

# Reproductive consequences of interactions between clonal growth and sexual reproduction in *Nymphoides peltata*: a distylous aquatic plant

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## Summary

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- Distyly is a sexual polymorphism in which plant populations contain two floral morphs differing in morphology and physiology. The dimorphism serves to promote animal-mediated cross-pollination between the floral morphs. Clonal propagation can interfere with the functioning of distyly by compromising intermorph pollinations, resulting in reduced fertility.
- Here, we investigate the relations between clonal growth and sexual reproduction in the aquatic macrophyte *Nymphoides peltata* (Menyanthaceae). Surveys of morph representation in 30 populations from five regions of China revealed that most populations exhibited strongly biased morph ratios and 30% contained a single floral morph.
- Experimental pollinations indicate that *N. peltata* possesses a strong dimorphic incompatibility system preventing self and intramorph fertilizations. An experiment involving the manipulation of morph ratios in an experimental population and an investigation in a natural population with strong morph substructure both provided evidence that compatible pollen dilution limits fertility.
- Despite constraints on the functioning of distyly in *N. peltata* we found no evidence for evolutionary changes to the heterostylous syndrome, as reported in *Nymphoides*, including populations of *N. peltata* in other parts of its geographical range.

**Key words:** aquatic macrophyte, clonal propagation, heterostyly, *Nymphoides peltata*, pollen dispersal, population substructure.

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## Introduction

Modular growth permits a wide range of clonal strategies in flowering plants (Harper, 1977). In clonal species the particular form of growth influences the spatial dispersion of flowers and opportunities for cross-pollination. Flowering shoots of a single genet may be spatially aggregated if clones remain intact and growth is extensive. Large clone size can interfere with effective pollen dispersal, with negative consequences for mating and fertility (Handel, 1985; Charpentier, 2002; Barrett, 2003). By contrast, clonal fragmentation can lead to considerable intermingling of genets at a local scale, promoting outcrossing between clones. However, the dispersal

of genets through clonal fragmentation can also result in extensive areas of genetic uniformity, particularly in aquatic plants (Barrett *et al.*, 1993). In populations of some clonal species sexual reproduction may be restricted because insufficient pollen delivery limits seed production.

The consequence of interactions between clonal strategies and sexual reproduction will be strongly influenced by the sexual system of species. For example, in self-incompatible plants large clone size may result in considerable wastage of male gametes and reduced fitness through male reproductive function as a result of pollen discounting (Harder & Barrett, 1996; Harder & Wilson, 1998). In self-compatible species geitonogamous pollen transfer may increase selfing rates

(Eckert, 2000; Reusch, 2001) resulting in reduced fitness because of inbreeding depression. Investigations of the relations between clonal growth and sexual reproduction are of particular interest in species with sexual polymorphisms (e.g. dioecy, Barrett & Thomson, 1982; heterostyly, Wyatt & Hellwig, 1979). This is because the small number of mating groups in populations and the ease with which they can be identified under field conditions provides an opportunity to investigate how reproductive success is influenced by the representation and spatial structure of mating groups.

Here we investigate interactions between clonal growth and sexual reproduction in the distylous aquatic macrophyte *Nymphoides peltata*. This widespread Eurasian insect-pollinated species is dispersed by seeds and rhizomes and has a prolific capacity for clonal growth (van der Velde & van der Heijden, 1981). As a result, populations can exhibit biased morph ratios or contain only a single floral morph (Ornduff, 1966; Marui & Washitani, 1993). This type of morph structure occurs commonly in other heterostylous plants with clonal growth and is particularly prevalent in aquatic species (Barrett & Forno, 1982; Eckert & Barrett, 1995; Thompson *et al.*, 1997; Shibayama & Kadono, 2003).

Constraints on the functioning of heterostyly as a result of clonal growth may have ecological and evolutionary consequences. For example, Ornduff (1966) proposed that strong population substructure resulting from clonal propagation may have led to the evolution of dioecism from distyly in species of *Nymphoides* capable of selfing because of weak self-incompatibility. He argued that under these conditions dioecism would provide a more effective outcrossing mechanism than heterostyly and used features of European populations of *N. peltata* to illustrate this idea. However, clonal growth can also disrupt the functioning of heterostyly and in pollen-limited environments may promote the evolution of selfing through homostyle (anthers and stigmas of equivalent height) formation (Barrett, 1979). Indeed, homostyles are reported in *Nymphoides* (Ornduff, 1970), including *N. peltata* (Marui & Washitani, 1993). Further studies are therefore clearly required to assess the impact of clonal growth on variation in the sexual systems of *Nymphoides* species.

Here we investigate the relations between morph ratios and reproductive success in clonal populations of distylous *N. peltata* from China and assess evidence for evolutionary modifications to the sexual system of this species. Through an extensive survey of 30 populations in lowland China we first determined the representation of floral morphs and how common the incidence of monomorphic populations are in this species. Since earlier studies of *N. peltata* reported a weak self-incompatibility system (Ornduff, 1966; van der Velde & van der Heijden, 1981), we also investigated the strength of dimorphic incompatibility in populations through a controlled pollination program. Finally, we manipulated floral morph ratios in an experimental population to determine whether reproductive success was frequency-dependent, and we inves-

tigated a natural population with strong morph structure to examine whether female fertility is influenced by the distance to the nearest mating partner.

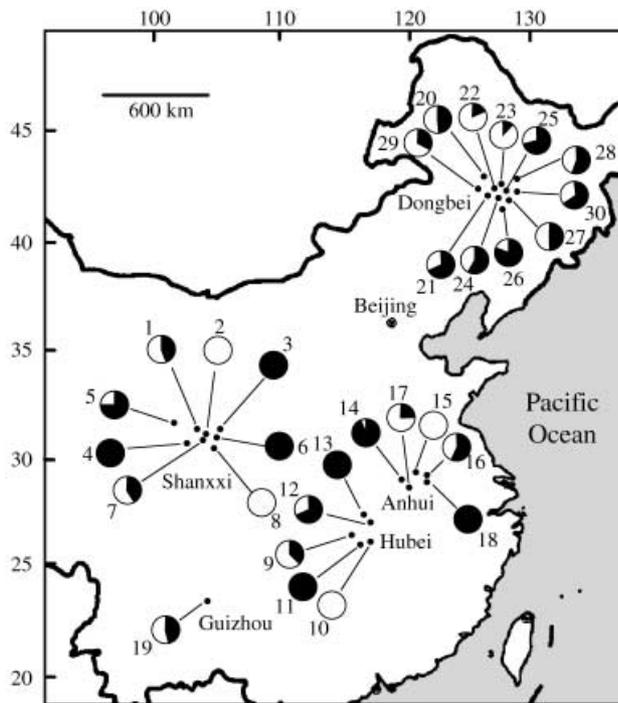
## Materials and Methods

### Study species

*Nymphoides peltata* (Gmel.) O. Kuntze (Menyanthaceae) is widely distributed in temperate regions of the north hemisphere but is native to Eurasia. Populations are typically limited to still waters or ponds, lakes and slow-moving streams. In lowland China where our studies were conducted the species is widespread but owing to extensive human activity, especially fisheries, large populations are relatively uncommon. *Nymphoides peltata* reproduces vegetatively by clonal fragmentation and sexually by seed. Maturation of floral buds is underwater. In the morning of anthesis the buds are raised above the water level and flowering occurs. After a few hours the corolla withers, the pedicels deflect and subsequent fruit development is underwater. The flowers are yellow and showy, consisting of a short basal tube and five spreading corolla lobes. In most parts of China, the species flowers from mid-April to October, but in central and eastern China, high temperatures interrupt blooming from the end of June to the beginning of September. Flowers of *N. peltata* are visited by a wide range of insect pollinators mostly bees and flies (Heukels, 1910; van der Velde & van der Heijden, 1981; Marui & Washitani, 1993). In China the main pollinators are solitary bees (especially *Halictus* spp.) and in Guizhou Province bumblebees (*Bombus* spp.). Seeds float for a short period of time before sinking because they are weakly hydrophobic (Cook, 1990) and germination and seedling establishment are restricted to exposed mud flats or very shallow water (Smits *et al.*, 1990).

### Morph ratios in natural populations

From 2001 to 2003, we investigated 30 populations of *N. peltata* in five regions of China ranging in altitude from 7 m to 2171 m. The locations of populations are mapped in Fig. 1. In each population we determined floral morph ratios by randomly sampling flowering ramets (mean sample size 78, range 10–562). The range of sample sizes reflects variation in the size of populations. In *N. peltata* there is usually one flowering shoot for each ramet and all ramets are attached to horizontal rhizomes anchored to the sediment. In most populations, it is difficult to identify individual genets and therefore the data we report are best considered floral morph ratios only. However, our own unpublished observations indicate that in common with other heterostylous plants there are no significant differences in the number of flowering stems, or flowers produced between the floral morphs, so that floral morph ratios probably approximate genet morph ratios. In our study, we obtained estimates of overall population size



**Fig. 1** The location and floral morph frequencies of the 30 populations of *Nymphaoides peltata* sampled in lowland China. Closed segments or circles represent flowers of the long-styled morph; open segments or circles represent flowers of the short-styled morph.

by multiplying mean flower shoot density per square meter by the total area of each population. We tested floral morph ratios in each population, and heterogeneity among populations against the expected equilibrium 1 : 1 ratio typical for distylous species using *G*-tests (Sokal & Rohlf, 1995).

### Controlled pollination experiments

We conducted controlled hand-pollinations on plants from eight populations of *N. peltata* from Dongbei, Shanxxi, Hubei and Anhui regions. The experiments were conducted both in the field on caged plants and on field transplants that were also caged and grown in large concrete tanks at Wuhan University. For each population sample we performed intermorph, intramorph and self pollinations shortly after the beginning of anthesis and harvested fruits 10–15 d after pollination. The sample sizes of flowers pollinated for the three classes of pollination in each population were: intermorph  $n = 12$ –21, intramorph  $n = 7$ –19, and self  $n = 8$ –13. We randomly selected flowers on each plant for each of the pollination treatments.

### The effect of floral morph ratios on fruit set

To investigate the effect of floral morph ratios on fruit set and obtain evidence for frequency-dependent reproductive success,

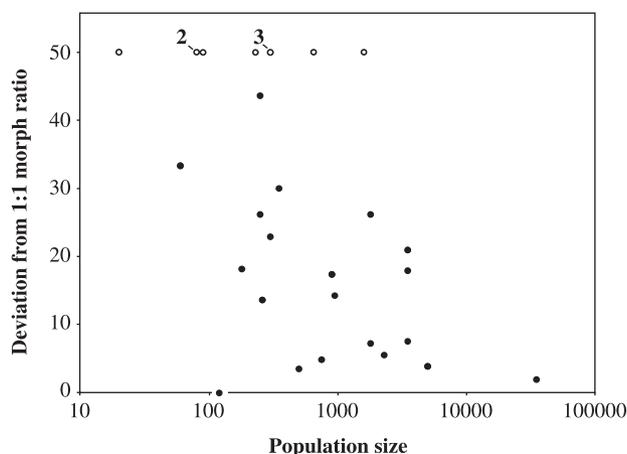
we used an experimental population of *N. peltata* growing in a concrete pond ( $3 \times 5$  m) at Wuhan University. Approximately 200 ramets of each floral morph were transplanted into the pond from a population on the outskirts of the city of Wuhan. Each day before anthesis we covered the entire population with a fine nylon screen that excluded pollinators and removed flowers to create the desired morph ratio. Flowers were removed so that each morph was evenly distributed throughout the experimental population. Flowers of *N. peltata* last for less than 1 d, so it was possible to conduct a different morph ratio treatment each day during the experiment. In total we conducted 11 morph-ratio treatments with four replicates per treatment over a 2-month period. We randomized treatments each day throughout the duration of the experiment and only ran trials on sunny days with fine weather. The treatments ranged from monomorphism through biased ratios (e.g. 0.1, 0.2, 0.3 frequency of the minority morph) to equal frequencies (0.5) of the floral morphs. We labeled all flowers with water-resistant tags and once the morph-ratio treatment was set up we removed the nylon screen to allow natural pollination by insects. Pollinator activity was consistently high throughout the experiment. Ten to 15 d after each daily treatment we harvested fruits and determined mean fruit set per treatment. We predicted that fruit set would be highest when the ratio of the floral morphs was close to unity and would decrease with increasingly biased morph ratios. This pattern should reflect the negative frequency-dependent reproductive success expected in a distylous species with strong dimorphic incompatibility.

To further investigate the influence of floral morph ratios on fertility we investigated a large population of *N. peltata* at Dongbei 20 occurring in a shallow lake with an area of  $1.5 \times 10^6$  m<sup>2</sup>. The population was characterized by extreme spatial segregation of the floral morphs as a result of prolific clonal growth. In the middle of the population we selected a large monomorphic circular patch of long-styled flowering ramets  $c. 13$  m diameter with the density of flowers roughly even throughout the patch. We sampled fruits from this monomorphic patch in concentric circular bands 0.5 m wide whose inner circles were 0–6 m in distance from the center of the patch using a  $0.5 \times 0.5$  m quadrat. Each distance class was replicated four times. Long-styled flowers at the periphery of the patch were close to short-styled flowers whereas long-styled flowers in the center of the patch were most distant from short-styled plants. We therefore predicted that fruit set should decrease from the periphery to the center of the patch.

## Results

### Variation in floral morph ratios

Floral morph ratios varied greatly among populations of *N. peltata* in China (Fig. 1). Of the 30 populations sampled, 70% were dimorphic and 30% were monomorphic for style



**Fig. 2** The relation between population size (log scale) and deviation from 1 : 1 floral morph ratio in 30 populations of *Nymphoides peltata* in lowland China. Closed circles, dimorphic populations; open circles, monomorphic populations.

representation. There was significant heterogeneity among populations in floral morph frequencies ( $G_{\text{het}} = 155.5$ ,  $df = 19$ ,  $P < 0.001$ ) although the frequencies of long- and short-styled morphs averaged for all dimorphic populations were close to a 1 : 1 morph ratio (L-morph = 0.52, S-morph = 0.48).  $G$ -tests using the sequential Bonferroni criteria at  $P = 0.05$  identified seven of the 20 dimorphic populations that deviated significantly from a 1 : 1 flower morph ratio, of these four were biased in favor of long-styled flowers and three short-styled flowers. Among the 10 monomorphic populations, six contained only flowers of the long-styled morph and the remaining four only flowers of the short-styled morph. Floral morph ratios were associated with considerable variation in population size. Small populations tended to exhibit greater deviations from a 1 : 1 floral morph ratio than large populations (Fig. 2). During our surveys of *N. peltata* we observed no homostylous or unisexual flowers in either dimorphic or monomorphic populations.

### Dimorphic incompatibility

Controlled pollinations clearly demonstrated that the populations of *N. peltata* we investigated in lowland China exhibited strong dimorphic incompatibility systems. In each

of the eight populations, self and intramorph pollinations of the floral morphs resulted in very little seed set whereas intermorph pollinations produced abundant seed in both morphs (Fig. 3). There was no evidence in our crossing program that individuals of *N. peltata* possessed weak self-incompatibility, as reported in earlier literature.

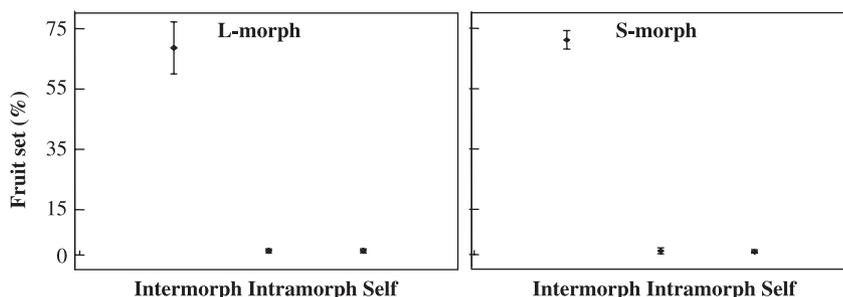
### Floral morph ratios and fruit set

There was a significant influence of variation in floral morph ratios on fruit set in the experimental population growing in the artificial pond at Wuhan (Fig. 4). The patterns of fruit set provide evidence for negative frequency-dependent reproductive success. On days in which the representation of floral morphs was near equal, fruit set averaged *c.* 90%. However, on days with strongly biased floral morph ratios fruit set declined until there was no fruit set in the monomorphic treatments. The pattern of decline in fruit set with morph-ratio bias was roughly symmetric and independent of which floral morph was the minority type.

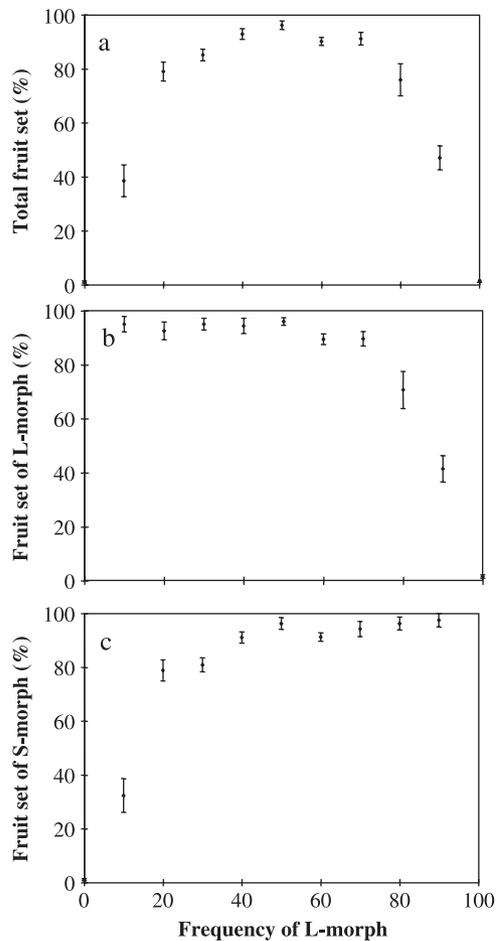
As predicted, there was a significant influence of distance to the nearest compatible mating partner on fruit set in the large population of *N. peltata* at Dongbei 20 (Fig. 5). The density of fruits in the long-styled patch was spatially structured and associated with the proximity of flowers of short-styled plants. There was a steep decrease in fruiting density for flowers that were further than 2 m from the periphery of the patch suggesting limited compatible pollen dispersal.

### Discussion

The number and spatial distribution of mating types within populations of self-incompatible, animal-pollinated plants with extensive clonal propagation can have important reproductive consequences. Because of local foraging by pollinators, most pollen dispersal is likely to be among flowers of a single mating type resulting in geitonogamous self-pollination and pollen wastage. Clonal plants with dimorphic incompatibility are particularly sensitive to these effects because unlike species with homomorphic incompatibility, the maximum number of mating types within populations is only two. We have demonstrated that extensive clonal propagation in the aquatic macrophyte *N. peltata* can affect the functioning of its



**Fig. 3** Fruit set following controlled intermorph, intramorph and self-pollinations in *Nymphoides peltata*. The means and SE are from pooled data from the eight populations used in the pollination experiments.

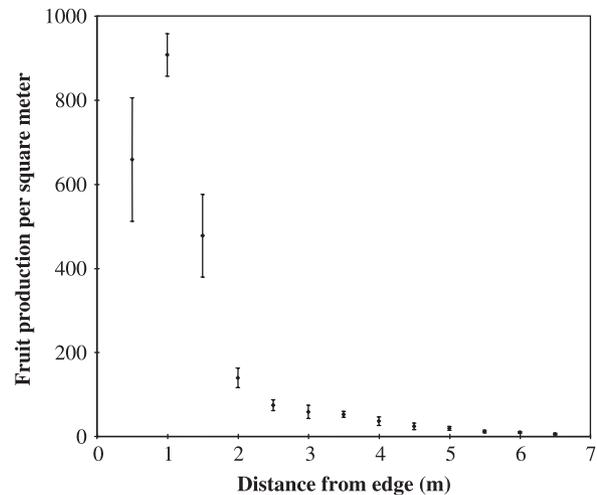


**Fig. 4** The influence of floral morph ratios (frequency of the L-morph) on mean per cent fruit set (SE) in an experimental population of *Nymphoides peltata* at Wuhan University (see text for details). (a) Total fruit set in the experimental population; (b) fruit set of flowers of the long-styled morph; (c) fruit set of flowers of the short-styled morph.

distylous sexual system resulting in reduced reproductive output. In the next section, we discuss characteristics of distyly in *N. peltata* and consider the ecological and evolutionary consequences of interactions between clonal growth and sexual reproduction in this species.

#### The distylous syndrome of *N. peltata*

Our investigations of *N. peltata* populations in China confirm several features of the floral biology of this species previously reported from the UK (Ornduff, 1966), The Netherlands (van der Velde & van der Heijden, 1981) and Japan (Marui & Washitani, 1993). *Nymphoides peltata* exhibits the typical reciprocal arrangement of stigmas and anthers that characterizes distylous species (Ganders, 1979; Barrett, 1992). The results of our controlled pollination studies indicate that the dimorphic incompatibility is strongly expressed in each of the eight populations that we examined (Fig. 3). Self and intramorph



**Fig. 5** The relation between the distance to the nearest compatible mating partner (short-styled morph) and fruiting density per square meter in a large patch of the long-styled morph of *Nymphoides peltata* at Dongbei (see text for details).

pollinations resulted in virtually no seed set whereas intermorph crosses were highly fertile. This result differs from the earlier study by Ornduff (1966) who found that ‘illegitimate pollinations invariably resulted in the formation of small capsules producing 10–20 seeds’. Variation in the strength of incompatibility is not uncommon in heterostylous plants, although most attention has focused on morph-specific differences in expression rather than geographical differentiation (reviewed in Barrett, & Cruzan, 1994). Because weak incompatibility in *N. peltata* is an important feature of the scenario of Ornduff (1966) for the evolution of dioecy from distyly in *Nymphoides* it would be worthwhile to examine populations from other regions to evaluate the nature of dimorphic incompatibility further.

#### Floral morph ratios and intermorph pollen dispersal

Thirty per cent of the *N. peltata* populations that we surveyed in China contained a single floral morph. In monomorphic populations no seed can be produced because of strong self-incompatibility (but see Marui & Washitani, 1993). Indeed, our own field observations of seed set confirmed this expectation. Seed was produced abundantly in dimorphic populations, but was near absent in monomorphic populations. Pollen dispersal between populations could provide compatible pollen for monomorphic populations but our own observations suggest that this rarely occurs. Two populations at Shanxxi separated by 1.2 km are instructive in this regard. Both flowered profusely at the same time but differed in that one contained only the short-styled morph whereas the floral morphs were equally represented in the other. Virtually no seeds were produced in the monomorphic population at Shanxxi, indicating that pollen dispersal by bees is generally quite restricted. The absence of seed production in monomorphic

populations has important implications for their long-term persistence. Habitat disturbance, or water level fluctuations resulting in the desiccation of clones, could lead to the local extirpation of monomorphic populations. This situation is in striking contrast to another clonal heterostylous aquatic, *Eichhornia crassipes* (Pontederiaceae). Monomorphic populations of this species can persist through seed reproduction despite habitat disturbance because populations are highly self-fertile (Barrett, 1980b,c).

Floral morph ratios in *N. peltata* were more likely to approach unity (1 : 1) in large rather than small populations (Fig. 2). This pattern probably occurs because small populations are more susceptible to the influence of stochastic forces on morph ratios, with implications for sexual recruitment. Founder effects followed by rapid clonal propagation probably play an important role in governing morph ratios in small populations of *N. peltata*. However, where habitat stability favors population persistence large populations can attain the equilibrium ratio of 1 : 1, as long as conditions favor regular recruitment from seed. In a distylous population in which only intermorph mating occurs (as in Chinese populations of *N. peltata*), the expected equilibrium ratio of 1 : 1 can be attained in a single cycle of sexual reproduction because of segregation at the distyly locus (Ganders, 1979; Barrett, 1992). However, in most populations of *N. peltata* founder effects, clonal propagation and limited sexual recruitment probably slow the approach to this equilibrium, despite the presence of both morphs in populations. Stochastic influences on morph ratios in heterostylous species are well documented and are especially evident in species with extensive clonal growth (Ornduff, 1972; Barrett & Forno, 1982; Eckert & Barrett, 1995; Thompson *et al.*, 1997).

We conducted two studies to investigate how floral morph representation in populations of *N. peltata* might influence reproductive success. By manipulating daily floral morph ratios in an experimental population we demonstrated that the percentage of flowers setting fruit was affected by the ratio of long- and short-styled flowers. Over a fairly wide range of morph ratios, fruit set averaged between 75% and 90%. However, once the most common floral morph approached a frequency of approx. 0.8, average fruit set declined steeply presumably because of insufficient compatible pollen for the fertilization of flowers. The rapid decline in fruit set was symmetrical indicating that regardless of which morph was in the majority, the same patterns of negative frequency-dependent fruit set occurred. Using manipulations of floral morph ratios in a wild population of *Narcissus assoanus*, a species with style-length dimorphism, Thompson *et al.* (2003) also demonstrated negative frequency-dependent patterns of reproductive success. The impact of frequency-dependent selection on morph ratios in heterostylous populations depends on the extent to which recruitment and population growth result from clonal vs sexual reproduction (Eckert & Barrett, 1995; Eckert *et al.*, 1996).

Our second experiment investigated the influence of distance to the nearest compatible morph on fruit set in a wild population at Dongbei which had strong spatial structuring of the floral morphs. We found that in a large circular patch of the long-styled morph, flowers positioned at the perimeter of the patch had a much higher probability of fruit set than flowers that were more centrally located. It was found that fruit set dropped precipitously for flowers that were more than 2–3 m from the patch boundary (Fig. 5). This pattern of fruit set could arise for two distinct reasons. It is possible that more fruit are produced at the edges of clones because ramet growth is more vigorous at the periphery and fruit production can be sustained more easily than at the center, where resources may be limiting. We have no direct evidence that the patch we examined is a single clone, or for the type of resource gradient that this hypothesis requires.

A second hypothesis, and the one we favor, concerns pollen limitation rather than resource limitation of reproductive success in *N. peltata*. We interpret the low fruit set of central flowers as resulting from the limited dispersal of compatible pollen from distant short-styled flowers. Based on our observations of foraging bees at the population, most compatible pollen is likely to be transferred to long-styled flowers close to short-styled pollen donors. As a result of pollen dilution, many central flowers would then receive insufficient pollen to mature fruit. It seems unlikely that this pattern of fruit set with distance would result from limited resources, as a gradient rather than a steep threshold would be more likely to occur. The steep decline in fruit set with distance parallels the classic leptokurtic distributions of pollen carryover and gene flow that are features of pollen dispersal in many animal-pollinated plants (reviewed by Levin & Kerster, 1974 and Harder & Barrett, 1996). Therefore, in our view, the pattern of fruit set with distance is more compatible with the pollen limitation hypothesis. Recently, Shibayama & Kadono (2003) reported that biased floral morph ratios were associated with reduced seed set in Japanese populations of clonal distylous *N. indica*. Pollen supplementation experiments provided experimental support for pollen limitation of seed set.

### Ecological and evolutionary considerations

In common with many clonal aquatic plants, the relative importance of clonal and sexual reproduction to population persistence in *N. peltata* is poorly understood. Although clonal growth may interfere with intermorph pollen transfer causing reduced seed production, we have no direct evidence that this limits sexual recruitment in distylous populations. Obviously, in monomorphic populations the absence of seed production poses problems for both persistence and seed dispersal to other sites. However, if these constraints were widespread and severe in *N. peltata* we might expect to see evidence for the breakdown of distyly and the evolution of self-pollination via homostyle formation. Although self-compatible

homostyles are common in other heterostylous groups (Ganders, 1979; Barrett, 1992), including *Nymphoides* (Ornduff, 1970), and have also been reported in a Japanese population of *N. peltata* (Marui & Washitani, 1993), we found no evidence that in Chinese populations the distylous syndrome has undergone evolutionary modifications promoting increased inbreeding. Moreover, despite Ornduff's use of *N. peltata* as an example of a species whose reproductive biology closely parallels that which has led to the evolution of dioecism in *Nymphoides*, our own investigations provide no evidence for the incipient gender specialization that is the hallmark of the evolution of separate sexes from cosexuality (Lloyd, 1979; Barrett, 1980a). However, because of the wide geographical range of *N. peltata*, further studies are required before it can be safely concluded that distyly is the exclusive sexual system of this species.

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