S-morphs with the stylar compatibility features of the L-morphs. Reciprocal crosses are required to explore compatibility relationships between the distylous morphs and the homostylous plants (Barrett and Shore 1987; Tamari *et al.* 2001).

A second way in which homostyly can evolve is when the action of modifier genes change morphological features of the heterostylous syndrome (Shore and Barrett 1986; Ornduff 1988; Barrett *et al.* 1989). These changes are also accompanied by a relaxation, or a complete loss of, the incompatibility systems. In Chapter 2, it was reported that in one of the distylous populations (TL), the close proximity of the stigma and the anthers of the S-morphs was associated with the partial self- and intramorph incompatibility. However, the equilibrium morph ratios within the distylous populations indicated disassortative mating maintained the populations despite the presence of the modified S-morphs (Chapter 2). Extensive surveys of the morph ratio, floral biology and mating system in other distylous

populations are now required to assess the breakdown of distyly in *Nymphoides* (Barrett *et al.* 1989).

4.5.2 Other floral traits adapted to autonomous self-pollination and fertilisation

Compared to the distylous flowers, the homostylous flowers have a lower pollen:ovule ratio (mean: 110.8 *vs.* 608.9) and a smaller corolla diameter (Figure 4.8). Homostylous flowers are even paler in colour relative to the distylous flowers. This suggests that traits that are no longer needed in the autonomous self-fertilisation system are reduced to limit costly expenditure on pollinator attraction and reward (Ornduff 1969). The lower pollen:ovule ratio in the homostylous flowers also indicates efficient pollination with the least amount of pollen required to fertilise the available ovules, as suggested by Cruden (1977) and Cruden and Jensen (1979). Alternatively, the autonomous flowers of the homostyle may allocate less resources to pollen production because the plants do not need to compete for pollen donation or find suitable mates for ovule fertilisation (Charnov 1987; Barrett *et al.* 1996). Redirection of the within-flower resources to the production of extra ovules and seeds (Figure 4.8), however, may increase the contribution of self-fertilising plant genes to the next generation (Lloyd 1979; Ramsey 1993).

4.5.3 Pollinator scarcity

Observations of insect visitors to the flowers of homostylous *Nymphoides geminata* were made during one flowering season, and very few, if any, pollinators were recorded. By contrast, flowers of the distylous *N. montana* were visited by native bees, introduced honeybees, flies and butterflies (Chapter 2). Although there were no detailed observation of floral visitors to the flowers of the distylous species in any populations, the high levels of open-pollinated fruit and seed set in the self-incompatible floral morphs indicate abundant pollinator activity (Chapter 2: section 2.4.8). The ability of homostylous plants to reproduce successfully during pollinator scarcity should provide reproductive assurance in their natural habitats. Autonomous self-pollination and/or fertilisation are known generally as an adaptation to insufficient pollinator service (Baker 1955; Ornduff 1972; Barrett 1985; Fausto *et al.* 2001; Carlson *et al.* 2007; Vaughton *et al.* 2007). However, a strong implication of pollinator scarcity in the present study cannot be confirmed with one observation period (see the following discussion) and warrants intensive observations of

pollinator visitation in several flowering seasons (Ramsey and Vaughton 1996; Kalisz and Vogler 2003).

Although the high proportion of open-pollinated fruit and seed set during pollinator scarcity indicates the ability of the homostylous plants to self-pollinate and/or self-fertilise autonomously, this may not indicate its importance or frequency in the natural populations. That is because, despite an apparent shortage of pollinator visitation, open-pollinated fruits produced more seeds than autonomous fruits grown under glasshouse conditions (Figure 4.11 and Figure 4.12). Pollinator-mediated self-pollination may have provided additional pollen for full seed set in addition to autonomous self-pollination in the natural populations, which often occurs upon pollinator's entry to small flowers with reduced herkogamy by brushing directly pollen onto the stigma (Dole 1992; Sun *et al.* 2005). Direct experimental tests of whether autonomous self-pollination increases seed set in the natural populations are required to assess the reproductive assurance benefit of autonomous self-fertilisation in nature (Eckert and Schaefer 1998; Kalisz and Vogler 2003; Sun *et al.* 2005; Vaughton *et al.* 2007). For example, in *Collinsia verna*, Kalisz and Vogler (2003) reported emasculated open-pollinated flowers produced fewer fruits than intact open-pollinated flowers, indicating autonomous self-fertilisation provided reproductive assurance in this species.

4.5.4 Polyploidy and no changes in the breeding system

It is generally accepted that switches to homostyly is associated with higher levels of polyploidy (Kelso 1992; Schoen *et al.* 1997; Tamari *et al.* 2001; Naiki and Nagamasu 2004; Guggisberg *et al.* 2006; Nakamura *et al.* 2007). This is because polyploidy could reduce the negative effects of inbreeding by having extra gene copies (Lande and Schemske 1985; Hedrick 1987; Barrett 1989b; Kelso 1992; but see Mable 2004; Barringer 2007). Also, self-compatible polyploids have advantages over self-incompatible diploids when colonising temporary or marginal habitats (Stebbins 1985; Petit and Thompson 1999). In *Nymphoides*, however, the study of chromosome number revealed that the evolution of homostyly was not associated with a change in ploidy level. Polyploidy was recorded in both homostylous (6*x*) and distylous species (4*x* and 6*x*) of the genus. Polyploid distyly has also been reported in other distylous *Nymphoides,* e.g. *N. indicia* and *N. peltata* (Ornduff 1970a), in the sister taxa *Villarsia*, e.g. *V. exaltata* and *V. capensis* (Ornduff 1974), and in unrelated taxa, e.g. *Turnera* and *Piriqueta* (Shore *et al.* 2006).

Since, both distylous and homostylous species are polyploids, it would be interesting to see if the polyploidy condition was derived from allopolyploidy which is caused by hybridisation between two (or more) genomes or autopolyploidy which is derived from conspecific parents (Guggisberg *et al.* 2006; Shore *et al.* 2006). Guggisberg *et al.* (2006) suggested that the higher rates of recombination that occur in hybrids, as in allopolyploids, would favour the disruption of the heterostyly supergene and origin of homostyly. So, the frequent association between homostyly and polyploidy could be primarily due to hybridisation rather than polyploidisation (Kelso 1992; Guggisberg *et al.* 2006; Mast and Conti 2006). Examination of meiosis and the use of codominant genetic markers could reveal whether there is an association between autopolyploidy and distyly *vs.* allopolyploidy and homostyly.

4.5.5 Wider distribution and higher colonisation potential

Habitat descriptions on herbarium (NE) specimen sheets of homostylous *Nymphoides geminata* and distylous *N. montana* and personal observations indicate that the habitats occupied by these species are different. The homostylous species occupies a diversity of habitats ranging from large water bodies to seasonal ponds and roadside depression (see for example Figure 4.2 and Figure 4.3), whereas the distylous species mostly appears in permanent water-bodies. The homostylous species also has a wider distribution than its congener distylous *N. montana* (Figure 4.1). Because the autonomous plants of homostylous *N. geminata* are independent of mate or pollinator to reproduce sexually, a single individual has the capacity to invade a new environment and found colonies (Baker 1955). By contrast, the distylous self-incompatible individuals which depend on pollinators for successful reproduction, inhabit a more stable habitat, such as a permanent water-body (Chapter 2). The origin of homostyly in heterostylous taxa is usually associated with the colonisation of ecologically, e.g. *Amsinckia* (Schoen *et al.* 1997) or geographically marginal areas, e.g. *Turnera ulmifolia* complex (Barrett and Shore 1987), *Eichhornia* (Barrett 1988a) and *Primula* (Kelso 1992; Guggisberg *et al.* 2006). In such situations, where opportunities for cross-fertilisation are limited by mate and/or pollinator availability, selection for reproductive assurance may favour mutations that increase self-fertilisation (Baker 1955; Barrett and Shore 1987; Barrett *et al.* 1989; Kelso 1992; Guggisberg *et al.* 2006).

4.6 Conclusions

As it was argued in the introduction of this chapter, the most frequent modification of distyly involves the evolutionary breakdown of distyly in the direction of increased self-fertilisation by the formation of homostyly. Alternatively, homostyly can be an ancestral condition for distyly. Charlesworth and Charlesworth (1979b) were the first to suggest this scenario; they argued the close proximity of stigmas and anthers within a flower increased the likelihood of receiving self pollen, and hence, diallelic incompatibility evolved first to reduce the detrimental effects of inbreeding which then followed by the evolution of reciprocal herkogamy. In Menyanthaceae, Ornduff (1988) proposed homostylous *Villarsia albiflora* could represent a situation in which distyly elsewhere in the genus has been derived from this type of monomorphism. The weight of evidence in this chapter, however, favours the most accepted hypothesis for the evolution of homostyly through a breakdown of distyly; these include the ability of the homostylous *Nymphoides geminata* to self-pollinate and self-fertilise autonomously which appears to be associated with its capacity for long-distance dispersal and persistence in temporary habitats with an unreliable water regime, e.g. roadsides and dry heath lands. In such ecological situations where pollinators are scarce, the homostyles may have been favoured because of their ability to produce seed in the absence of pollinators. On the other hand, if *N. geminata* represents an ancestral condition of distyly in *Nymphoides*, distyly has gone extinct in such ecologically marginal environments. Clearly, phylogeny of the genus would be required to resolve this issue.

If the self-compatible homostyly evolved from a self-incompatible distyly in *Nymphoides*, as it appears to be a general evolutionary pathway in other heterostylous taxa, then this transformation may have involved evolution of several characters. First, fertilisation by self-pollen became possible with genetic modifications to the incompatibility systems of distyly. Second, morphological traits that promote self-pollen deposition evolved concomitantly, e.g. reduced stigma–anther separation. Finally, selection favoured reduction in the floral traits no longer required for pollinator attraction and reward, e.g. smaller corolla size and lower pollen:ovule ratio. Taken together, these results suggest that the homostylous condition should provide an opportunity for reproductive

assurance in unfavourable pollination environments. Reproductive success of emasculated open-pollinated flowers *vs.* control open-pollinated flowers needs to be compared to determine the reproductive assurance benefit of autonomous self-fertilisation in nature (Eckert and Schaefer 1998; Kalisz and Vogler 2003; Sun *et al.* 2005; Vaughton *et al.* 2007).