Chapter 3 Loss of sex in a clonal distylous species

3.1 Introduction

In animal-pollinated clonal species, extensive clonal propagation can interfere with efficient pollen transfer and may have negative consequences on sexual reproduction (Handel 1985; Wang *et al.* 2005). With large clones, individual flowers are more likely to be surrounded by flowers of the same ramet or different ramets of the same genet resulting in more geitonogamous self-pollinations (Eckert and Barrett 1994a; Kennington and James 1997; Eckert 2000; Reusch 2001; Jacquemyn and Honnay 2007). This can result in low seed production and reduced fitness through increased self-fertilisation and expression of inbreeding depression in self-compatible species, (Handel 1985; Harder and Barrett 1995; Eckert 2000; Reusch 2001). However, the consequences of extensive clonal reproduction in self-incompatible species should be more pronounced. In large clones, self-incompatible pollen is typically transferred among ramets of the same genet and seed production may, therefore, be reduced (Harder and Barrett 1996). In such species, successful reproduction is limited to pollen transfer between different genotypes, i.e. genetically different individuals (Worthen and Stiles 1988; Wilcock and Jennings 1999; Charpentier 2002; Honnay *et al.* 2006; Jacquemyn and Honnay 2007).

Most heterostylous aquatic species have a great capacity for clonal reproduction through some form of vegetative propagation as well as sexual reproduction through seed production (Eckert and Barrett 1995; Dorken and Eckert 2001; Wang *et al.* 2005). The balance between sexual and clonal reproduction can vary among populations where populations exhibit a biased morph ratio (Brys *et al.* 2007) or contain only a single floral morph, i.e. monomorphic populations (Dorken and Eckert 2001). Because self-incompatible species require disassortative (intermorph) pollination for successful mating and fertility, both increased distance to the opposite flower type and lack of mating partners can contribute to limited seed production (Wang *et al.* 2005; Brys *et al.* 2007). In self-incompatible distylous *Nymphoides peltata* (Wang *et al.* 2005) and *Hottonia palustris* (Brys *et al.* 2007), for example, individuals from the most abundant morph type were less likely to produce fruits and seeds than were individuals from the rarer morph type. In extremely

unbalanced situations, such as in monomorphic populations, seed is not produced because of the presence of self-incompatibility systems which reduces the fertilisation rate following assortative (self- and intramorph) pollinations. Severely reduced, or an absence of, seed production has been reported in monomorphic populations of several heterostylous taxa, including *Eichhornia crassipes* (Barrett 1992b), *Lythrum salicaria* (Eckert and Barrett 1992), *Decodon verticillatus* (Eckert and Barrett 1992; Eckert *et al.* 1999; Dorken and Eckert 2001) and *Nymphoides peltata* (Uesugi *et al.* 2004; Wang *et al.* 2005). Barrett (1980b; 1980c) reported an exception to this in monomorphic populations of *Eichhornia crassipes* which were highly self-fertile.

In addition to limited numbers of compatible mates causing sexual sterility at the population level, sterile polyploids are also known to reduce seed production at the genet level (Les and Philbrick 1993; Eckert 2002; Lui *et al.* 2005). The former is defined as a transient genetic factor reducing seed production, because reproductive success can be restored by the reintroduction of compatible mating partners (Wilcock and Jennings 1999; Eckert 2002), whereas the later is a permanent genetic factor (Eckert 2002). Limited sexual reproduction can also be due to ecological factors such as, altitudinal variation (Eckert and Barrett 1993), repeated disturbance (Schaal and Leverich 1996), reduced light levels (Kudoh *et al.* 1999), and frequent fire events (Gross and Caddy 2006) impairing pollination, seed development and/or seedling establishment (Eckert and Barrett 1993). Identifying the ecological and/or genetic factors underlying variation in sexual reproduction may be crucial in exploring the evolutionary causes and consequences of the wide reproductive variation in clonal plants (Eckert 2002).

Populations where there is exclusive clonal reproduction over many generations may lose their capacity for sexual reproduction through the accumulation of sterility mutations that disable the many traits involved in sex (Barrett 1980b; Eckert *et al.* 1999; Lui *et al.* 2005; Gross and Caddy 2006). Trait loss can be expected with the relaxation of selection on the traits that no longer increase fitness, i.e. the 'use it or lose it' hypothesis (Fong *et al.* 1995; Eckert 2002; Gross and Caddy 2006). The loss of male (Warburton *et al.* 2000; Sharma 2001; Kimpton *et al.* 2002; Gross and Caddy 2006) and female (Eckert *et al.* 1999; Gross and Caddy 2006) function has been reported in several clonal species that appear to have lost their ability to reproduce sexually. Sexual sterility can also be associated with

degeneration of other floral traits involved in seed production, such as reduced number of flowers (Lui *et al.* 2005) and weak dichogamy (Eckert 2002).

For sterile plants to persist and then increase in a sexual population, they must have some fitness advantages that compensate for their loss of sexual function (Eckert 2002). It has been hypothesised that once sex is lost, evolution of genetic sterility may be facilitated if sterility is associated with enhanced vegetative vigour through resource reallocation or antagonistic pleiotropy (Eckert *et al.* 1999; Eckert 2002). However, a reduction in sexual reproduction along with a greater investment in clonal reproduction is not a general trend. In *Decodon verticillatus*, Eckert *et al.* (1999) reported no obvious changes in survival and vegetative vigour with reduced sexual reproduction, i.e. the neutral mutation hypothesis. In this hypothesis, the loss of sexual reproduction could precipitate through fixation of neutral mutations that cause trait degradation via genetic drift (Eckert 2002; Dorken *et al.* 2004; Gross and Caddy 2006). Sexual sterility can also be associated with a decrease in overall vigour, including both sexual and clonal reproduction, due to the cumulative build up of deleterious mutations in the absence of sex, i.e. the mutational meltdown hypothesis (Lynch *et al.* 1993; Eckert 2002).

In clonal species, the relative importance of sexual *vs.* asexual reproduction may lead to variation in the amount of genetic diversity among populations (Piquot *et al.* 1996; Dorken and Eckert 2001; Eckert 2002). Because most clonal plants are able to reproduce sexually, and because even low levels of sexual recruitment can maintain high levels of within-population genetic diversity, clonal plant populations may be as genetically diverse as sexual populations (Ellstrand and Roose 1987; Hamrick and Godt 1990; Kimpton *et al.* 2002; Rottenberg and Parker 2004). However, in clonal populations where sexual reproduction is totally suppressed, prolonged clonal growth is expected to affect negatively the level of genetic diversity (Godt and Hamrick 1991; Eckert and Barrett 1993; Dorken and Eckert 2001; Rossetto *et al.* 2004). There are two non-mutually exclusive reasons why clonal growth is associated with the low level of within-population genetic diversity: first, clonally produced offspring are genetically identical to each other and to the parent plant, and second, clonal plants often lack the capacity for long-distance dispersal (Eckert 2002; Honnay *et al.* 2006). In sexually polymorphic species, the low level of within-population genetic diversity is believed to cause complete sexual extinction due to a deficiency in

compatible pollen and mating partner (Eckert and Barrett 1993; Charpentier *et al.* 2000; Honnay *et al.* 2006).

The impact of clonal growth on variation in sexual reproduction has been reported in other species of *Nymphoides* (Barrett 1980a; Uesugi *et al.* 2004; Wang *et al.* 2005). In *N. peltata*, Wang *et al.* (2005) found that a low number of mating partners and their increased spatial distributions resulting from extensive clonal growth disrupted the functioning of distyly in monomorphic populations. Ornduff (1966) argued that weak self-incompatibility and an unbalanced morph ratio resulting from clonal growth may favour replacement of distyly by dioecy to provide a more effective outcrossing system in *N. peltata*. However, Barrett (1980a) suggested that clonal reproduction allows persistence of *N. indica* populations where sexual reproduction is limited by self-incompatibility and monomorphy. Transition from sexual reproduction to exclusively clonal, asexual reproduction has also been reported in monomorphic populations of Eurasian *N. peltata* (Ornduff 1966; Uesugi *et al.* 2004), and some populations of North American *N. cordata* and *N. aquatica* and Colombian *N. flaccida* (Ornduff 1970a).

In this study, population surveys of distylous *Nymphoides montana* in the Northern Tablelands of NSW revealed one population to be monomorphic for style length, i.e. the population contained only one floral morph. The absence of the opposite morph (or mating partner) in the population may have consequences on sexual and clonal reproduction, as low fertility is reported to be a common feature of other monomorphic populations (Barrett 1992a; Eckert and Barrett 1993; Eckert *et al.* 1999; Dorken and Eckert 2001; Uesugi *et al.* 2004; Wang *et al.* 2005).

3.2 Aims

This study investigates the causes and consequences of an absence of mating partner on sexual and clonal reproduction in a monomorphic population of distylous *Nymphoides montana*. 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?

3.3 Material and Methods

3.3.1 Study species and sites

Nymphoides montana (Menyanthaceae) is an aquatic clonal distylous plant inhabiting shallow water around the margins of lakes and swamps (Chapter 2). Distylous populations contain two floral morphs that differ in the reciprocal positioning of stigma and anthers. In the short-styled morphs (S-morph), the stigma is positioned below the anthers, whereas in the long-styled morphs (L-morph), the stigma is positioned above the anthers. The flowers are insect-pollinated, with five yellow showy petals, five stamens and 2-fused carpels; the ovary contains approximately 75 ovules. *Nymphoides montana* possesses self- and intramorph incompatibility systems found in most distylous species. The two morphs, however, differ in the extent of their incompatibility systems. The S-morphs show partial self-incompatibility, whereas the L-morphs are highly self-incompatible (Chapter 2). Crosses between plants of the opposite morph types produce approximately a 95–98% seed set (Chapter 2). As with many clonal species, *N. montana* combines sexual reproduction with clonal reproduction via spreading stolons and broken leaves. Each individual plant produces both reproductive shoots and vegetative shoots. The reproductive shoots include reproductive nodes borne on the stolon with pairs of floral buds at each node. The vegetative shoots include vegetative nodes borne on the stolon with newly grown roots and leaves at each node. Clonal reproduction occurs when the aboveground shoots die over winter and the vegetative fragments, which are no longer connected to the parent plant, are transported to favourable sites where they become established.

The Cooney Creek population (CC) of *Nymphoides montana* is situated in the Northern Tablelands of New South Wales, Australia (Figure 3.1; 1000 m elevation, 30° 32′ 06″ S, 151° 50′ 20″ E). During March 2005–2007, c. 600 flowers were scored throughout the whole population. Flowers of this population are much like those of the S-morphs; the anthers are positioned at the mouth of the corolla tube, slightly above the stigma. No flower of the opposite morph (L-morph) was sighted within the population during the three study years, and therefore, the CC population was regarded as a monomorphic population.

This population is separated from three distylous populations of *N. montana*, the Dumaresq Dam (DD) and Thomas Lagoon (TL) and Glencoe (GC) populations, by 26, 27 and 70 km respectively, and is located in the shallow water habitat typical of the distylous populations.

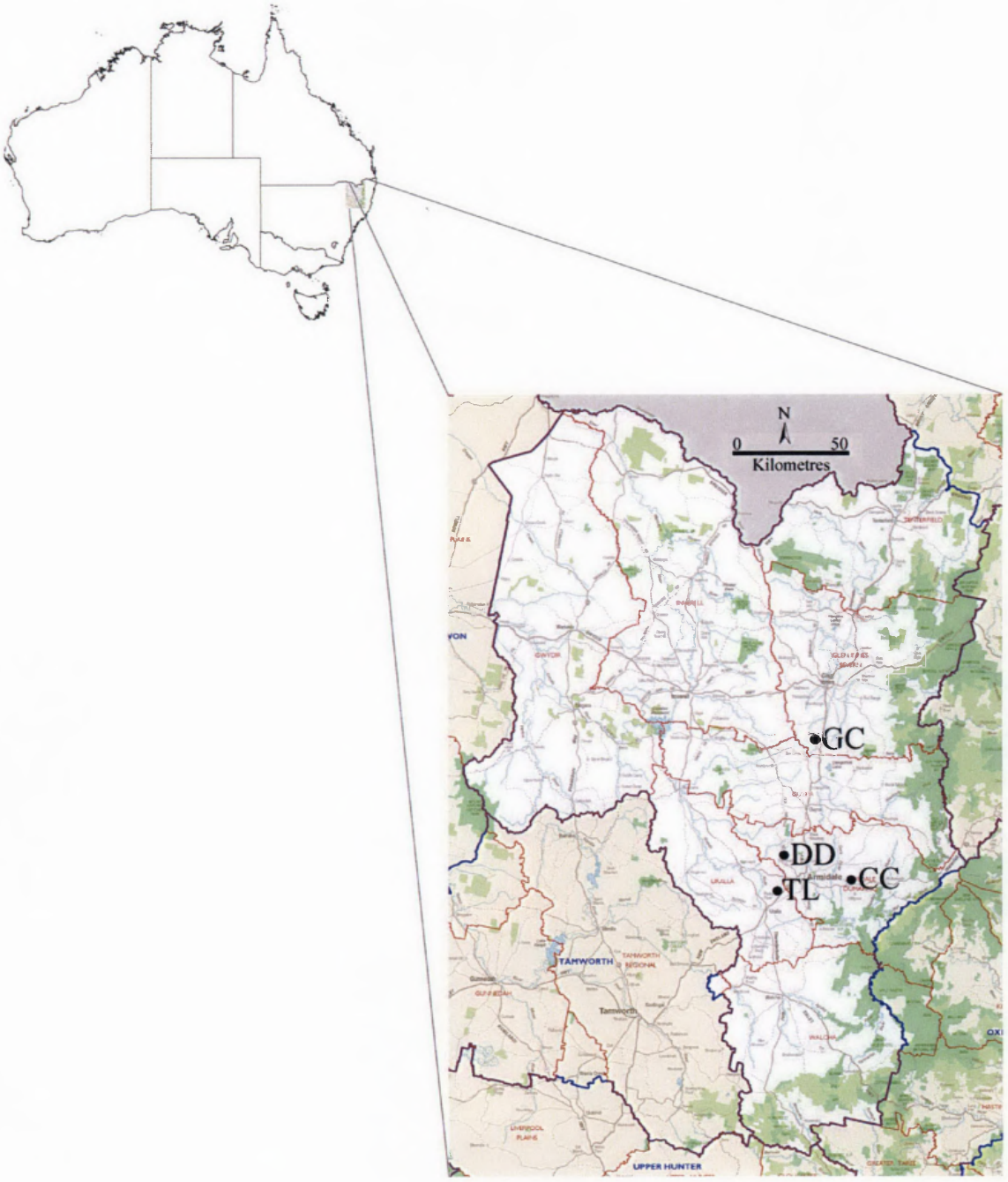

Figure 3.1. Location of the monomorphic population (Cooney Creek, CC) of *Nymphoides montana* in the Northern Tablelands, NSW, Australia. The monomorphic population is located close to the fertile distylous populations (DD, Dumaresq Dam; TL, Thomas Lagoon; GC, Glencoe) of this species. Source: www.aec.gov.au

3.3.2 Floral measurements

This study sought to characterise the floral morphology, specifically the positions of the stigmas and anthers, of the monomorphic plants of *N. montana*. One flower from each of 20 different plants was randomly collected, each at least 3 m apart; this minimised the likelihood of selecting ramets. The samples were preserved in 70% ethanol until measured. Measurements of the floral traits were taken from the base of the superior ovary as described in Chapter 2: section 2.3.5. 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. Principal Component Analysis (PCA) and Discriminant Function Analysis (DFA) were performed on all floral traits from the monomorphic population (CC), the two distylous populations (DD and TL) and another distylous population (Glencoe, GC) from the previous chapter. A covariance matrix was used to extract eigenvalues. Traits that contributed the most in distinguishing the type of the floral morph were identified by the DFAs. In addition, stigma height, anther height and stigma–anther separation were compared between the monomorphs and each of the distylous morphs using one-way ANOVAs. The DD population was used as representative of other distylous populations. Here and elsewhere, 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.

3.3.3 Open pollination and pollinations within and between populations

In February 2005 and February 2006, open-pollinated fruit set was estimated to assess the level of fertility in the natural environment. For each year, 40–45 mature flowers were tagged in at least 3 m intervals, and the number of flowers forming to mature fruits was scored two weeks later.

In March 2006, 16 ramets, including newly grown plants from stolon pieces, were collected; collections were at least 3 m apart. Ramets were transferred to the Botany glasshouse, University of New England (UNE), and planted as described in Chapter 2: section 2.3.11. The levels of sexual fertility as well as self-incompatibility were determined by conducting a series of hand pollination experiments. On each monomorphic plant (M), one flower was allocated to each of the following treatments: within-flower self-pollination, cross-pollination with another plant in the same population (M × M), and cross-pollination

with the S-morph (M × S) and the L-morph (M × L) of the two distylous populations (DD and TL). Controlled cross-pollinations were also performed between the S-morphs and the L-morphs from the two distylous populations, 10 plants per morph per population, and the number of mature fruits containing at least one seed was scored to calculate percent fruit set. In addition, to test male fertility, one flower from each of five plants of each morph of the two distylous populations received pollen from the monomorphic population. Mature fruits were collected and seeds were counted. Percent seed set sired by pollen from the two distylous morphs from the two populations was compared using a random block ANOVA with plant as a random factor and both morph and population as fixed factors. Population was considered as a fixed factor, since the two distylous populations were the only fertile populations in this study.

3.3.4 Chromosome count

To assess whether the variation in sexual fertility may have a basis in ploidy, root tip meristems were obtained from 22 young ramets from the monomorphic population (CC) and 25 ramets from each of the two distylous populations, DD and TL. The ramets were placed in small plastic containers filled with rain water in the Botany glasshouse (UNE). Additional light (Wotan Power Star HQI-R 250 W/NDL, Wotan Lamps Ltd., London) was supplied for a period of 6 h per day to encourage rooting. After 10 days, roots 5-mm-long were harvested and placed in 2 mM 8-hydroxyquinoline for 3–5 h. The material was fixed in absolute ethanol and glacial acetic acid (3:1) for at least 24 h at 4°C. Samples were macerated in 1 mol/L hydrochloric acid at 60° C for 15–20 min, washed and then suspended in 45% acetic acid for 10 min. After hydrolysis, the root tip meristems were transferred to a drop of 0.5% carbon-fuchsin solution on a microscope slide. The solution was prepared following Gounaris (2005). The root tip meristem was squashed under a coverslip and the slide observed using a light microscope at 100x magnification. A total of 220 cells (5 cells/2 root tips/22 ramets) from the CC population and a total of 250 cells (5 cell/2 root tips/25 ramets) from each of the DD and TL populations were obtained at metaphase stage. From each population, ten cells with a good spread of chromosomes were enlarged to 400x magnifications on a Nikon digital sight DS-L1 attached to the microscope and the chromosomes 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.

3.3.5 Male sterility

Male sterility was assessed by examining pollen morphology, the quantity of aborted pollen produced per flower, variation in pollen size, and viability by stainability and germinability experiments.

3.3.5.1 Pollen morphology

Variation in anther and pollen morphology between the monomorphic and distylous populations was assessed using a scanning electron microscope (SEM). Pollen and anthers from three plants from each of the CC and DD populations were collected and mounted onto SEM stubs. The air-dried samples were coated with gold and observed under various magnifications with a JEOL JSM-5800LV (Tokyo, Japan) SEM.

3.3.5.2 Size and proportion of aborted pollen

The sterility of pollen based on pollen size and production was assessed by collecting pollen from two flowers from each of 10 randomly chosen plants from the monomorphic population. Pollen grains were prepared semi-permanently on microscope slides (Beattie 1971). Healthy pollen appeared swollen and triangular, whereas aborted pollen appeared shrivelled with a convoluted surface. The proportion of healthy and aborted pollen was counted for a total of 100 pollen grains per flower. The area (height × base; Chapter 2: Figure 2.6) of 15 healthy pollen and 15 aborted pollen grains from each flower was also measured. Pollen grain sizes among the plants and between the two types of pollen were compared using a random block ANOVA with plant and pollen type as random and fixed factors, respectively.

3.3.5.3 Pollen stainability and germinability

Pollen viability was assessed by two methods: pollen grain germinability (PGG) and pollen grain stainability (PGS). For the PGG experiment, pollen of known age was removed from newly dehisced anthers from each of 10 different flowers from each of the monomorphic population (CC) and the two distylous populations (DD and TL). To find the optimal sucrose percentage and time for pollen germination, five different sucrose germination media (5%, 10%, 15%, 20% and 25%) and two different time intervals for duration of pollen germination (after 2 h and 4 h) were examined on one flower from each of the three populations. Pollen was incubated on a slide at room temperature (22–24° C)

after which a drop of acetocarmine stain (Radford *et al.* 1974) and a coverslip were applied. The pollen grains showed the highest germinability on the 15% sucrose germination medium after 4 h after the commencement of the experiment. Viable pollen was able to grow a tube, *in vitro*, whereas inviable pollen did not germinate. For the rest of the experiment, the percentage of the number of pollen tubes to emerge from a total of 100 pollen grains was determined. Mean (± SE) percentages of germinated and ungerminated pollen are presented here.

For the PGS experiment, pollen from all five anthers from 10 flowers on different plants was collected from each of the three populations. The collected pollen was placed onto a microscope slide and stained with acetocarmine. Pollen was considered viable if stained light-red to red, whereas non-coloured pollen was considered inviable. The percentage of stained pollen was calculated from a total of 100 pollen grains. Mean (± SE) percentages of stained and unstained pollen are presented here.

3.3.6 Clonal growth experiment

The clonal growth experiment was taken to evaluate Eckert's (2002) four hypotheses concerning the loss of sex and its association with clonal growth to see how sterile individuals increase in frequency in a natural population. Considerations included: is the reduction in sexual reproduction associated with a decrease in overall vigour (mutational meltdown hypothesis), or an increase in vegetative growth and clonal propagation (resource reallocation and antagonistic pleiotropy hypotheses)? Has sexual sterility spread through the population because it has no impact on the vegetative performance (neutral mutation hypothesis)?

The vegetative growth and clonal propagation of the monomorphic population CC were compared to those of the fertile distylous populations, DD and TL, by collecting 14 individuals from each of the populations and transferring them to the Botany, UNE glasshouse. The last vegetative nodes on stolons, containing apical buds and a pair of leaves, of the individuals collected were selected and weighed. The young ramets were placed in 10-cm pots with a medium to heavy soil mixture (2 loam:1 sand:1 clay). The experiment was set up in a complete randomized block to account for any variation in the glasshouse, e.g. light, humidity and temperature, which could affect plant growth. A total of 70 plants were randomly assigned to seven blocks. Each block contained two white

trays (70 × 50 × 10 cm). In each tray, one plant from the CC population and one plant from each of the distylous morphs from each of the DD and TL populations were positioned randomly. The experiment commenced in February 2006 and lasted for five months during the cool seasons of the year.

The total number of leaves, flowers and vegetative nodes borne on stolons were counted every week for one flowering season. The total plant dry mass allocated to vegetative growth (leaves and roots), sexual reproduction (flowers) and clonal reproduction (stolons) was also calculated. Flowers were harvested throughout the season and placed in an oven at 80°C to dry. At the end of the flowering season, just as the above-ground structures began to senesce, all ramets were harvested and leaves, roots and stolons were placed separately in paper bags, dried to a constant weight at 80°C for at least 72 h and weighed.

An analysis of covariance (ANCOVA) was used to detect differences between the two sexual systems, asexual *vs.* sexual, in the number of leaves, number of vegetative nodes, number of flowers and total plant dry mass, while controlling for variation in the fresh mass (primary weight) character. An ANCOVA based on type III sum of squares was conducted with: fresh mass as a covariate, block, sexual system, population nested within sexual system, and morph nested within sexual system and population. Block was considered as a random factor and population as a fixed factor.

3.3.7 Sampling, DNA isolation and ISSR analysis

During March and November 2007, the monomorphic CC population and the two distylous populations, DD and TL, were sampled to determine the extent of clonality in each study populations using inter-simple sequence repeats (ISSR). In various ISSR studies the number of sampled individuals has been reported to vary between 4–20 (Ye *et al.* 2004), 14–25 (Li *et al.* 2006), 15–30 (Ge *et al.* 2003) and 15–31 (Meloni *et al.* 2006). In March, 15–23 individual ramets were sampled from each study population. In November, 24 more samples were collected from different part of the CC population to increase the sample size. Samples were collected along transects at intervals of at least 5 m; this minimised the likelihood of selecting ramets. Because the TL population was dry in March 2007, the collected samples were from juvenile ramets growing on the surface of the soil, whereas in the DD and CC populations the samples were collected from floating shoots.

Young leaves and petioles were taken for each sample, placed in plastic bags and stored at −20°C until the DNA was extracted.

Fresh plant tissue was used to extract total genomic DNA using the Qiagen DNeasy kit and CTAB (cetyltrimethylammonium bromide) method. In the latter, 100–150 mg of material was crushed in a 2 mL microcentrifuge tube using a tissue crusher for 15 min. One millilitre of lysis buffer (Doyle and Doyle 1987; Table 3.1) (Table 3.1) was added to the tube and the mixture was incubated at 65°C for 1 h, then centrifuged at 12 000 rpm for 5 min. The resulting supernatant was transferred to a new centrifuge tube and washed twice with chloroform:isoamyl alcohol (24:1 v/v). The aqueous phase was transferred to a new tube to which 300 µL of cold isopropanol was added. The mixture was incubated in the −20°C freezer for 15 min or more for DNA precipitation. The DNA was pelleted by centrifuging at 14 000 rpm for 15 min and then washed twice with 70% and 90% ethanol. The DNA pellet was air-dried for 1 h and resuspended in 200 µL of TE buffer.

Table 3.1. Components required for lysis buffer for 30 samples

Component	Amount
CTAB	30 mL
PVP	1.2 g
β-mercaptoethanol	150 µL
DIECA	0.03 g
Ascorbic Acid	0.03 g

(Doyle and Doyle 1987)

Forty five ISSR primers, 15–23 nucleotides in length (based on the UBC ISSR primer set No. 9, Biotechnology Laboratory, University of British Columbia), were obtained for screening polymorphisms. Nine primers (Table 3.2) that produced reproducible bands were selected for amplification for all of the DNA samples. Thermal Gradient PCR (Corbett Research, Australia) was used to uncover optimal annealing temperatures within a range of ± 1°C. The best temperatures for obtaining products of outstanding quality were in the range of 41°C to 49°C (Table 3.2).

PCR reactions contained 100 ng of genomic DNA, 0.2 µM of primer, 1 unit *Taq* polymerase, 1x PCR buffer, 4mM magnesium chloride, 400 µM dNTPs. The amplification was performed in a PC-960C Thermal Cycler (Corbett). Initial denaturation was for 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 46°C, 1.5 min at 72°C, and a final extension of 10 min at 72°C. Nine µL of each PCR reaction was resolved using 1% agarose gel electrophoresis in 1x TBE buffer. HypperLadder I (Bioline) molecular weight standard was added. The electrophoresis was run by 200 V and 90 mA for about an hour. Bands of DNA in the gel were stained with ethidium bromide. Digital images of the gel taken under UV transilluminator were used to score the presence or absence of bands. Duplicate PCR reactions were run for all primer per specimen combinations, and a blank control included in each set of PCR reactions and gel electrophoresis. Only bands that were clear and reproducible were included in the analysis.

Table 3.2. Sequences of nine ISSR primers and optimum annealing temperature for each ISSR primer used in this study. The number of polymorphic (*P*) bands obtained from 83 individuals of *Nymphoides montana* sampled across the three populations are also indicated.

ISSR primer	Sequence	Temperature (°C)	No. of bands (*P*)
808	AGAGAGAGAGAGAGAGC	41	3(3)
818	CACACACACACACACAG	47	6(6)
826	ACACACACACACACACC	49	7(6)
827	ACACACACACACACACG	44	4(3)
834	AGAGAGAGAGAGAGAGYT	41	5(5)
841	GAGAGAGAGAGAGAGAYC	41	6(6)
855	ACACACACACACACACYT	41	4(4)
880	GGAGAGGAGAGGAGA	49	10(6)
900	ACTTCCCCACAGGTTAACACA	44	8(5)

All the amplified ISSR bands were treated as dominant genetic markers. ISSR bands were scored as 1 (present) or 0 (absent) and these binary data were used to assemble a rectangular matrix. Assuming Hardy-Weinberg equilibrium, genetic diversity was analysed using POPGENE version 1.31 (Yeh *et al.* 1999) to assess the proportion of polymorphic loci (*P*%), number of alleles (*n*a), number of effective alleles (*n*e), Shannon's diversity index (*I*), Nei's (1987) total genetic diversity (H_T), diversity within populations (H_S), diversity among populations ($D_{ST} = H_T - H_S$), and the proportion of among population differentiation ($G_{ST} = H_T - H_S/ H_T$). G_{ST} is equivalent to Wright's (1965) between population differentiation coefficient (F_{ST}). The Nei's estimates of genetic diversity vary from 0 to 1.

Analysis of molecular variance was conducted using GenAlEx version 6 (Peakall and Smouse 2006) to calculate variance components and statistical significance level for variation among and within populations. Pair-wise genetic distance (PhiPT) values among the three populations were obtained from the AMOVA. The test of significance for the AMOVA was carried out on 999 permutations of the data. Nei's (1972) unbiased genetic distance (*D*) between the three populations was estimated using POPGENE version 1.31 (Yeh *et al.* 1999). *D* varies from 0 (total similarity) to infinity (total dissimilarity). Closely related populations tend to show *D* values < 0.1, whereas divergent populations tend to have *D* values > 0.2 (Bader 1998). A dendrogram for the three populations was also created using UPGMA method modified from NEIGHBOUR procedure of PHYLIP version 3.5.

Clonal or genotypic diversity was estimated by calculating the following parameters in the monomorphic and each of the distylous populations: 1) The proportion of distinguishable genotypes (Ellstrand and Roose 1987; Eckert and Barrett 1993) was measured as G/N, where G is the number of genotypes detected and N is the total number of individuals (ramets) sampled. 2) Simpson's diversity index (D) modified for finite sample size by Pielou (1969) was calculated as:

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

where n_i is the number of ramets of ith genotype and N is the sample size. The index D varies from 1 in a population where every individual sampled is a different genotype to 0 in a population composed of a single clone. 3) Genotypic evenness measure of Fager (1972) was calculated as:

$$E = (D - D_{min})/(D_{max} - D_{min}),$$

where

$$D_{min} = (G - 1)(2N - G)/N(N - 1)$$

and

$$D_{max} = (G - 1)N/G(N - 1).$$

The index E varies from 1 in a population with a completely uniform genotype frequency to 0 for a population in which all individuals possess the same genotype. A clustering analysis of all genotypes was done using a neighbour-joining method in PAUP ver. 4.0b2a (Swofford 1999). The bootstrap procedure, with 2000 random samplings, was also employed to confirm the topology of the neighbour-joining dendrogram based on ISSR data among 83 individuals.

3.4 Results

3.4.1 Floral measurements

Flowers of the monomorphic population are more similar to the distylous S-morph in which the stigma is located below the anthers than the distylous L-morph. In the PCA, the monomorphs formed a distinct group that clustered in close proximity to the S-morph

group along the PC1 axis (Figure 3.2). Stigma height and stigma–anther separation had strong loadings on the PC1 suggesting this axis was an index of female character and herkogamy (Table 3.3). Anther height and stamen insertion height had strong loadings on the PC2 suggesting this axis was an index of male traits (Table 3.3). The loadings of the traits of the PCA are given in Table 3.3. The first three principal components explained ≥ 96% of the variation in the traits. In the DFA, cross validation verified this result and 89% of the total variation among the individuals was correctly classified.

Results of one-way ANOVAs comparing the monomorphs and the distylous S-morphs and L-morphs for the male and female floral traits are given in Table 3.4. Stigma height of the monomorphs showed no significant difference to that of the S-morphs but was significantly shorter than that of the L-morphs (Figure 3.3). Anthers of the monomorphs were intermediate in size compared with the anthers of the distylous morphs. Anther height of the monomorphs was significantly shorter than that of the S-morphs and longer than that of the L-morphs (Figure 3.3). Consequently, the monomorphs showed a significantly reduced stigma–anther separation compared with the distylous morphs (Figure 3.3).

Table 3.3. Loading of traits on the first three scores in the Principal Component Analysis. Eigenvalues and percent total variance are given in parentheses and brackets, respectively. Character loadings of an absolute value > 0.50 are given in bold.

Character	PC1 (14.30) [83.9]	PC2 (1.69) [10.0]	PC3 (0.38) [2.2]
Stigma height	**−0.58**	−0.21	−0.15
Stigma width	−0.15	−0.04	−0.10
Stigma length	−0.23	−0.01	**−0.55**
Style length	−0.34	−0.20	0.40
Anther height	0.25	**−0.60**	−0.34
Filament length	0.16	−0.19	−0.01
Anther length	0.05	−0.06	**−0.52**
Stamen insertion height	0.20	**−0.54**	0.17
Stigma–anther separation	**−0.56**	−0.20	0.05
Corolla tube width	0.05	−0.41	0.24

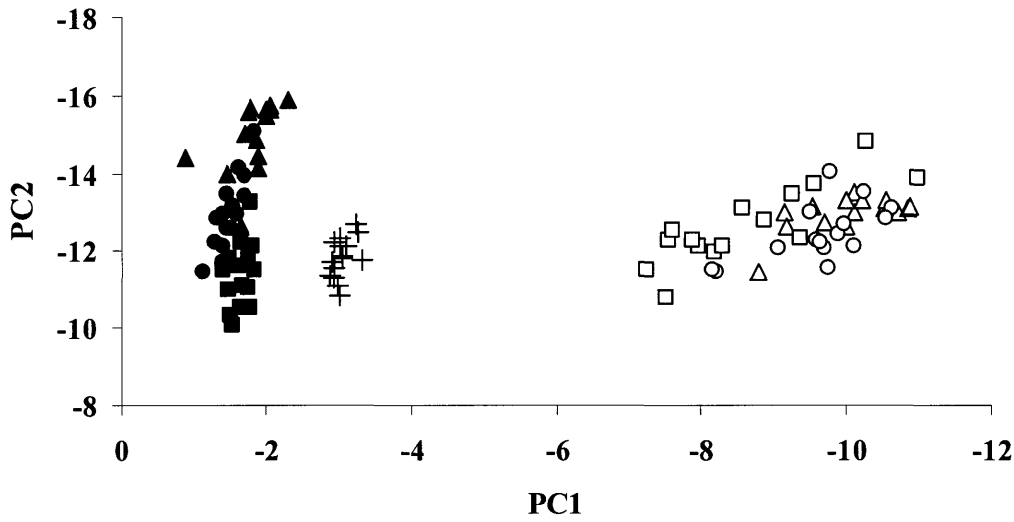

Figure 3.2. Plot of the first and second PCA scores for the distylous S-morphs (left, filled symbols), the monomorphs (middle, crosses) and the distylous L-morphs (right, open symbols) of *Nymphoides montana*. PC1 and PC2 explained 83.9% and 10% of the variation among the individuals, respectively. Symbols referring to the distylous populations are: ▲Δ, Dumaresq Dam (DD); ■□, Thomas Lagoon (TL); ○●, Glencoe (GC).

Table 3.4. Results of one-way ANOVAs for stigma height, anther height and stigma–anther separation of the monomorphs, distylous S-morphs and distylous L-morphs of *Nymphoides montana*. For all parameters, floral traits were significant except for the stigma height of the monomorphs and distylous S-morphs. The Dumaresq Dam (DD) population was representative of other distylous populations. Differences between the two distylous morphs have been previously examined in Chapter 2. Analyses refer to data in Figure 3.3.

		Stigma height			**Anther height**			**Stigma–anther separation**		
Source	df	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*
Monomorph *vs.* S-morph	1	0.09	0.54	0.468	28.71	117.58	<0.001	32.03	159.97	<0.001
Error	28	0.17			0.24			0.20		
Monomorph *vs.* L-morph	1	146.78	1098.13	<0.001	9.26	92.85	<0.001	122.61	739.94	<0.001
Error	28	0.13			0.09			0.16		

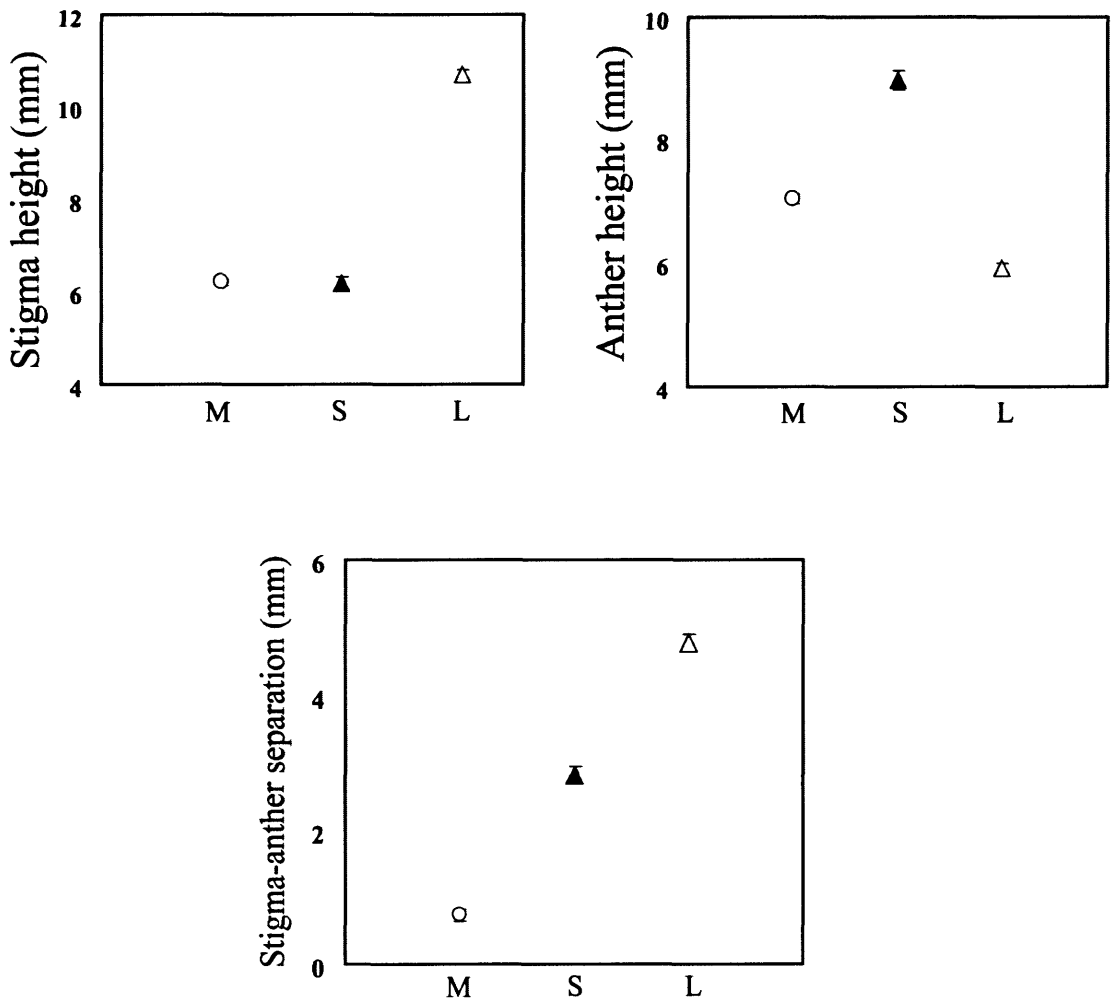

Figure 3.3. Mean (± SE) stigma height, anther height and stigma–anther separation of the monomorphs (M, open circle), distylous S-morphs (S, filled triangle) and distylous L-morphs (L, open triangle) of *Nymphoides montana*. The functional distance between the stigma and anthers of the monomorphic flowers was near zero. The Dumaresq Dam (DD) population was representative of other distylous populations.

3.4.2 Sexual infertility

In the natural environment, no open-pollinated flowers in the monomorphic population set fruits or seeds during the two years of the study. In the glasshouse, from a total of 32 monomorphic flowers that received either self-pollen or cross-pollen from another plant within the same population, no fruits were formed (Figure 3.4). In the between-population pollination treatments, the 64 monomorphic flowers that received fertile pollen from the distylous morphs produced very few seeds (< 4% seed set; Figure 3.4). However, seed production in the monomorphic population improved slightly with the L-morph pollen from the distylous populations (Figure 3.4). The monomorphs had significantly more seed set when they were crossed with the distylous L-morphs compared with the distylous S-morphs ($F_{1,45}$ = 5.12, P = 0.029). The effect of plant was not significant ($F_{15,45}$ = 0.48, P = 0.938). There were no significant differences in percent seed set sired by pollen from the two distylous populations ($F_{1,45}$ = 2.10, P = 0.154). The population × morph interaction was also not significant ($F_{1,45}$ = 0.03, P = 0.863). In addition, in the monomorphic population the pollen donor failed to produce any seeds in either of the two pollen-recipient distylous populations as pollen recipients. With the cross-pollination experiment between the two distylous populations, the control experiment, fruit set for each morph was 100%.

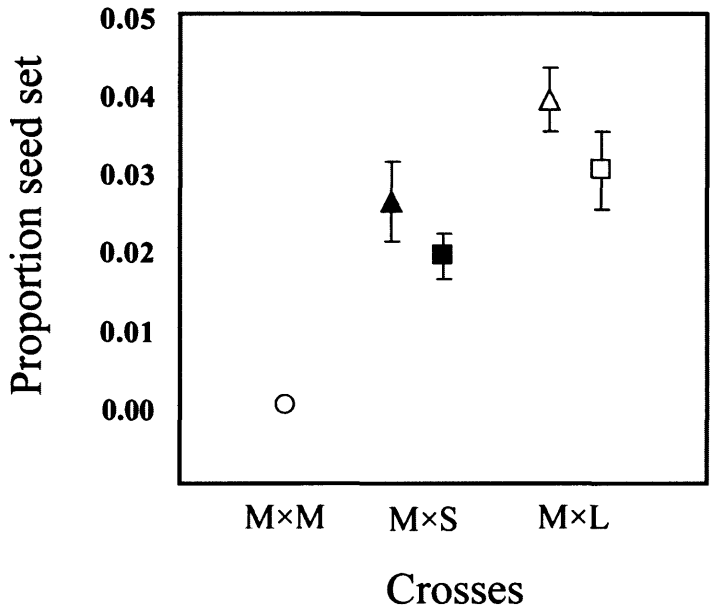

Figure 3.4 Comparison of percentage seed set after within-population cross-pollination (M × M, open circle), between-population cross-pollination with the distylous S-morphs (M × S, filled symbols) and with the distylous L-morphs (M × L, open symbols), involving *Nymphoides montana* from the monomorphic population and the two distylous populations (triangle, DD and square, TL). In the within-population crosses, no seed was set. In the between-population crosses, the monomorphs produced more seeds when crossed with the distylous L-morphs compared with the distylous S-morphs. Means (±SE) of proportion seed set per population have been plotted.

3.4.3 Chromosome count

The basic chromosome number of *Nymphoides* (Menyanthaceae) is $x = 9$ (Ornduff 1970). Somatic chromosome counts in the monomorphic population of *N. montana* showed average counts close to the hexaploid number $6x = 54$ (Figure 3.5; mean ± SD = 52.4 ± 2.9). The distylous populations showed more than one cytotype ($4x$ and $6x$). Tetraploid $4x = 36$ (Figure 3.5; mean ± SD = 34.58 ± 1.88) and hexaploid (mean ± SD = 52.1 ± 2.2) counts were obtained for the distylous TL and DD populations, respectively.

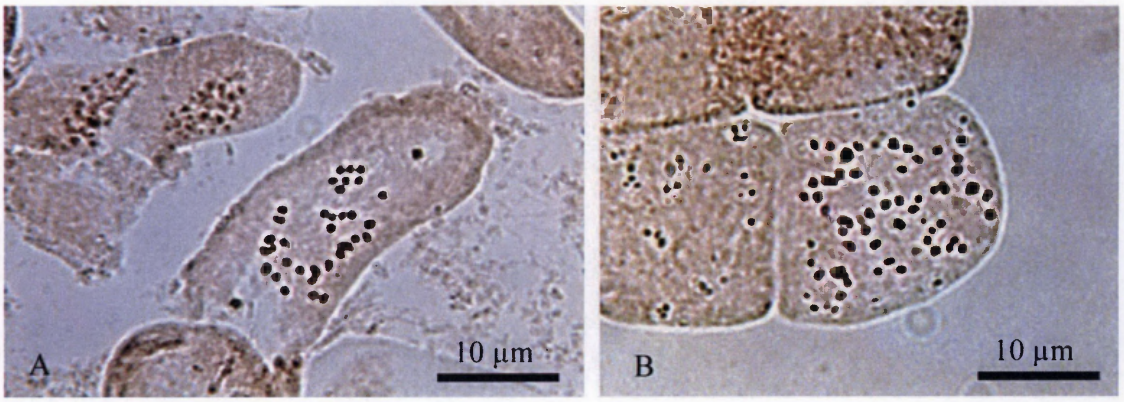

Figure 3.5. Microphotographs of somatic chromosomes of A) the distylous Thomas Lagoon ($4x$ = 36) and B) the monomorphic Cooney Creek ($6x$ = 54) populations of *Nymphoides montana*.

3.4.4 Male sterility

In the monomorphic flowers, most anthers did not dehisce and their wall layers remained intact, although the proportion of the anthers that did not dehisce was not quantified (Figure 3.6 A and B). This is in contrast with the fertile distylous morphs where anthers dehisced longitudinally and released pollen (Figure 3.6 C). The dehisced anthers of the monomorphic flowers showed two different pollen grain shapes; swollen healthy pollen and shrunken aborted pollen with collapsed exine layers (Figure 3.6 D).

On average, 17% of pollen produced in the monomorphic population looked healthy, whereas aborted pollen accounted for 83% of the pollen (Figure 3.7). Healthy pollen grains were significantly larger than the aborted pollen in the monomorphic population (Figure 3.7; $F_{1,9} = 100.36$, $P < 0.001$). The effect of plant was not significant ($F_{9,9} = 0.99$, $P = 0.507$).

To further investigate pollen viability, the proportions of germinated pollen and stained pollen were examined. Approximately 1% of pollen grains germinated and 7% stained in the monomorphic population (Figure 3.8). In contrast, the distylous populations showed high rates of pollen stainability and germinability (Figure 3.8).

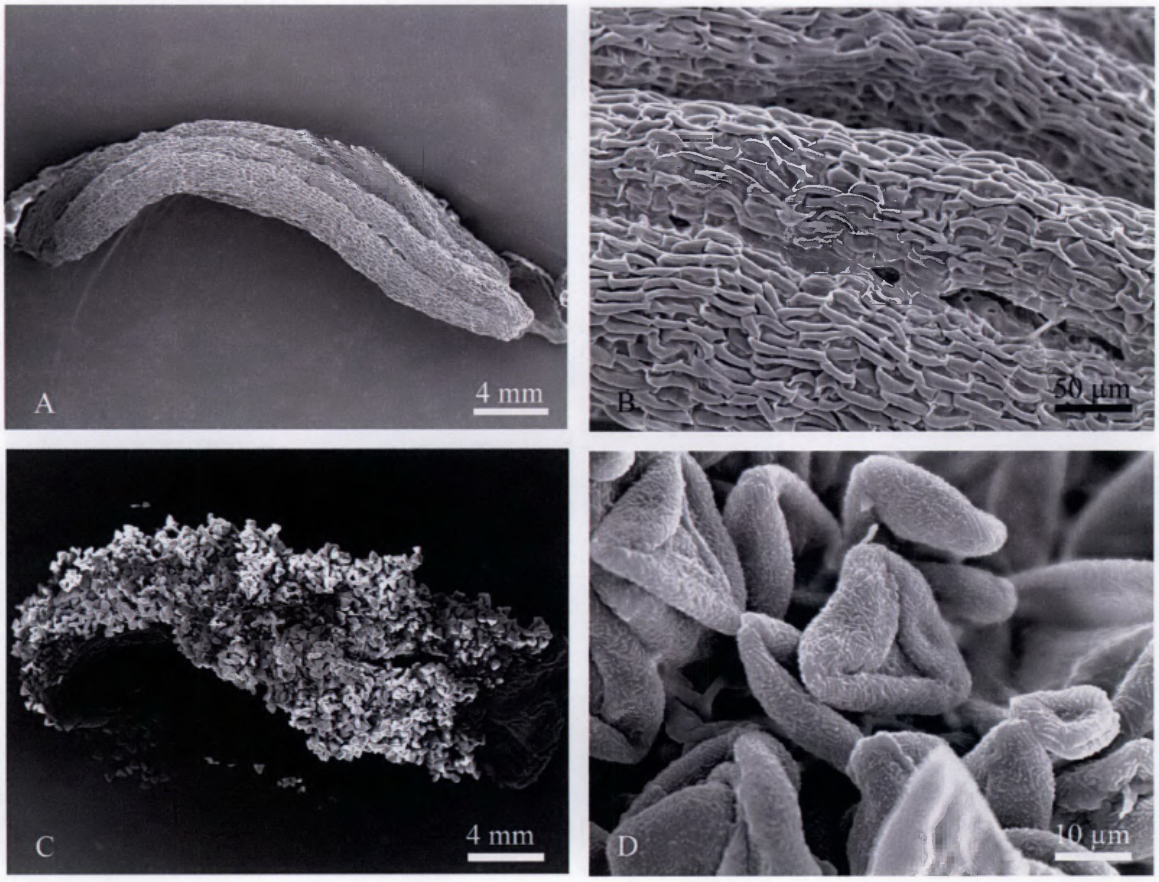

Figure 3.6. SEM micrographs of air-dried anthers and pollen of *Nymphoides montana*, comparing an indehisced anther (A) with intact anther walls (B) from the monomorphic population and a dehisced anther (C) from a distylous population at the time of anthesis. D) illustrates the degenerating pollen grains with collapsed exine from the monomorphic population.

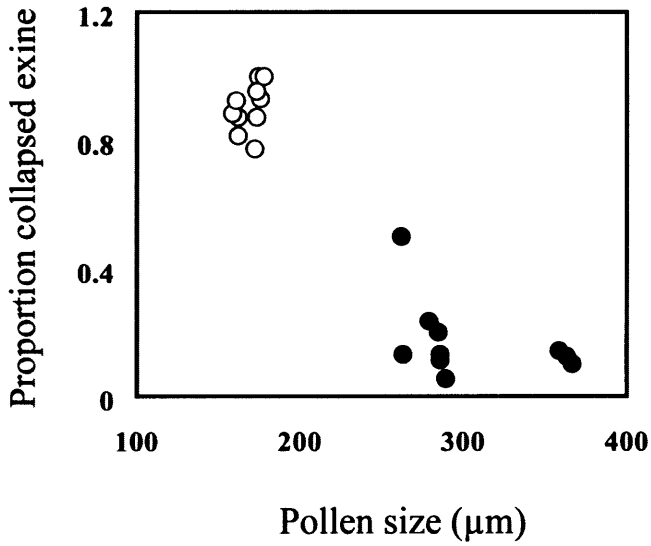

Figure 3.7. Comparison of proportion of pollen exhibiting collapsed exine and pollen size in the monomorphic population of *Nymphoides montana*. Shrivelled aborted pollen (open symbol) was smaller and produced in greater proportion compared with healthy pollen (filled symbol). The above graph is plotted for 10 individual plants.

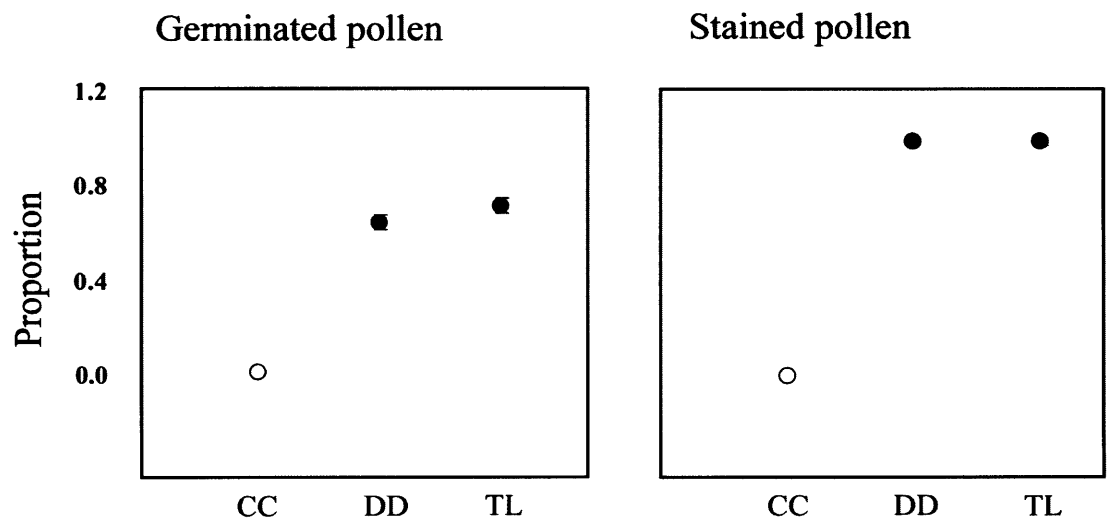

Figure 3.8. Variation in pollen viability between plants from the monomorphic (CC, open circles) and the two distylous (DD and TL, filled circles) populations of *Nymphoides montana*. Near zero percent pollen germinated (left panel) or stained (right panel) in the monomorphic population which is in contrast to the high rate of pollen germinability and stainability of the distylous populations. Means (± SE) of germinated and stained pollen per population have been plotted.

3.4.5 Clonal growth experiment

Results of the ANCOVAs showed that the monomorphic population exhibited higher vegetative performance than the distylous populations. The mean values of the number of leaves, vegetative nodes and flowers, and total plant dry mass of the Monomorphs were significantly increased compared to those of the distylous S-morphs and L-morphs (Table 3.5, Figure 3.9). The effects of fresh mass and block were not significant (Table 3.5). Comparing the two distylous populations, the number of vegetative nodes and flowers did not differ significantly (Table 3.5). The number of leaves and final biomass, however, varied between the distylous populations (Table 3.5). Comparison of the distylous floral morphs revealed significantly greater numbers of leaves for the S-morphs than the L-morphs (Table 3.5). However, there were no significant differences in the number of vegetative nodes, the number of flowers and final biomass between the distylous morphs (Table 3.5).

Table 3.5. Analyses of covariance (ANCOVAs) of the effect of sexual system on the number of leaves, vegetative nodes and flowers and total plant dry mass. All parameters differed significantly between the two sexual systems. Sexual system refers to the fertile distylous populations and the sterile monomorphic population. Morph refers to the monomorph, the distylous S-morph and the distylous L-morph. Fresh mass of plant at the start of the experiment was the covariate.

		No. of Leaves			**No. of Vegetative nodes**			**No. of Flowers**			**Total plant dry mass**		
Source	df	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*	*MS*	*F*	*P*
Fresh mass	1	0.03	2.25	0.140	0.33	2.59	0.114	0.97	3.16	0.081	0.02	1.10	0.299
Block	6	0.01	1.18	0.330	0.15	1.20	0.320	0.31	1.01	0.428	0.03	1.87	0.104
Sexual system	1	1.01	82.43	0.000	5.25	41.28	0.000	9.13	29.80	0.000	0.32	18.17	0.000
Population (sexual system)	1	0.06	4.69	0.035	0.07	0.51	0.477	0.67	2.19	0.145	0.13	7.62	0.008
Morph (sexual system, population)	2	0.08	6.19	0.004	0.28	2.17	0.124	0.52	1.70	0.193	0.01	0.33	0.723
Error	52	0.01			0.13			0.31			0.02		

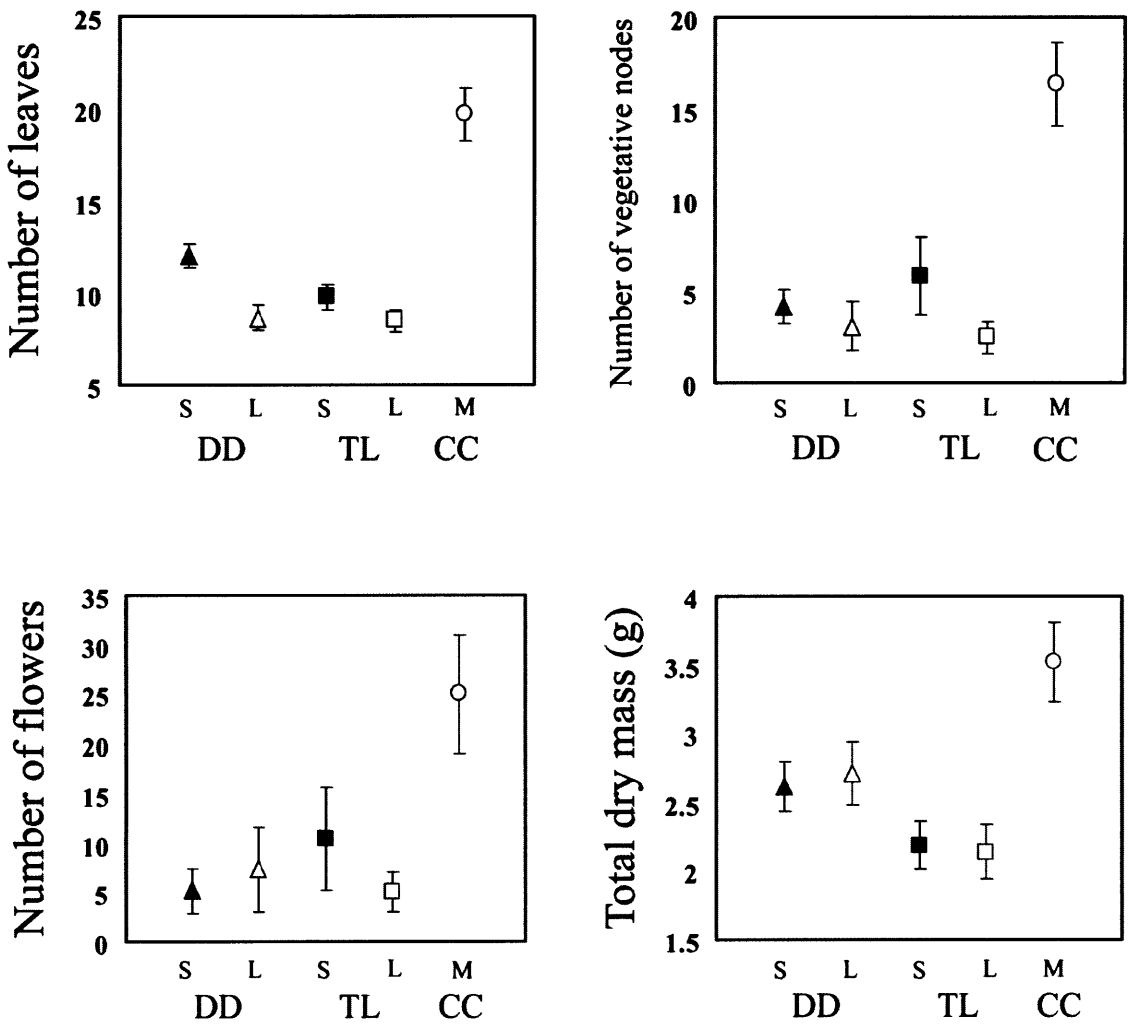

Figure 3.9. Variation in the capacity for the clonal propagation between plants from the distylous populations (triangle: DD and rectangle: TL; black: S-morph and white: L-morph) and the monomorphic population (circle, CC) of *Nymphoides montana* grown in a common glasshouse environment. The monomorphic population exhibited higher vegetative performance and flower production than the distylous populations. Means (± SE) of vegetative and reproductive traits and plant total dry mass per morph per population have been plotted.

3.4.6 Genetic diversity and differentiation

Following an initial screening of 45 primers, nine primers that produced reproducible ISSR bands were selected to amplify DNA samples of all *N. montana* collections. A total of 53 different ISSR bands were scored. Among the 53 loci, 44 (83.01%) were polymorphic among the populations. The number of polymorphic bands for each primer is given in Table 3.2. The population diversity analysis using POPGENE revealed that the distylous populations exhibited a high level of variability (Nei's $H = 0.166–0.255$ and $P\% = 88.68–90.57\%$; Table 3.6 and Figure 3.10), whereas the monomorphic population exhibited no genetic variation (Table 3.6 and Figure 3.11). The average number of observed and effective alleles is given in Table 3.6. At the species level, Shannon's index of diversity (I) was 0.428 and Nei's total genetic diversity (H_T) was 0.294. An average genetic diversity within populations (H_S) was estimated to be 0.141 and among populations (D_{ST}) 0.154. The proportion of genetic variation contributed by differences among populations (G_{ST}) was 0.523, which means that about 50% of the total genetic variation existed within population. This result was further confirmed by molecular variance analysis (AMOVA) within populations (Table 3.7).

AMOVA analysis indicated that most genetic variability resides among populations (57%), whereas a lesser portion of the variance was found among individuals within populations (43%). The PhiPT value for within-population genetic variability was 0.575, which was significant at a probability of 0.001 (Table 3.7). Pair-wise PhiPT values derived from AMOVA showed that the genetic distance between the monomorphic population and each of the distylous populations was higher than the genetic distance between the two distylous populations (Table 3.8). The UPGMA dendrogram of Nei's genetic distance provides a more coherent clustering based on the sexual systems. The two distylous populations grouped together, whereas the monomorphic population formed an isolate (Figure 3.12).

3.4.7 Genotypic (or clonal) diversity

Genotypic structure and the number of clones detected differed between the distylous and monomorphic populations. A total of 35 different clones or genotypes were identified among the 83 ramets analysed. Of these, 18 and 17 putative genotypes were specific to the

distylous DD and TL populations, respectively, whereas only one genotype was detected in the monomorphic population that was unique to the population (Figure 3.13). The number of observed genotypes expressed as a proportion of the sample size (G/N = 0.782–0.809) and the Simpson's genotypic diversity (D = 0.980–0.990) were relatively high in the distylous populations, whereas the monomorphic population showed no diversity (Table 3.6; G/N = 0.026, D = 0.000). Therefore, the monomorphic population, which was spread over an area of m^2, was composed of a single genotype. Genotypes were evenly distributed spatially in the distylous populations (Table 3.6; E = 0.666–0.851). However, since only one genotype dominated the monomorphic population, the E value was 0.

The neighbour-joining dendrogram revealed that the monomorphic population was differentiated into one cluster of similar genotype (Figure 3.13). For each of the distylous populations, however, most individuals were differentiated by their genotype (Figure 3.13).

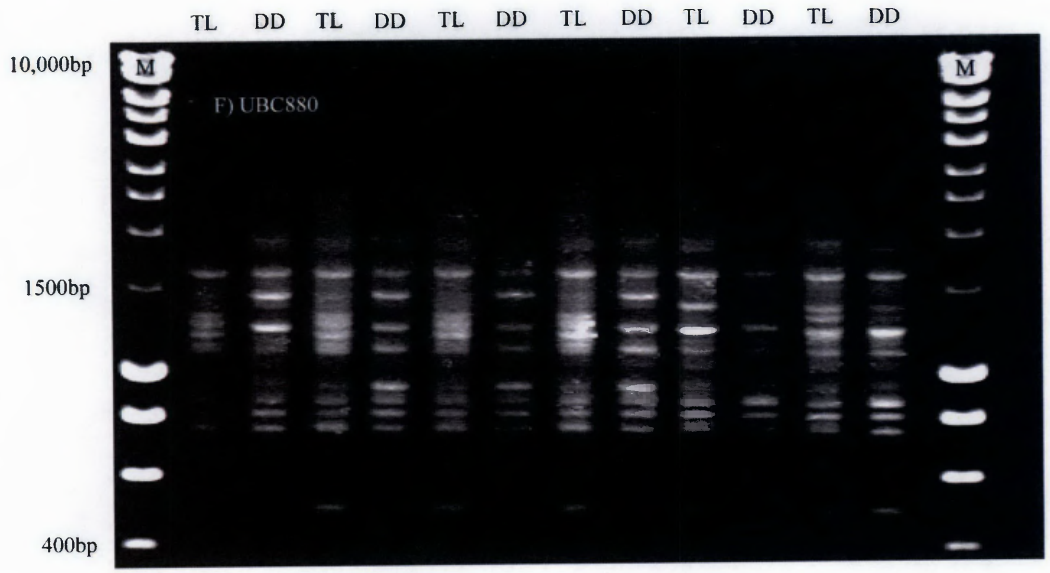

Figure 3.10. Electrophoresis profile of PCR products amplified with primer UBC880 for the distylous populations (DD, Dumaresq Dam; TL, Thomas Lagoon) of *Nymphoides montana* showing polymorphic loci. M: molecular size marker

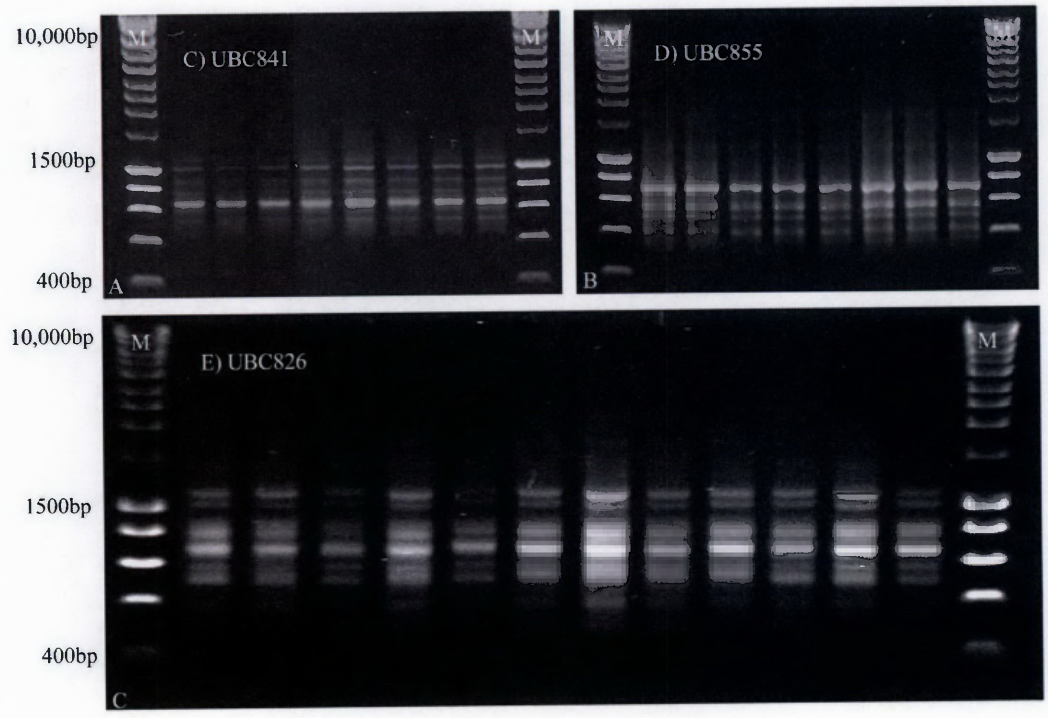

Figure 3.11. Electrophoresis profile of PCR products amplified with primers UBC841, UBC855 and UBC826 for the monomorphic (Cooney Creek; CC) population of *Nymphoides montana*. (A) and (B) illustrate similar patterns between the same eight individuals, although some bands were presented weakly in some individuals due to possible variation in PCR. (C) shows a similar pattern between 12 more individuals from a different part of the population. M: molecular size marker

Table 3.6 Genetic and genotypic (or clonal) diversity in the monomorphic (CC) and the two distylous (DD and TL) populations of *Nymphoides montana*.

Populations	***N***	***na***	***ne***		***H***	***I***	***P%***	***G***	***G/N***	***D***	***E***
Cooney Creek (CC)	39	1.000	1.000		0.000	0.000	0.00	1	0.026	0.000	0.000
Dumaresq Dam (DD)	23	1.886	1.401		0.255	0.399	88.68	18	0.782	0.980	0.851
Thomas Lagoon (TL)	21	1.905	1.219		0.166	0.291	90.57	17	0.809	0.990	0.666
				H_T:	0.294						
				H_S:	0.141						
				D_{ST}:	0.154						
				G_{ST}:	0.523						

N, number of individuals sampled; number of observed alleles, number of effective alleles; *H,* Nei's genetic diversity; H_T, total genetic diversity; H_S, diversity within populations, D_{ST}, diversity among populations; G_{ST}, degree of population differentiation; *I,* Shannon's information index; *P%*, percentage of polymorphic loci; *G,* number of genotypes detected; *G/N*, proportion of distinguishable genotypes; *D,* Simpson's genotypic diversity; and *E,* Fager's genotypic evenness.

Table 3.7. Analysis of molecular variance (AMOVA) based on 53 ISSR markers for the monomorphic population (Cooney Creek, CC) and the two distylous populations (Dumaresq Dam, DD and Thomas Lagoon, TL) of *Nymphoides montana*. Probability values are based on 999 permutations.

Source	df	Sum of squares	Mean squares	Variance component	% of total variance	*P*-value
Among population	2	328.33	164.17	6.03	57%	
Within population	80	357.02	4.46	4.46	43%	0.001
Total	82	685.35	168.63	10.49		

Table 3.8. Pair-wise PhiPT values calculated by AMOVA illustrating differences between the monomorphic population (Cooney Creek, CC) and each of the two distylous populations (Dumaresq Dam, DD and Thomas Lagoon, TL) of *Nymphoides montana*. PhiPT values are given below the diagonal, and the associated *P*-values based on 999 permutations are given above the diagonal.

Populations	DD	TL	CC
DD	—	0.033	0.001
TL	0.063	—	0.001
CC	0.655	0.770	—

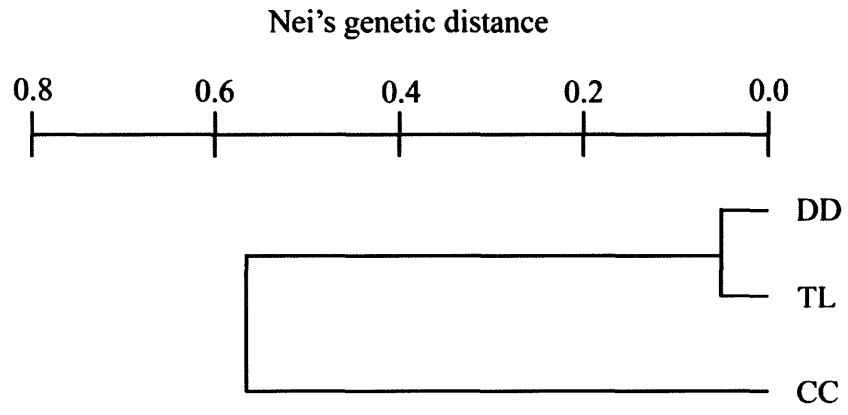

Figure 3.12. UPGMA dendrogram of Nei's genetic distances (*D*) between the monomorphic (Cooney Creek, CC) population and the two distylous (Dumaresq Dam, DD and Thomas Lagoon, TL) populations of *Nymphoides montana*.

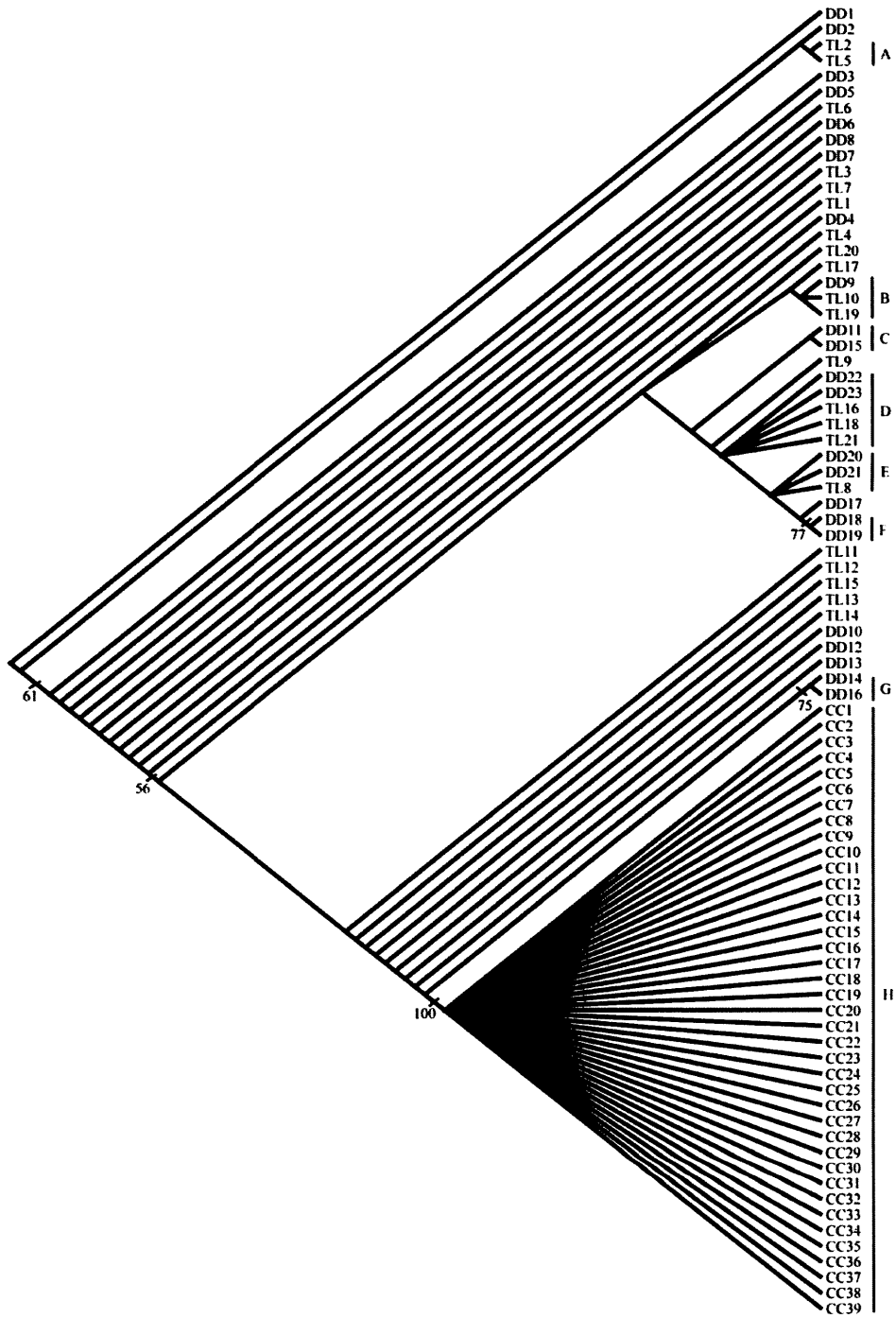

Figure 3.13. Neighbour-joining dendrogram based on ISSR data among 83 individuals of the monomorphic (CC, Cooney Creek) and the two distylous (DD, Dumaresq Dam and TL, Thomas Lagoon) populations of *Nymphoides montana*. In the monomorphic population all individuals belong to the same clone, whereas in the distylous populations most individuals were differentiated by their genotype. Numbers on branches are bootstrap frequency values for 2000 bootstrap replicates. A, B, C–H are individual ramets each from the same genotype.

3.5 Discussion

Flowers of the monomorphic population of distylous *Nymphoides montana* resemble the S-morph plants morphologically but with much reduced stigma–anther separation. A strong link between severely reduced seed production and the failure of sexual recruitment is revealed in this monomorphic population. The monomorphic plants failed to produce any seeds in both natural and glasshouse environments. This indicates that the causes of sexual sterility are more likely to be genetic factors (Barrett *et al.* 1993; Eckert 2002) rather than ecological factors (Dorken and Eckert 2001; Gross and Caddy 2006). Comparisons of the performance of the plants from the monomorphic population as both pollen and seed parents confirm that genetic factors negatively affected both male and female reproductive function. Male sterility was further detected with pollen morphology, the quantity of aborted pollen produced per flower, variation in pollen size and viability experiments. In addition, the clonal growth experiment revealed that genetic sterility could be facilitated in the clonal population once it became established because sexual infertility was associated with enhanced vegetative growth and clonal propagation through either resource reallocation or antagonistic pleiotropy (Eckert 2002). Moreover, the absence of sexual reproduction in the monomorphic population was associated with extensive genetic uniformity suggesting that the population may consist of a single clone maintained by clonal propagation.

3.5.1 Basis of sterility (environment vs. *genetic)*

Several studies have commented on the basis of sexual sterility associated with clonal reproduction in aquatic (Eckert *et al.* 2000; Dorken and Eckert 2001; Eckert 2002) and terrestrial plants (Gross and Caddy 2006) and whether genetic or environmental factors cause this sterility. The monomorphic population of *Nymphoides montana*, as both pollen donor and recipient in the cross-pollination experiments with the fertile populations, failed to produce any fruits or seeds under glasshouse conditions (Figure 3.4). These results together with the absence of open-pollinated fruit set in the natural environment indicate genetic factors rather than environmental factors have contributed to the reduced sexual fertility (Eckert 2002). A genetic basis to sterility of a population is confirmed when a sterile population shows reduced, or a lack of, seed production following cross-pollination experiments involving fertile and sterile populations even under benign glasshouse conditions (Eckert and Barrett 1993; Eckert *et al.* 1999; Dorken and Eckert 2001; Eckert

2002). In contrast, unfavourable environmental conditions are expected to reduce pollinator activity (Barrett 1980c; Eckert and Barrett 1993) and limit sexual reproduction (Mooney and Billings 1961; Barrett 1980c; Eckert and Barrett 1993; Schaal and Leverich 1996) in geographically peripheral populations (Eckert *et al.* 1999; Dorken and Eckert 2001; Dorken *et al.* 2004). The study populations of *N. montana* are located at the northern periphery of the species' distribution range. To determine whether northern populations are more likely to be monomorphic and sterile than southern populations, widespread sampling is required across the geographical range.

3.5.1.1 What is the genetic basis of sexual sterility?

A change in ploidy, known as a permanent genetic sterility factor, can cause variation in sexual fertility among populations within species. Eckert *et al.* (2000) found sexual sterility is associated with triploidy in an aquatic plant *Butomus umbellatus*. In *Nymphoides montana*, however, chromosome counts revealed that the sterile monomorphic population is at the same ploidy level as one of the two distylous populations studied (Figure 3.5). Both the sterile and fertile populations had average counts close to the hexaploid number $6x = 54$ (mean SD = 52.4 ± 2.9 for sterile population and 52.1 ± 2.2 for fertile population). Sterile polyploidy, therefore, can be ruled out as the possible cause of sexual infertility in this monomorphic population. The results of the chromosome count also showed a difference in ploidy ($4x$ and $6x$) between the two distylous populations studied. Once the tetraploid population has become established, the hexaploids could originate from the union of a haploid (n) gamete with an unreduced ($2n$) gamete (Ornduff 1987). Chromosome counts of pollen mother cells needs to be examined.

Low mating partner diversity, known as a transient genetic sterility factor, has been shown to diminish the capacity for sexual reproduction in other heterostylous species (Ornduff 1970a; Barrett 1977; Barrett 1980a; Barrett *et al.* 1993). Sexual fertility in the sterile monomorphic population of *Nymphoides montana* was restored to some extent by experimental glasshouse pollination with viable pollen imported from the distylous populations (Figure 3.4). The monomorphic plants exhibited significantly higher percent seed set in crosses using the L-morph (or the rare mating partner) pollen than did in crosses using the S-morph pollen. Although seed set estimates of 3–4% were not high enough to restore the reproduction completely with the viable pollen, the incompatibility system could have been the main factor that obstructs fruit and seed set in the monomorphic population

following the initial loss of compatible partners. Repeated founder events and periods of small population size could lead to the loss of compatible mating partners carrying different *S* alleles in self incompatibility systems (Byers and Meaghert 1992; Eckert and Barrett 1992; Reinartz and Les 1994). In such situations where a small self-incompatible population suffer from a lower number, and eventually a loss, of *S* alleles, the availability of mating partners becomes a critical factor for sexual reproduction (Barrett *et al.* 1993; Demauro 1993; Charpentier *et al.* 2000).

3.5.2 Degeneration of sexual floral traits

Traits no longer increasing fitness should tend to be lost by mutations (Haldane 1933). The 'use it or lose it' hypothesis has been a likely explanation for the loss of sex in some sterile taxa, including *Decodon verticillatus* (Eckert *et al.* 1999), *Butomus umbellatus* (Thompson and Eckert 2004) and *Grevillea rhizomatosa* (Gross and Caddy 2006) in all of which traits with no function in sexual reproduction have been degraded. The sterile population of *Nymphoides montana*, also documents the degeneration of sexual floral traits which seem to have lost their function in sexual reproduction. In most flowers, the sterile anthers could not dehisce to release pollen grains (Figure 3.6). Furthermore, pollen germinability and stainability were very low and about 85% of pollen produced in the population exhibited collapsed exine (Figure 3.7 and Figure 3.8). Failure in the mechanical release of pollen from anthers has been also reported in two sterile populations of *Grevillea rhizomatosa* (Gross and Caddy 2006). Pollen sterility has been reported in other clonal species that appear to reproduce asexually, including *Santalum lanceolatum* (Warburton *et al.* 2000) *Zieria baeuerlenii* (Sharma 2001) and *Grevillea infecunda* (Kimpton *et al.* 2002).

It seems, however, that pollen sterility is not the ultimate cause of sterility in the monomorphic population of *N. montana*, since a very low level of seed (< 4%) was produced in the cross-pollination treatments using fertile pollen from the two distylous populations (Figure 3.4). Therefore, infertility in this sterile population is caused by the loss of both male and female function, also observed in *Decodon verticillatus* (Eckert *et al.* 1999) and *Grevillea rhizomatosa* (Gross and Caddy 2006). Studies that reveal the exact stage or stages at which female infertility is most evident, including the structural characteristic/s of the stigmatic grooves (Matthews *et al.* 1999), ovule development (Eckert *et al.* 2000) and stigma receptivity at anthesis (Gross and Caddy 2006) are required.

Furthermore, the results of this study showed the level of pollen and ovule sterility could differ between the individual ramets, since the percentage of pollen germinability and pollen stainability varied between 0–4% and 0–12%, respectively, and a seed fertility of 1–12% was recorded following between-population pollinations. Clearly, this warrants further investigations and may provide insight into possible spatial differences in pollen and ovule sterility.

In *Nymphoides montana*, plants in the sterile population also exhibited weak stigma–anther separation (herkogamy) when compared with the fertile distylous plants (Figure 3.3). Stigma–anther separation has been shown to be an adaptive feature in the fertile populations to promote cross-pollination and reduce self-pollination (Chapter 2). Degeneration of such a floral mechanism in the sterile population appears to be corroborated by the loss of the male and female sex organs that no longer function to increase fitness. Eckert (2002) has also reported reduced dichogamy (temporal stigma–anther separation) within and among flowers of sterile *Butomus umbellatus*.

3.5.3 Establishment of sterility (clonal vs. *sexual reproduction)*

When sex contributes little to fitness, mutations that inhibit seed production would tend to reallocate resources from non-functional sexual floral traits to vegetative growth and clonal propagation (Eckert *et al.* 1999; Eckert *et al.* 2000; Eckert 2002; Dorken *et al.* 2004). In *Nymphoides montana*, the genetic sterility is associated with enhanced vegetative growth, clonal propagation and also flower production (Figure 3.9). These results do not reveal any association between sterility and a decrease in overall vigour (mutational meltdown hypothesis) or null expectations of the neutral mutation hypothesis. The observed increase in total plant mass of the sterile plants, however, indicates that either the sterile plants use the resources saved from not producing viable pollen and ovules for the non-functional flowers in vegetative (roots, leaves) and clonal (stolons) structures, or other pleiotropic effects of the sterility mutations are involved (Eckert 2002). Therefore, it is possible that the sterile plants, once they have originated, have spread in the natural population through the direct selection of advantages associated with reduced investment in seed production or indirect selection resulting from antagonistic pleiotropy (Eckert *et al.* 1999; Dorken *et al.* 2004). Experimental removal of flower buds is needed to distinguish whether the advantages of the sterile plants over the fertile plants are the result of resource reallocation or antagonistic pleiotropy (Eckert 2002). Dorken *et al.* (2004) found an

increase in ramet survival of sterile populations of *Decodon verticillatus* over fertile populations where all plants were intentionally prevented from investing in sex. They argued genetic trade-offs between sexual and clonal reproduction via pleiotropy may have promoted sexual sterility among populations of *D. verticillatus*.

The question, however, remains: why are the non-functional flowers still produced in the sterile population of *Nymphoides montana*? Increasing flower production indicates a) only high fitness genotypes are preserved through clonal propagation regardless of whether they are sterile (Dorken *et al.* 2004), or b) flowers and clonal structures do not compete for either resources or meristems (Eckert *et al.* 2000), since in *N. montana*, the stolons produce both vegetative and reproductive nodes.

3.5.4 Lack of genetic and genotypic diversity within the sterile monomorphic population

It is predicted that extended clonal growth may have a negative impact on genetic diversity and the species' ability to reproduce sexually (Honnay, Jacquemyn *et al.* 2006) despite the clonal plant population being as genetically diverse as the sexual population (Eckert, Dorken *et al.* 1999). Comparisons of genetic and clonal diversity using ISSR markers in the sterile *vs.* fertile populations of *Nymphoides montana* gave some useful insights on the impact of clonal growth on sexual reproduction, although the number of ISSR loci were lower than similar ISSR-based studies in other species (Ge *et al.* 2003; Qiu *et al.* 2004; Ye *et al.* 2004; Dong *et al.* 2006). In the distylous populations, recruitment of sexually outcrossed seedlings may have accounted for the high level of genetic (Nei's H = 0.17–0.26 $P\%$ = 69.23–75.00%) and clonal diversity (G/N = 0.78–0.81, Simpson's D = 0.98–0.99). In both populations, genotypes were evenly distributed (Fager's E = 0.666-0.890). The observed proportions of distinguishable genotypes and genotypic diversity in the distylous populations were much higher than the average reported for other clonal plant species by Ellstrand and Roose (1987; G/N = 0.17 and Simpson's D = 0.62). By contrast, in the monomorphic population, the evidence on genetic (Nei's H = 0.00 $P\%$ = 0.00) and clonal diversity (G/N = 0.03, Simpson's D = 0.00) indicates this population relies solely on clonal propagation. This population is dominated by one large clone, indicating a lack of sexual recruitment and extensive clonal propagation have affected negatively on within-population genetic diversity. Loss of sexual reproduction and its association with genetic uniformity has been reported in several studies (Barrett *et al.* 1993; Eckert and Barrett

1993; Uesugi *et al.* 2004). In *N. peltata* (Uesugi *et al.* 2004) and *N. indica* (Shibayama and Kadono 2007), sub-populations or populations with equal morph ratios and frequent sexual reproduction were genotypically diverse, whereas monomorphic sub-populations or populations were composed of a single genotype and appeared to be sterile. Similar results have been reported for sterile monomorphic and fertile tristylous populations of *Decodon verticillatus* (Eckert and Barrett 1993; Dorken and Eckert 2001). Elsewhere, Piquot *et al.* (1996; 1998) showed that fertile and newly established populations of *Sparganium erectum* contained several genotypes, whereas less fertile and old populations contained a single genotype.

3.5.4.1 Population structure

A high level of genetic differentiation between populations was detected in *Nymphoides montana* based on Nei's coefficient of genetic differentiation (52%), Shannon's information index (45%) and the AMOVA analysis (41%). This could be explained by the *N. montana* populations having variable modes of reproduction: primarily asexual in the monomorphic population *vs.* sexual and asexual in the distylous populations. The UPGMA dendrogram of Nei's genetic distance also showed that the sterile monomorphic population was genetically distanced from the two distylous populations, whereas the two fertile populations were grouped together (Figure 3.12). The isolated position of the sterile population in the dendrogram may further indicate that there has been enough evolutionary time for the loss of male and female function since the origin of sterility in this species (Figure 3.12). Low, or an absence of, inter-population gene flow can not be discounted as a possible explanation for the observed population differentiation.

3.6 Conclusions

The results provide evidence for the evolutionary transition from predominantly sexual reproduction to predominantly asexual clonal reproduction in distylous species of *Nymphoides montana*. This could be explained in the following way. The monomorphic population is founded by a single genotype, or alternatively, several genotypes occurred and all but one genotype is eliminated following a bottleneck. In such situations, sexual reproduction necessarily involves seed production following auto- and/or geitonogamous self-pollination. The self-incompatible plant loses its ability to produce viable seeds in the absence of a compatible mating partner and/or pollen, and only propagates clonally. Natural selection can no longer retain male (anther and pollen) and female (ovule)

reproductive function because the contribution of sex to fitness is reduced in the population. Finally, clonal reproduction becomes favoured and further expands the population when the sterile plants divert resources from the inviable pollen and ovules to clonal propagation and vegetative growth. Release from investment in sexual reproduction and its association with enhanced clonal reproduction needs to be studied further by experimental removal of the flower buds (Eckert 2002; Dorken *et al.* 2004).