

Flower Phenology and Sexual Maturation: Partial Protandrous Behavior in Three Species of Orchids

RAYMOND L. TREMBLAY*, GRIZEL POMALES-HERNÁNDEZ AND
MARÍA DE LOURDES MÉNDEZ-CINTRÓN

Department of Biology, University of Puerto Rico—Humacao, 100 carr. 908, Humacao, P.R., 00791

*Corresponding author: raymond@hpcf.upr.edu

ABSTRACT.—Plants have theoretically multiple alternatives for preventing self pollination and consequently the effect of inbreeding, such as sequential flowering, dichogamy and self-incompatibility to name a few. We investigated the reproductive biology of three sequentially flowering (acropetal) endemic orchids from Puerto Rico. Since sequential flowering is present in the studied species and very rarely (1.0%) is there more than one flower open simultaneously on an inflorescence, we hypothesized that the orchids should be self-compatible and show no effect of protandry (dichogamy). We performed hand self—and cross-pollinations and evaluated whether the species are self-compatible and whether the receptivity to pollination success (fruit set) is influenced by the age of flowers (protandry). We define protandry as pertaining to a hermaphroditic organism that assumes a functional male condition prior to shifting to a functional female state. We found that all three species are self-incompatible. Furthermore, flower age is important for predicting the likelihood of fruits set. Older flowers (6+ days) are significantly more likely to produce fruits (functional protandry). The multiple mechanisms for preventing self-pollination (sequential flowering, dichogamy and self-incompatibility) that are noted for these species suggest that the historical evolutionary processes for preventing inbreeding may be complex. We hypothesized that because multiple mechanisms are present for preventing self-pollination inbreeding depression is likely to be high.

KEYWORDS.—*Lepanthes*, Puerto Rico, reproductive success, dichogamy, self-incompatible

INTRODUCTION

Low fruit set in many plant species is a consequence of pollinator limitation (Tremblay et al. 2005), or self-incompatibility, which are perceived as mechanisms to promote cross-pollination (Marshall and Folsom 1991). Promoting cross-pollination is believed to increase fitness by increasing genetic diversity, while self-pollination decreases genetic diversity and can result in inbreeding depression (Lande and Schemske 1985; Dudash 1990; Jarne and Charlesworth 1993). Consequently, many species probably evolved complex mechanisms in order to prevent self-pollination, including self-incompatibility through sporophytic or gametophytic processes, and through pre-visitation mechanisms, including sequential flowering, dichogamy (protandry, pro-

togyny) and solitary flowers (Delaporta and Calderón-Urrea 1993; Walker-Larson and Harder 2000).

In the Orchidaceae, pollinator limitation is frequently invoked as the cause of low fruit set, but in self-compatible species low fruit set may additionally be attributed to incompatible self—and geitonogamous pollinations, resulting in flower or fruit abortion (Delaporta and Calderón-Urrea 1993; Walker-Larson and Harder 2000; Tremblay et al. 2005). In self-compatible orchids a number of mechanisms for preventing self-pollination (Johnson and Edwards 2000) have been detected, including: sequential flowering (*Psychilis* spp., Ackerman (1989); *Malaxis massonii* (Ridl.) Kuntze, Aragón and Ackerman (2001)), dichogamy/protandry (*Goodyera oblongifolia* Raf., Ackerman (1975); *Listera cordata*, Ackerman and Mesler (1979); *Spiranthes*, Catling (1983); Sipes and Tepedino (1995); *Prescottia stachyodes* (Sw.) Lindl., Singer and Sazima

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(2001a); *Erythrodes arietina* (Rchb. F. & Warm.) Ames, Singer and Sazima (2001b); *Sauroglossum elatum* Lindl., Singer (2002); *Mesadenella cuspidata* (Lindl.) Garay, Singer (2002); *Notylia nemorosa* Barb. Rodr., Singer and Koehler (2002); Warford (1992); *Manniella cypripedoides* Salazar, T. Franke, Zapfack & Beenken, Salazar et al. (2002)), temporal variation in pollinarium size (*Bulbophyllum weddellii* (Lindl.) Rchb. f., *B. ipanemense* Hoehne and *B. involutum* Borba, Semir and F. Barros, Borba and Semir (1999a); *Trigonidium obtusum* Lindl., Singer (2002)), and change in position of lip and column after pollinarium removal or after a few days if pollinarium is not removed (Sosa and Rodríguez-Angulo 2000).

We investigated whether or not self-incompatibility is present in three sequentially flowering tropical species of *Lepanthes* (Orchidaceae). In addition we determined whether their flowers are protandrous. Protandry is a dichogamous mechanism; it is defined as the maturation of the male (androecium) structure before the maturation of the female (gynoecium) structures (Kearns and Inouye 1993).

MATERIALS AND METHODS

We determined the breeding system of three endemic species of *Lepanthes* from Puerto Rico: *L. woodburyana* Stimson (53 individuals), *L. rupestris* Stimson (56 individuals), and *L. rubripetala* Stimson (35 individuals). Each species was grown in a Wardian case (Orchidarium CaseTM) in the laboratory at the same temperature and humidity (18–24°C, 45–100% humidity).

The phenology of flower buds, flowers (including anthesis), and fruits for each plant was recorded. The age of flower buds, flowers and viable fruits along each inflorescence was recorded daily. We considered a fruit viable as soon as we noticed the expansion of the ovary. As a control we segregated four specimens of *Lepanthes rupestris* and *L. rubripetala* and *L. woodburyana* to determine the effect of flower longevity on non-manipulated flowers, the effect of pollinia removal on flower longevity and the ability to self-fertilize without

manipulation (apomixis). No fruits were set unless flowers were hand pollinated.

Determination of the breeding system

Cross pollination vs. self pollination.—Flowers of the three species of *Lepanthes* studied do not show any evidence of self-pollination, but to determine if autogamous pollinations produce viable fruits, we performed a series of hand self-pollinations. We self-pollinated 3-day old flowers of *L. rupestris* (n = 33) and *L. woodburyana* (n = 50), and 6-day old flowers for *L. rubripetala* (n = 14).

Determination of protandry.—To test if protandry is present in *Lepanthes* we made cross-pollinations at different time intervals of the flower age from one to eight days. We compared the percentage of fruit production per pollination for two age treatments (1–5 days and 6+ days) and among the three species. We used contingency tables to evaluate the null hypothesis that the pattern was random among days and species.

RESULTS

Flower development and fruit phenology

From the time we noticed flower bud set, these took 11 to 15 days to open in all three species. Open flowers lasted up to 10 days in *L. rupestris* and *L. woodburyana*, and 11 days in *L. rubripetala* when not manipulated. However, flowers manipulated through pollinia removal or pollination reduced the life span of open flowers. In general, removal of pollinia caused flowers to close and wither within 1 to 2 days, moreover, pollination (deposition of pollinia onto the stigma) resulted in flower closure the next day (Table 1). Mean life span (until dehiscence) of fruits was estimated at 31 and 39 days for *L. rubripetala* and *L. rupestris*, respectively. None of the fruits that were set aborted (abscission of fruit in early stages of development).

Hand self and cross-pollinations

Hand self pollination did not produce any fruits for any of the three species of

TABLE 1. Flower phenology of *Lepanthes* flowers. Time (in days with s.e., sample size in parentheses).

Characteristics	<i>L. rubripetala</i>	<i>L. rupestris</i>
Development of flower bud to anthesis	14.6 ± 0.3 (104)	11.7 ± 0.2 (150)
Flower lifespan without manipulation	6.9 ± 0.5 (32)	5.8 ± 0.4 (36)
Flower lifespan with pollinia removal only	1.2 ± 0.1 (11)	1.4 ± 0.1 (46)
Flower lifespan when flower is pollinated	1.0 ± 0.0 (55)	1.0 ± 0.0 (61)
Fruits life span	31.2 ± 2.2 (23)	39.3 ± 1.3 (34)

orchids. On the other hand, fruit set by cross-pollinations (irrespective of flower age) was 23.2% for *L. rubripetala*, 23.1% for *L. rupestris* and 41.1% for *L. woodburyana*.

The effect of flower age on fruit set

The number of hand cross-pollination varied among species and was dependent on flower production. Three hundred seventy-three pollinations were performed in *L. rupestris*, 142 in *L. rubripetala* and 192 in *L. woodburyana*. The pattern of fruit set and flower age was consistent among species. Older flowers (6+ days) had significantly higher fruit set in all three species as compared from the expected proportion (Table 2). Under the null hypothesis, the expected number of fruits (observed in brackets) were all significantly lower than observed fruit set, 12 (17) for *L. rubripetala*, 22 (31) for *L. rupestris*, 38 (56) for *L. woodburyana*.

DISCUSSION

Flower longevity is often short (a few days) in plants (Primack 1985). In orchids, Primack (1985) noted that these have characteristically long-lived flowers and that

the flowers of *Habenaria orbiculata* (Pursch) Torr. last about 12.7 days in the field. However, flowers of species such as *Dendrobium crumenatum* Sw. and *Sobralia macrantha* Lindl. last only one day, while an extreme case are the flowers of *Grammatophyllum multiflorum* Lindl. which last nine months in greenhouses. In *Lepanthes* flower longevity is in the range of 6 – 8 days. When flowers are manipulated (pollinaria removal and pollinaria deposited) flower age is drastically reduce to one day. This phenomenon of shorten flower life span when flowers are manipulated is well known in orchids. Given the small size of the flowers, a reduction in the life span of open flowers may not simply be due to flower manipulation but also to actual physical damage to delicate sexual structures.

The effect of protandry has been observed in orchids. In *Goodyera oblongifolia* pollen deposition is prevented by the proximity of the column to the lip in young flowers. As flowers age the column becomes exposed and thus receptive to pollen deposition (Ackerman 1975). In the genus *Spiranthes*, outcrossing is promoted by sequential flowering but also by protandry (Catling 1983; Sipes and Tepedino 1995).

TABLE 2. Hand cross-pollinations of three species of orchids with different flower age categories (in days). Numbers in parenthesis are observed proportion. test performed to determine if fruit set is flower age independent.

Flower	Hand Cross Pollination					
	<i>L. rubripetala</i>		<i>L. rupestris</i>		<i>L. woodburyana</i>	
	No Fruit	Fruit	No Fruit	Fruit	No Fruit	Fruit
Age						
1-5	75 (52.8%)	16 (11.2%)	222 (59.5%)	55 (14.7%)	77 (40.1%)	23 (11.9%)
6+	34 (23.9%)	17 (11.9%)	65 (17.4%)	31 (8.3%)	36 (18.8%)	56 (29.2%)
Chi Square	4.55		6.22		28.4	
P-value	0.033		0.013		< 0.001	

Protandry in *Spiranthes* is mechanical, as in *G. oblongifolia*, and the time for male maturation to female stage last from 5 to 30 days (depending on the species of *Spiranthes*). Furthermore, the probability of cross-pollination is influenced by the behavior of foraging insects that move up the inflorescence (acropetal). A similar pattern of flower phenology along the inflorescence and pollinator behavior was also found in *Prescottia stachyodes* (Singer and Sazima 2001a). In *Erythodes arietina* the male phase lasts 1–2 days and is a mechanical function of the column (Singer and Sazima 2001b) as in *Sauroglossum elatum* and *Notylia nemorosa* from Brazil (Singer 2002; Singer and Koehler 2002). In many of these species the viscidium dries out in older flowers and the pollinaria are consequently not removed if further visits occur (Singer and Koehler 2002). Therefore, in some species of orchids flowers do go through fully separate male and female phases.

In *Lepanthes* the shift to receptive female is clearly not as abrupt as in the previous examples. The change in receptivity is one of proportion of increase fruit set as flowers age, with a more pronounced effect of flower age in *L. woodburyana* as compared to the other two species. Temporal overlap in the male and female flower function is present in many species (Bertin 1993; Bertin and Newman 1993; Navarro 1997; Fetscher 2001; Offord 2004). Interestingly, the general explanation for the presence of dichogamy in plants is to reduce geitonogamous pollinations in self-compatible plants; however, dichogamy is as common in self-compatibility and self-incompatibility species (Bertin 1993).

It is interesting to note that in *Lepanthes*, a group of over 800 neotropical species, almost all species have sequential flowers. If the process of sequential flowering is a primary mechanism for reducing self-pollination then why should plants be self-incompatible and protandry (at least partially) be present? Which one of the mechanisms, self-incompatibility, sequential flowering or protandry was the first to evolve? It seems that since self-incompatibility is present, one might argue that sequential flowering and protandry were

not sufficiently effective for preventing self-pollination.

We would predict that inbreeding depression (mating among related individuals) may be high in *Lepanthes* because of the multiple mechanism that are apparently present to prevent self-pollination, thus mating with unrelated individuals (example, from other populations) should result in higher fitness. This hypothesis does rely on the fact that self-incompatibility, sequential flowering, and protandry were evolutionarily selected and not random processes. A complicating issue is that sequential flowering has evolved numerous times in the Orchidaceae and may be linked to other evolutionary processes such as pollinator attraction and not to minimize self-pollination.

Previous population genetic studies using allozymes in *Lepanthes* showed that populations are usually more inbred (F_{IS}) than one would expect from a random mating system and rarely at Hardy-Weinburg equilibrium (Tremblay and Ackerman 2001; Carronero, Tremblay, and Ackerman, unpublished data). Consequently, the opportunity for breeding with closely related individuals is present and may be a common event (Tremblay and Ackerman 2001, 2003).

Recent phylogenetic analyses suggest that the most likely sister groups to *Lepanthes* are *Fronitaria*, *Trichosalpax*, *Zootrophion*, *Acianthera* and *Lepanthopsis* (Pridgeon, Solano, and Chase 2001; Pridgeon and Chase 2001). The relationships between the six genera are poorly resolved in the consensus tree. However, the flowers on inflorescences of *Lepanthopsis*, *Trichosalpax*, *Zootrophion*, *Acianthera* and *Fronitaria* (monotypic) usually open simultaneously; although single flower species are present in some of the genera (Luer 1986, 1991, 1997). Unfortunately we have no information in regards to reproductive biology of these five sister genera and thus we do not yet know if these species are self-incompatible and if flower age is important for fruit production. In addition, we do not know if simultaneous flowering is derived or ancestral.

Our results show that flowers of the

three species of *Lepanthes* are self-incompatible and a tendency to increase female receptivity as flower age. The partial protandrous behavior and sequential flowering may be a relic character prior to the development of self-incompatibility. However, this assumes that self-incompatibility is more efficient as a mechanism for preventing self-pollination than protandry and sequential flowering. A further assumption is that inbreeding depression (reduce fitness) is present when mating among related individuals, otherwise there would be no fitness advantage for any of the previous mechanisms. Because of the small effective populations size of *Lepanthes* the opportunity for inbreeding is likely to be high (Tremblay and Ackerman 2001) and thus mechanisms for promoting outcrossing may be advantageous.

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