

BRIEF COMMUNICATION

POLLINATION BY FLIES, BEES, AND BEETLES OF
NUPHAR OZARKANA AND *N. ADVENA* (NYMPHAEACEAE)¹

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Nuphar comprises 13 species of aquatic perennials distributed in the temperate Northern Hemisphere. The European species *N. lutea* and *N. pumila* in Norway, the Netherlands, and Germany are pollinated by bees and flies, including apparent *Nuphar* specialists. This contrasts with reports of predominant beetle pollination in American *N. advena* and *N. polysepala*. We studied pollination in *N. ozarkana* in Missouri and *N. advena* in Texas to assess whether (1) there is evidence of pollinator shifts associated with floral-morphological differences between Old World and New World species as hypothesized by Padgett, Les, and Crow (*American Journal of Botany* 86: 1316–1324. 1999) and (2) whether beetle pollination characterizes American species of *Nuphar*. Ninety-seven and 67% of flower visits in the two species were by sweat bees, especially *Lasioglossum* (*Evylaeus*) *nelumbonis*. Syrphid fly species visiting both species were *Paragus* sp., *Chalcosyrphus metallicus*, and *Toxomerus geminatus*. The long-horned leaf beetle *Donacia piscatrix* was common on leaves and stems of *N. ozarkana* but rarely visited flowers. Fifteen percent of visits to *N. advena* flowers were by *D. piscatrix* and *D. texana*. The beetles' role as pollinators was investigated experimentally by placing floating mesh cages that excluded flies and bees over *N. advena* buds about to open and adding beetles. Beetles visited 40% of the flowers in cages, and flowers that received visits had 69% seed set, likely due to beetle-mediated geitonogamy of 1st-d flowers. Experimentally outcrossed 1st-d flowers had 62% seed set, and open-pollinated flowers 76%; 2nd-d selfed or outcrossed flowers had low seed sets (9 and 12%, respectively). Flowers are strongly protogynous and do not self spontaneously. Flowers shielded from pollinators set no seeds. A comparison of pollinator spectra in the two Old World and three New World *Nuphar* species studied so far suggests that the relative contribution of flies, bees, and beetles to pollen transfer in any one population depends more on these insects' relative abundances (and in the case of *Donacia*, presence) and alternative food sources than on stamen length differences between Old World and New World pond-lilies.

Key words: bee pollination; beetle pollination; fly pollination; *Nuphar*; Nymphaeaceae; pollinator spectra; self-compatibility.

The genus *Nuphar*, spatterdock or yellow pond-lilies, consists of aquatic perennials distributed throughout the temperate Northern Hemisphere. Molecular studies of the North American and European members of the genus led to the recognition of 13 distinct lineages (excluding hybrids), five in Europe and Asia and eight in North America (Padgett, 1997, 1998; Padgett, Les, and Crow, 1999). An earlier assessment (Beal, 1956) that recognized only two species worldwide cannot be upheld (see also Wiersma and Hellquist, 1997). Pollination has been studied in *N. advena* (Aiton) W. T. Aiton from the southeastern United States (Schneider and Moore, 1977, and earlier

work summarized therein), *N. lutea* (L.) Sm. from Europe and southern Eurasia (Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997, and earlier work summarized therein), and *N. pumila* (Timm) DC. from eastern Scandinavia and northern Eurasia (Lippok and Renner, 1997). A few observations on the northwestern American species *N. polysepala* Engelm. by V. Grant and B. Meeuse were summarized by Schneider and Moore (1977). Below (Results and Discussion) we add new details of Grant's observations on *N. polysepala* based on correspondence with him (letters to SSR of 12 and 25 August, 1999).

These four species appear to have different pollinator spectra, with the European species pollinated by flies and bees, the American species by beetles, with flies and bees playing but a secondary role (Schneider and Moore, 1977; Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997). The view that *Nuphar* as a genus is closely adapted for pollination by beetles comes from a study of *N. advena* in central Texas (Schneider and Moore, 1977; following then prevailing broad species concepts

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[Beal, 1956], Schneider and Moore treated *N. advena* as ssp. *macrophylla* (Small) Beal. of the European species *N. lutea*). Schneider and Moore found an average of three long-horned leaf beetles, *Donacia piscatrix* Lac., in 32 *N. advena* 1st-d (female stage) flowers. The beetles carried copious amounts of pollen, and Schneider and Moore judged them more effective pollinators than the sweat bees, honey bees, and flies that also visited the flowers “because of [the beetles’] abundance and the length of time which they remain in the flowers, whereas the bees soon departed . . . [.]” Whether such an assessment holds would depend on the plant’s mating system, especially on the long-term effects of selfing, but nothing is known about genetic neighborhood sizes in *Nuphar*.

Robertson (1889), studying the same species, *N. advena*, in Illinois and Florida, found it mainly pollinated by sweat bees (*Halictus pectoralis*) and shore flies (*Notiphila* sp.), and he regarded *Donacia piscatrix* as “worse than useless when it comes to pollination.”

The fossil record of Donaciinae in North America goes back about 58 million years, with one of the fossils from Alberta belonging to subgenus *Donacia* (*Donacia*), the most derived group (ten Nearctic species and five Old World species), which feeds and oviposits exclusively on submerged portions of nymphaeaceous leaves, stems, and peduncles (Hoffman, 1940; Askevold, 1988, 1990, 1991). Perhaps influenced by the beetles’ exclusive dependence on Nymphaeaceae, which in many areas translates as *Nuphar*, there has been a tendency to assume reciprocal dependence on the part of the flowers on the beetles for pollination. Thus, the numerous stamens, flat stigmatic surfaces, nocturnal flower closure, and intense sweet scent of *Nuphar* flower have been interpreted as “primary adaptations to assure pollination by beetles,” and it was suggested that pollination by bees and flies developed later: “the evolution of other insects (e.g., bees) and other aquatic plants [. . .] has brought about the appearance of new pollinators of *Nuphar* and the adaptive radiation of *Donacia*” (Schneider and Moore, 1977). That beetle pollination is the ancestral condition in *Nuphar*, as well as being the predominant pollination mode in the genus today, became widely accepted in the literature and is reflected in statements such as “the flowers of *Nuphar* have a close relationship with beetles of the genus *Donacia*, which complete their life cycle in association with the plant, during which time they facilitate pollination” (Schneider and Williamson, 1993).

However, investigations of *N. lutea* and *N. pumila* in the Netherlands, Norway, and Germany showed that these species are pollinated by bees and flies rather than by *Donacia* or other beetles. The flies include several apparent *Nuphar* specialists, such as the scatophagid *Hydromyza livens* and ephydriids of the genera *Hydrellia* and *Notiphila* (van der Velde, 1986; Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997). Another species of *Notiphila* was found as a *N. advena* pollinator in Florida (Robertson, 1889).

The different role of *Donacia* beetles in the pollination of *Nuphar* in Europe and (apparently) different parts of North America led to the present study. By comparing beetle, bee, and fly abundances and behaviors on two American species with the European findings (Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997)

we wanted to assess whether different floral morphologies of Old and New World species are associated with different pollinators as suggested by Padgett, Les, and Crow (1999). These authors have shown that *Nuphar* comprises two major clades. Of the four species whose pollination has been studied, *N. advena* and *N. polysepala* belong to the New World clade, and *N. pumila* and *N. lutea* to the Old World clade. A fifth species investigated here, *N. ozarkana* (G. S. Miller & Standley) Standley, also represents the New World clade. [*Nuphar ozarkana* is considered a synonym of *N. advena* by Wiersema and Hellquist (1997), whereas Padgett, Les, and Crow (1999) accord it species status based on relative *matK* and internal transcribed spacer (ITS) sequence divergence among 13 species and two hybrid lineages of *Nuphar*.] All these species have bowl-shaped flowers with seemingly easily accessible pollen and nectar rewards. However, New World and Old World flowers differ markedly in anther lengths and stigmatic disk sizes (Padgett, Les, and Crow, 1999). Different-sized reproductive parts or different amounts of nectar might be differentially attractive to flies, bees, or beetles. Given the widely different assessments of the role of *Donacia* beetles as *Nuphar* pollinators (Robertson, 1889; Schneider and Moore, 1977), we also investigated these beetles’ effectiveness as pollinators experimentally at the same site as Schneider and Moore (1977).

MATERIALS AND METHODS

Observations on *N. ozarkana* were made between 4 and 14 July, 1997, in southeastern Missouri, USA at Poplar Bluff (latitude 36°43' N, longitude 90°23' W) along the Beaver Dam Creek, a clear, slow-running creek with a maximum depth of 1.30 m. The average water temperature at the study site was 28°C. Observations on *N. advena* were made between 18 and 23 July 1997, near San Marcos, Texas, USA (29°53' N, 97°57' W) along the Aquarena slough, a streamlet flowing slowly into Spring Lake of the San Marcos River. The depth of the extremely clear water ranged between 50 and 90 cm, and the average water temperature during the study period was 25°C. Flowers of *N. ozarkana* were observed (by BL) for a total of 22 h over 6 d, usually between 1000 and 1700, and for 2 h during one night. Insect visits to a total of 26 flowers (1–7/d) were recorded over the entire observation period. Similarly, 26 flowers of *N. advena* were monitored for a total of 20 h during 6 d between 1000 and 1900, i.e., from opening to closure, and for 2 h during one night. At both sites, stigma and nectary secretions were tested for glucose with diabetes test paper (Diastix, Bayer, Germany), and intact flowers of different ages were tested for terpenoid content indicating floral scent production by immersion in a watery solution of neutral red (Vogel, 1990).

To evaluate the mating system of *N. advena* we bagged flowers just prior to opening, subjected them to one of the following treatments, and then rebagged them. (1) Flowers were emasculated prior to anther dehiscence to test for the presence of agamospermy. (2) Flower buds were bagged without further manipulation to test for spontaneous selfing. (3) First-day flowers were emasculated and cross-pollinated with pollen from a plant 200 m away. (4) Early 2nd-d flowers, still in the receptive stage, were emasculated and cross-pollinated with pollen from a plant 200 m away. (5) Early 2nd-d flowers were self-pollinated with pollen from their own freshly dehiscent anthers. Seed set in naturally pollinated flowers was quantified for comparison with that in experimental flowers. Hand-pollinations were carried out by excising stamens and rubbing their anthers over the stigmatic disks. Because of the clonal nature of pond-lilies, widely spaced plants were used to ensure cross-pollination. Bags were removed after anthesis so as not to hinder fruit development.

Developing fruits were collected ~10 d to 2 wk later and fixed in 70% ethanol. Developing seeds and aborted ovules were counted to determine percentage seed set.

Donacia beetles' role as pollinator was tested by placing floating mesh cages over *N. advena* plants with buds ready to open and adding *D. piscatrix* and/or *D. texana* individuals to each cage; we could not securely distinguish these species in the field. Beetles were inspected for visible pollen loads, but it is nevertheless possible that they had pollen grains from earlier visits to *Nuphar* flowers on them. Because flowers at San Marcos usually had two or three beetles in them, we added 2–3 beetles per flower per cage; in total, 30 beetles were added to ten caged flowers. Beetles were placed inside cages, but not directly onto flowers, which resulted in some flowers being visited by several beetles, others by none. The cages completely excluded bees and flies.

RESULTS AND DISCUSSION

The only species of *Nuphar* that occurs in Texas is *N. advena* (Wiersema and Hellquist, 1997). The entity occurring in Missouri is considered a species, *N. ozarkana*, by Padgett, Les, and Crow (1999) based on relative *matK* and ITS sequence divergence among 13 species and two hybrid lineages of *Nuphar*. Based on morphology it was considered a form of *N. advena* not worthy of taxonomic recognition (Wiersema and Hellquist, 1997). Our measurements (below) and ecological observations suggest to us that the genetic distinctness of *N. ozarkana* from *N. advena* is paralleled by floral-morphological differences.

Flowers of *N. ozarkana* measure ~2.5 cm in diameter and are borne on rigid peduncles ~2 cm above the water surface. They have six broadly spatulate sepals ($N = 9$ flowers), which are tinged green on the outside and dark red on the inside, and on average ten petals that are completely yellow. The ~119 stamens and petaloid stamens are oblong-linear, 7 mm long, and packed below the stigmatic disk before anthesis. They open centripetally. As is typical of Nymphaeaceae, there is a gradual transition from stamens to petals, with petaloid and staminoid intermediates. The ovary is ovoid and crowned by a circular flattened disk with typically 11 rays of stigmatic tissue. There are 25–90 ovules per ovary ($N = 8$ ovaries), and the mature fruits contain on average 41 seeds ($N = 6$ fruits; range 43–72).

Flowers of *N. advena* are distinctly larger than those of *N. ozarkana*, measuring ~3.5 cm in diameter (vs. 2.5 cm in *N. ozarkana*) and having on average 204 stamens and petaloid stamens (vs. 119). The sepals are slightly obovate, and there are 18 petals (vs. 10 in *N. ozarkana*; $N = 5$ flowers). Red-tinged sepal insides, common in *N. ozarkana*, are not seen in *N. advena*. The stigmatic disk has 16, rather than 11, rays. On average, there are 327 ovules per ovary (vs. 25–90 in *N. ozarkana*; $N = 10$ ovaries), and the mature fruits contain on average 252 seeds ($N = 11$ fruits; range 186–353).

First-day (female-stage) flowers of both species remain almost completely closed except for a small triangular opening just above the stigmatic disk. The only way for insects to enter these flowers and reach the petal nectaries is to land on, or crawl over, the stigma, which on the first day is covered with a sticky mucus. The mucus is devoid of glucose as assayed by diabetes test strips and does not appear to serve as a pollinator reward. Flowers of *N. ozarkana* emitted no perceptible odor, even after fresh flowers had been placed in a clean glass vial for ~20

min. By contrast, those of *N. advena* smelled like immature apricots. Fresh petal nectaries of *N. ozarkana* did not produce visible nectar drops. However, when the same glucose test strip was touched to all ten nectaries of a flower it stained weakly. In *N. advena*, by contrast, single nectaries produced visible drops that strongly stained the glucose test strip. In neither species did the nectaries stain with neutral red (as also reported by Schneider and Moore, 1977).

Flowers of both species opened around 0800, depending on sun intensity. They were functionally female on the first day, because pollen sacs only open on the second day. First-day flowers closed completely between 1800 and 1900, while 2nd- and 3rd-d flowers remained partially open. On the second day, the outer row of stamens bent backwards, presenting pollen, while the mucus on the stigmatic rays gradually dried out. During each of the following three days, one or two rows of anthers matured and presented pollen, while the now completely dry stigma changed color from dark yellow to brown. Following the maturation of the last row of anthers, the petals and stamens withered, peduncles lost their rigidity, and fruit ripening proceeded.

Flowers of *N. ozarkana* were visited by three species of sweat bees, *Lasioglossum* (*Evylaeus*) *nelumbonis* Robertson, *Dialictus bruneri* (Crawford), and *Augochlora pura pura* (Say), that together made up 97% of all visits. The syrphid flies *Paragus* sp., *Chalcosyrphus metallicus* Wiedemann, and *Toxomerus geminatus* Say together accounted for 3% of the visits. There was a single visit by a species of *Bombus*. The bees and syrphids collected or ate pollen and occasionally sat on the stigmatic disk to groom themselves. Pollen-seeking bees predominantly visited older, pollen-presenting flowers. *Donacia piscatrix* Lac. was observed only on 2- or 3-d old flowers that were in late male stage, and of 38 flowers checked during one night, five contained one beetle each.

Flowers of *N. advena* at San Marcos received fewer insect visits than those of *N. ozarkana* at Poplar Bluff (279 vs. 591 visits to a total of 26 flowers studied for comparable lengths of time). Sixty-nine percent of all visits were by *Lasioglossum* (*Evylaeus*) *nelumbonis*, the same bee that was the most abundant visitor to *Nuphar* at Poplar Bluff. Syrphids made up 3.9% of all visits and *Xylocopa* bees 11%. Two species of *Donacia* were found inside the flowers, *D. piscatrix* and *D. texana* Crotch. Together they accounted for 15% of all visits to flowers during the 20 h of observation. Sometimes, beetles were observed pushing their way through the sepals of *N. advena* and into a flower before it fully opened, apparently to feed on nectar. Of 22 flowers checked during one night (some being completely closed 1st-d flowers, others partially open 2nd- and 3rd-d flowers), each contained at least one beetle, and several contained copulating pairs (a total of 29 beetles were seen in these flowers). At both study sites, a few of the outer sepals were visibly gnawed by *Donacia*. One beetle gnawing on a petal of *N. advena* had pollen attached to its abdomen, and several dissected beetles had abundant *N. advena* pollen in their guts.

Results of breeding system experiments (Table 1) indicate that *N. advena* is incapable of agamospermy and does not spontaneously self-pollinate. Experimental selfing of 2nd-d flowers yielded 9% seed set; 1st-d flowers

TABLE 1. Mean seed sets (%) in *Nuphar advena* in Texas and *N. lutea* in southwestern Germany after different experimental treatments to determine breeding systems. Figures for *N. lutea* from Lippok and Renner (1997).

Treatment	Bagged	Emasculated	<i>N. advena</i>	<i>N. lutea</i>
Natural pollination	No	No	77.5 ^a (11)	64.5 ^a (17)
1st-day outcrossing	Yes	Yes	62.3 ^a (10)	60.5 ^a (18)
2nd-day outcrossing	Yes	Yes	12.0 ^b (10)	42.2 ^b (18)
2nd-day selfing	Yes	No	8.6 ^b (16)	50.2 ^b (20)
Agamospermy	Yes	Yes	0 (10)	0 (15)
Spontaneous selfing	Yes	No	0 (10)	0 (15)
Caged with beetles	No	No	27.6 (10)	n.a.
Beetle-pollinated ¹	No	No	68.7 ^a (4)	n.a.

¹ Of the flowers that opened within the bee-and-fly-exclusion cages, only four were pollinated by the experimentally introduced *Donacia* beetles; the other flowers in the cages set no or few seeds.

^{a,b} Values with the same letter are not significantly different at the $P = 0.05$ level (Tukey's test).

cannot be autogamously pollinated because of the flowers' strong protogyny. Experimental outcrossing of 2nd-d flowers resulted in a similarly low seed set (12%), indicating that stigmas are less receptive on the second day. Experimental cross-pollination of 1st-d flowers and natural pollination yielded high seed sets (62 and 78%, respectively). Six of ten flowers that bloomed inside the mesh cages with "beetles-only" as potential pollinators set no or few seeds and appeared not to have been visited. Four had an average seed set of 69% due to beetle pollination. Since experimental crossing and selfing of 2nd-d flowers resulted in much lower seed sets (9 and 12%), the 69% seed set in the cages likely results from beetle-mediated geitonogamous pollination of 1st-d flowers (flowers inside cages belonged to single plants). Taken together, these figures suggest that natural seed set in *N. advena* is mostly due to xenogamy or geitonogamy of 1st-d flowers. In European *N. lutea* and *N. pumila*, by contrast, experimental selfing and outcrossing of 2nd-d flowers yielded seed sets almost as high as those in 1st-d flowers (Lippok and Renner, 1997; Table 1).

During the study period, the visitors responsible for most pollen transfer between flowers in *N. ozarkana* and *N. advena* were pollen-collecting sweat bees, especially *Lasioglossum nelumbonis*. Schneider and Moore (1977) during a 12-mo study of *N. advena* found halictids (*Halictus* sp., which may be identical with *Lasioglossum nelumbonis*) and honey bees to be the most frequent visitors, together with *Donacia piscatrix*. They observed the bees to "visit more flowers in a given amount of time [than the *Donacia* beetles]." We found *Donacia* to be infrequent on *N. ozarkana*, where they were only seen in 2nd- or 3rd-d flowers whose stigmas had dried up and to be abundant on *N. advena* as earlier observed by Schneider and Moore (1977). Possibly the larger size of *N. advena* flowers, intense apricot smell, and more copious nectar (each flower has 18, rather than 10, petals and therefore nectaries) contribute to that species' higher attractivity for beetles. Also, two species of *Donacia* occur at San Marcos, but only one at Poplar Bluff, and this may explain why more beetles were found on San Marcos flowers. In a monograph of *Donacia*, Askevold (1990, p.610) reports that *D. piscatrix* and *D. texana* have mouthparts adapted

for feeding on plant tissue, including, but not restricted to Nymphaeaceae, and for cutting holes into leaf or peduncle surfaces into which they oviposit, rather than for feeding on pollen or nectar. However, *D. piscatrix* and *D. texana* individuals we dissected contained abundant *Nuphar* pollen. We also agree with Schneider and Moore (1977) that beetles appear to be taking up nectar from the petal nectaries. In addition to feeding on nectar and pollen, *D. piscatrix* also mates in *Nuphar* flowers (Marx, 1957, p.242).

Verne Grant (personal communication in Schneider and Moore [1977] and letters to SSR of 12 and 25 August, 1999), who studied two populations of *N. polysepala* Engelm. on two days in July 1949 near Echo Lake, El Dorado County, Sierra Nevada, California, found the flowers frequented by *Donacia* beetles, syrphids, and muscid-like flies; no bee visits were observed. *Donacia* beetles were abundant on flowers in one population, but only one beetle was found in the other population. Beetles, syrphids, and muscid-like flies had their venters covered with *Nuphar* pollen, and since they usually landed on the stigmas were effective pollinators.

The precise role of *Donacia* as pollinators in nine species of *Nuphar* remains to be investigated. The species occurring in China (*N. sinensis* Hand.-Mazz.) and Japan (*N. oguraensis* Miki, *N. japonica* DC., and *N. pumila*) have flowers very similar to those of *N. lutea*, and indeed *Donacia gracilipes* Jacoby and *D. nitidor* (Nakane) have been recorded as visitors to the last two species (Kimoto, 1981). Of the New World species yet to be studied, *N. ulvacea* (Mill. & Standl.) Standl., *N. orbiculata* (Small) Mill. & Standl., *N. variegata* Durand, and *N. sagittifolia* (Walt.) Pursh are similar to *N. advena*, *N. polysepala*, and *N. ozarkana* (Padgett, Les, and Crow, 1999). The North American *N. microphylla* (Pers.) Fern. is part of the Old World clade, and its flowers are similar to those of *N. pumila*. All these species possess petal nectaries and, on the second day, abundant pollen, and both rewards are accessible to flies, bees, and beetles.

Padgett, Les, and Crow (1999) suggested that the different anther lengths of Old World and New World species may influence pollinator effectiveness and selection (i.e., result in shifts in pollinator spectra). Analyses of pollen deposition on pollinators' body surfaces (and subsequent deposition patterns on stigmas) would be necessary to substantiate a correlation between anther length and pollinator effectiveness in *Nuphar*. However, Padgett, Les, and Crow's hypothesis can be assessed preliminarily by comparing pollinator spectra in the two Old World and three New World species studied so far (keeping in mind the unequal and in the case of *N. polysepala* short observation periods). *Nuphar lutea* and *N. pumila* (three populations studied) are mainly visited by flies, with bees playing a secondary role; *N. polysepala* (two populations) is visited by flies and *Donacia*; *N. advena* (one population studied in detail) is mainly visited by halictids, flies, and *Donacia*; and *N. ozarkana* (one population) is mainly visited by halictids and flies. Flies are thus the only insects visiting all species, while visitation by bees and beetles varies among species and sites (for example, *Donacia* is absent from *N. lutea* populations studied in Germany, but is an occasional visitor in Norway; Lippok and Renner, 1997). The relative contribution of flies, bees,

and beetles in any one population of *Nuphar* thus appears to depend more on these insects' relative abundances (and in the case of *Donacia*, presence) and alternative food sources than on stamen length differences between Old World and New World pond-lilies.

An earlier study of *N. advena* found that bagged flowers produced some seeds (Schneider and Moore, 1977; seed set was not quantified), while our bagged flowers set no seeds. All species of *Nuphar* are protogynous, with the only overlap between the female and male stages occurring during the morning of the second day when the stigmatic rays are still receptive (Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997; this report). This would be the time when spontaneous selfing might occur were it not for the position of the stamens below the stigmatic disk and their extrorse dehiscence, which together preclude pollen from reaching the stigma unless via some outside agent. Insect-mediated selfing of 2nd-d flowers is possible because, like other pond-lilies studied (Ervik, Renner, and Johanson, 1995; Lippok and Renner, 1997; Table 1), *N. advena* is self-compatible. Whether species of *Nuphar*, like many clonal plants, are adapted to constant selfing (in the form of geitonogamy) or maintain mixed-mating systems is unknown.

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