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Sex allocation in inflorescences of the self-compatible *Polygonatum verticillatum* (Liliaceae)

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Abstract. The modularity and sequential flower production in hermaphroditic plants enable them to modify sex allocation in response to biotic or abiotic environmental variation. To test three predictions derived from the relationship between sexual specialization and compatibility levels, we analyzed the breeding system (self-incompatibility) of *Polygonatum verticillatum* and the variation in sexual investment to stamens, ovules, pollen grains and P:O ratio among flowers at different positions of the inflorescence. Our results show that in self-compatible *P. verticillatum* sexual investment varies throughout the inflorescence with an increase in the number of pollen grains but does not show male specialization in top flowers, unlike other self-incompatible species of the genus previously studied. Comparative studies of the variation in floral sex allocation between taxonomically close species should contribute to a better interpretation of the relationships between sexual investment and the breeding system.

Keywords: breeding system, self-compatibility, sex allocation, P:O ratio variation, *Polygonatum*.

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Introduction

The modularity and sequential flower production in hermaphroditic plants enables them to modify sex allocation over time in response to biotic or abiotic environmental variation (Lloyd, 1980; Charnov, 1982; Lloyd & Webb, 1986; Brunet & Charlesworth, 1995). Temporal variation among flowers in sex allocation is not necessarily adaptive, however, a few studies have analyzed the selective, genetic, or ecological factors that generate within-individual temporal changes in sex expression (Brunet, 1992; Mazer *et al.*, 2009).

One of the potential advantages of modularity in hermaphrodites is the ability to adjust sex allocation over time. Flexibility in the allocation of resources to male and female functions per flower may allow plants to respond to changes in environmental conditions that differentially affect either the costs or fitness gains through these functions (Delesalle *et al.*, 2008). Additionally, mating system should influence the evolution of sex allocation strategies in flowering plants beyond simply reducing allocation to male function and to secondary sexual traits (Mazer & Delesalle, 1998).

P:O (pollen:ovule) ratio is a conservative indicator of breeding system (Cruden, 1977). Variation in P:O ratio may occur within individuals, within or among populations, and among species (Cruden, 2000). Studies of temporal variation among flowers in the P:O

ratio have detected high variation within individuals of many species as a result of genetically determined developmental patterns and/or plastic responses to resource availability (Delesalle *et al.*, 2008).

Application of sex allocation theory to P:O ratios has allowed to formulate three theoretical predictions about variation of sexual investment in selfing versus outcrossing taxa:

(1) Outcrossing taxa generally evolve high P:O ratios compared to autogamous taxa. As selfing levels increase, the P:O ratio should decline because of decreased allocation to male function (Charlesworth & Charlesworth, 1981; Charnov, 1982; Schoen, 1982; Ritland & Ritland, 1989). Despite the somewhat varying ranges of the P:O values, a substantial decrease in the P:O ratio usually follows the shift from xenogamous to autogamous breeding systems (Erbar & Langlotz, 2004).

(2) Autogamous taxa are expected to produce sequential flowers with a highly stable P:O ratio because mating opportunities among the flowers of highly autogamous selfing individuals remain constant throughout the flowering period (Mazer & Delesalle, 1998; Delesalle & Mazer, 2009). By contrast, related animal pollinated outcrossing taxa are expected to evolve floral P:O ratios that change over the lifetime of an individual plant (Mazer *et al.,* 2009).

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(3) Patterns of variation in P:O ratio are affected by temporal separation of the male and female phase (dichogamy) (Brunet & Charlesworth, 1995; Delesalle *et al.*, 2008). Protandrous outcrossers should exhibit temporal increases in the P: O ratio among flowers produced sequentially along the primary axis. In plants with inflorescences with protandrous flowers, the first flowers may have a low proportion of ovules compared to the later flowers. In this case, protandry would be expected to select for female-biased assignment in early-opening compared to late-opening flowers, and protogyny would show the opposite pattern (Brunet & Charlesworth, 1995).

The genus *Polygonatum* is represented in the Iberian Peninsula by 3 species: *P. odoratum* (Mill.) Druce, *P. multiflorum* (L.) All. and *P. verticillatum* (L.) All. In previous works, Guitián *et al.* (2001, 2004) have shown that self-incompatible *P. odoratum* flowering stems present sequential opening of flowers and pollinators have a clear directional foraging upward on inflorescences. Thus, lower female-phase flowers should receive pollen from other plants, whereas upper male-phase flowers should disperse pollen to other plants. Brunet and Charlesworth (1995) demonstrated that large differences in the pollen transfer probabilities from different flower positions can lead to the evolution of sexual inversion in sequential bloomers, like in *P. odoratum* (Guitián *et al.*, 2004) and *P. multiflorum* (Kosinski, 2012). These models support the view that enhancement of male fitness through pollen donation plays a key role in the evolution of the reproductive system in *P. odoratum* and *P. multiflorum* (andromonoecy).

Our prediction is that plant-pollinator interactions in vertical inflorescences of *Polygonatum* species with different breeding system can modulate the variation patterns in the evolution of sex allocation. To test this prediction, we analyzed: the breeding system (self-incompatibility) of *P. verticillatum* and the variation in sexual investment to stamens, ovules, pollen grains, and P:O ratio between flowers at different positions of the inflorescence. Finally, we analyzed to what extent the three previous predictions were fulfilled between *Polygonatum* species.

Material and Methods

The plant and Study site

Polygonatum verticillatum (L.) All. (Whorled Solomon's Seal) is a perennial herb with a thick and long creeping rhizome. The stem is robust, angled, grooved, 30–90 cm tall, with many whorls of 4–8 lance-shaped, 9–20 cm long leaves. Clusters of 2–3 small tubular whitish flowers with green tips hang from the axils of the leaves. Flowers are 8–12 mm long, with perianth parts fused into a broad tube below at the base and spreading into short triangular tepals at the tip. Fruit is a red berry, which eventually turns dark purple.

Polygonatum verticillatum blooms at the end of June and bears fruit at the end of July. It is pollinated by *Bombus* species. At the time of sampling plants had 2–15 flowers open simultaneously and 80% of the plants had more than one whorl open simultaneously (range 2–6) enabling the transfer of pollen between flowers of the same plant. It is an incomplete dichogamous species: the maturation of the stigma precedes the male phase. The stigma begins to be receptive in the bud phase and remains receptive at the beginning of the male phase (J. Guitián, unpublished data).

The study was conducted in A Rogueira forest (1500 m a.s.l.), in the Sierra de O Caurel (Lugo, Spain), located in the NW of the Iberian Peninsula. The plants used for experiments grew in 2 isolated patches (populations henceforth), 4 km apart (coordinates 42.61674 -7.0939 and 42.67570 -7.15619) in a deciduous forest of *Fagus sylvatica* L., *Betula alba* L. and *Sorbus aucuparia* L.

Breeding system

For the study of the breeding system, we placed in one of the populations a tulle cage of 2 x 2 x 2 m that prevented the access of the insects. The plants (n = 21) were marked in the interior, and the number of flowers per whorl counted (number of flowers/plant: x̄±sd = 15 ± 7.9; n = 293 flowers). Every morning we performed the following treatments: A) Pollination with pollen from the same plant (facilitated autogamy; n = 98 flowers). Using a brush, pollen was taken from a flower of the plant and different flowers of the same plant were pollinated. B) Spontaneous autogamy (n = 52 flowers). The plants were not pollinated, remaining isolated from the insects. At the same time, 10 plants outside the cage were marked with free access to the pollinators, which were used as a control (n = 143 flowers).

At the time of fruiting, we counted the fruits and calculated the fruit/flower ratio in each of the treatments. To avoid missing fruits due to dispersal by mammals in the controls (outside the tulle cage) we considered the green fruits that had reached the final size in all treatments.

Pollen and ovule production

Twenty plants were randomly selected per population and all flowering buds produced were collected 1 or 2 days before anthesis (n = 137 and n = 157, respectively). Position of each bud in the inflorescence was recorded. Buds were kept in individual vials with 70% ethanol until examination in the laboratory. Flower buds were dissected to determine ovule number per flower with the aid of a stereomicroscope. Pollen was counted using a particle counter (Coulter Counter® Z2, Beckman Inc.) equipped with a 100 μm aperture tube and a particle size channelizer accessory. For estimation of pollen production, two anthers from each flower bud (one from each stamen whorl) were examined. Anther content was released into a vial containing 0.5 ml of electrolytic solution

(Isoton II®), by mechanical destruction of the theca walls and subsequent vortexing. The wall fragments were then removed, and the remaining solution poured into a beaker containing 50 ml of Isoton II. The vial was then refilled with 0.5 ml of Isoton II and shaken to collect any remaining pollen, then emptied into the beaker. The final volume was thus 51 ml (0.5 + 50 + 0.5). For each flower five counts were performed, each in 0.5 ml of the pollen suspension sample. Counter accuracy was previously checked by manual counting under a light microscope: for these counts the pollen from two anthers per flower was suspended in 1 ml of a solution of detergent and safranin, from which ten 5 μl replicates were then obtained for counting. To verify that the Coulter technique is adequate we used a counting chamber (Experimental Bioscience) to estimate the number of pollen grains in 20 flowers.

We calculated the value of the P:O ratio as well and recorded the position of each flower on the inflorescence. To consider that inflorescences had different numbers of flowers, flower position on the inflorescence was in all cases defined as "base" (the earliest third to open), "middle" (the middle third), or "top" (the last third). Additionally, we analyzed the stability in the relationship P:O as the ratio of its value at top vs basal flowers (P:O top-base = P:O top/P:O base).

Data analysis

Differences in fruit set among the treatments in the breeding system experiment were analyzed with chi-squared test for 2 x 2 contingency tables. The effects of flower position on pollen number, ovule number and P:O ratios were analyzed with the Friedman ANOVA test. All statistical analyses were performed with IBM SPSS (2020).

Results

Breeding system

Polygonatum verticillatum produced fruits both in spontaneous autogamy and in facilitated geitonogamy. Fruit set did not differ between the two pollination treatments (chi-square χ^2: 0.05, p = 0.882). Spontaneous and facilitated autogamy (geitonogamy) led to lower fruit set than control plants freely exposed to the insects (Figure 1) (control vs facilitated autogamy: chi-square χ^2: 9.28, p < 0.001; control vs spontaneous autogamy: chi-square χ^2: 7.13, p < 0.001). In control plants freely exposed to the insect's fruit set varied consistently between the different positions of the inflorescences, from 70–80% in the base to 0–1% in the top.
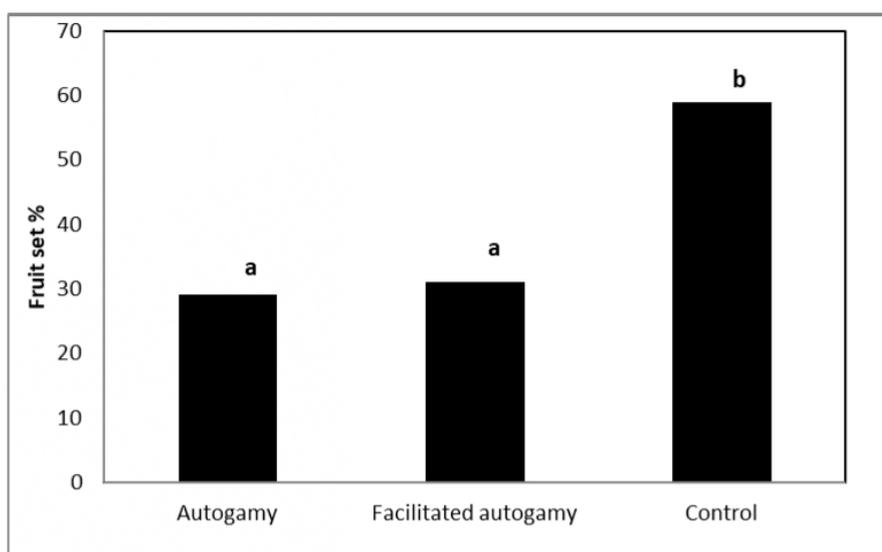

Figure 1. Fruit set in pollination treatments of *Polygonatum verticillatum*.

Pollen and ovule production

The number of stamens was six in all the flowers and did not show any variations in relation to the position of the flower in the inflorescence. The average number of pollen grains per flower obtained with the Coulter was 17,897 (n = 20), nad with the counting chamber the number was 18,076 (p = 0.55) which show that the technique used was adequate.

The number of ovules varied from 13.8 in the base to 11 in the top ($\bar{x}\pm sd$ = 12.2 ± 2.2; n = 279); the number of pollen grains varied from 22,958 in the base to 33,617

in the top ($\bar{x}\pm sd$ = 26,919 ± 19,519) (n = 208); P:O ratio varied between 1,614 in the base to 2,992 in the top (total P:O = 1,820).

The number of ovules per flower decreased with the position in the inflorescence (Friedman Anova Test = 36.55; p = 0.000) (Figure 2). The top position flowers have shown the highest number of pollen grains (Friedman Anova Test = 10.51; p = 0.05) (Figure 2). The highest P:O ratio was in the top position (Friedman Anova Test = 17.37; p = 0.0001) (Figure 2). The increase in the P:O between the flowers on the top and at the base was $\Delta P:O_{top-base}$ = 1.86.
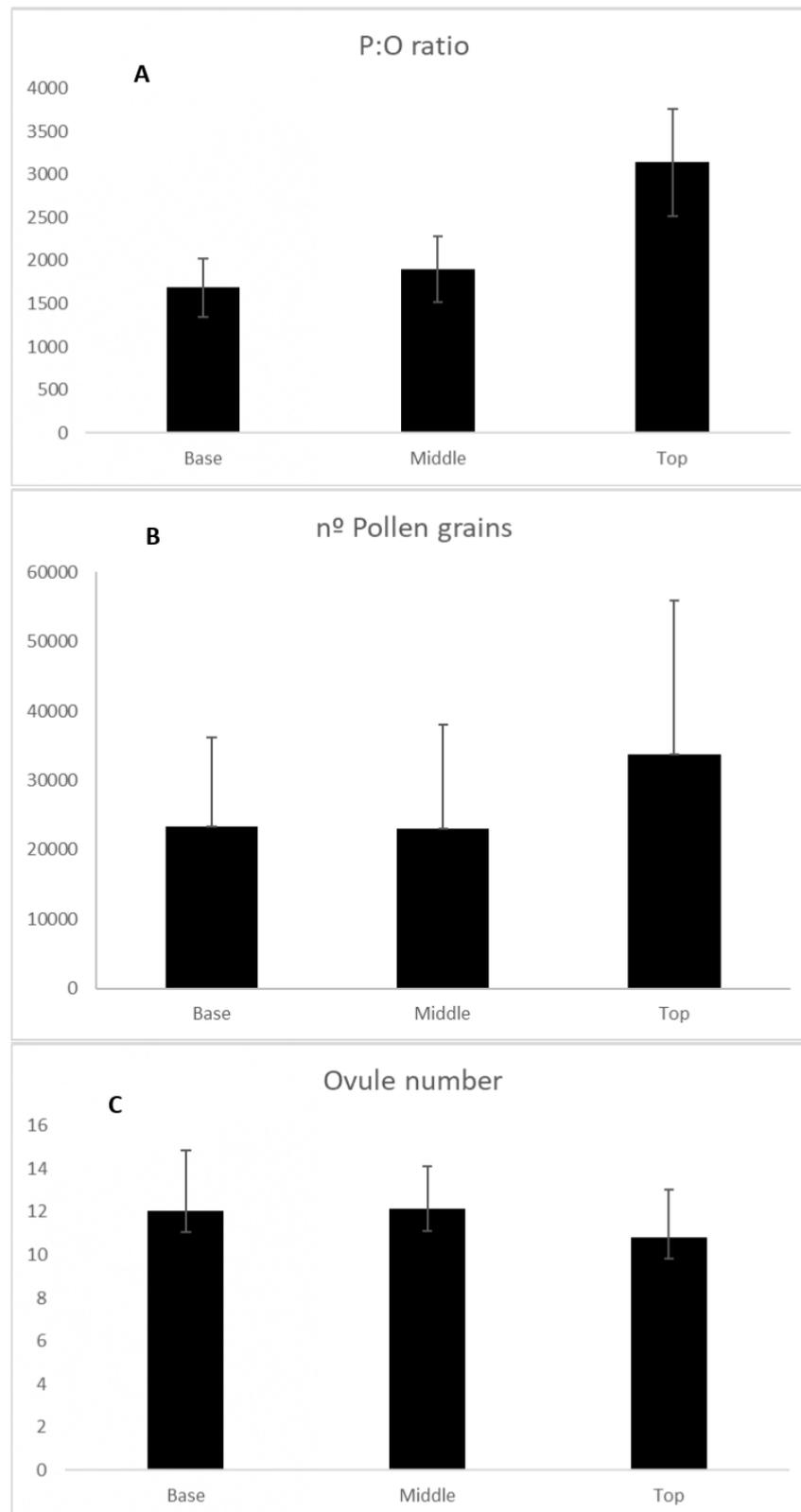
Figure 2. Variation between flowers in different positions in the inflorescences of *Polygonatum verticillatum*
(1: base; 2: middle; 3: top). A, P:O ratio; B, Pollen number; C, Ovule number.

Discussion

Our results show that *Polygonatum verticillatum* is a self-compatible, facultatively autogamous species. This contrasts with the breeding system of the other Iberian species of the genus, *P. odoratum* and *P. multiflorum,* both self-incompatible (Guitián *et. al.*, 2004; Kosinski, 2012). In addition, the floral opening pattern within *P. verticillatum* inflorescences and the behavior of the pollinators allowed the transfer of geitonogamous pollen. *P. verticillatum* did not show male specialization in distal flowers. All flowers were cosexual, by contrast

with self-autoincompatible *P. odoratum* in which both hermaphrodite and male flowers are present (andromonoecious; Guitián *et al.*, 2004).

Our results support the prediction (1), since the P:O ratio of self-compatible *Polygonatum verticillatum* is lower than in self-incompatible *P. odoratum* (1,820 vs 4,389) or in other species of the genus (e.g. facultative xenogamous *P. cyrtonema;* Liu *et al.*, 2017). However, numerous studies have shown since Cruden (1977) that the shift in P:O ratio in self-incompatible species is achieved by producing more pollen grains than closely related self-compatible species, not by a decrease in the number of ovules. In both species the number of ovules decreases from the base to the top, although this decrease is greater in *P. odoratum*: 11.8 to 6 vs 12 to 10.8 (Guitián *et al.*, 2004).

Contrary to the prediction (2), in *Polygonatum verticillatum* the increase in the P:O between the top flowers vs the base flowers was Δ P:O $_{top\text{-}base}$ = 1.86 (*P. odoratum* Δ P:O $_{top\text{-}base}$ = 1.51; Guitián *et al.*, 2004). Delesalle *et al.* (2008) found little evidence to support the prediction that the P:O ratio of the selfing taxa will consistently vary less than in outcrossing taxa in the genus *Clarkia*. However, it is important to emphasize that *P. verticillatum* is a facultative autogamous and although the full selfers and outcrossers do differ in mating opportunities, these differences may not be sufficiently strong or consistent to select for different patterns of change in sex allocation.

Our results were contrary to the prediction (3). There is an increase in P:O ratio towards the distal positions and therefore there is no increase in femaleness towards the top flowers of the inflorescences, as would be expected. However, although theory predicts that selection will favor temporal decrease in the P:O ratio in protogynous outcrossing species (Brunet & Charlesworth, 1995), this may not apply if flowers become so highly pollen-limited towards the end of an individual´s flowering period that selection favors autogamous self-pollination and lower P:O ratios (Mazer *et al.*, 2009).

Despite the differences in sexual assignment between *Polygonatum* species, a similar pattern of increased male function in the terminal positions of the inflorescence is present. These variations in P:O may be affected, in addition to differences in the reproductive system or pollinators behavior, by environmental factors, space-time variations in the availability of resources, or architectural effects (Diggle, 1995; Guitián *et al.*, 2001; Scalone *et al.*, 2013). Altogether, our results show that only the first prediction (1) is fulfilled here: because differences observed in the mean P:O ratio were consistent with sex allocation theory and typical of outcrossing vs selfing congeners (Parachnowitsch & Elle, 2004).

The change from outcrossing to selfing is a repeated evolutionary transition, occurring in many unrelated genera apparently associated with a reallocation of resources (see Charlesworth, 2006). *Polygonatum odoratum* produces more ovules per ovary in late buds than their outcrossing counterparts. This increase in ovule production is also coupled with the development of smaller floral structures (i.e., petals) and it suggests that resources not used for pollinator attraction may be allocated to additional ovule production in this species.

Additionally, *Polygonatum verticillatum* is an incomplete protogynous species (functional adichogamy according to Eckert & Schaefer, 1998). Incomplete protogyny can play a role in breeding system transitions from self-incompatibility (SI) to self-compatibility (SC) (Goodwillie & Weber, 2018) and a broad phylogenetic analysis of angiosperms found that transitions from SI to SC were especially frequent in protogynous species (Routley *et al.*, 2004), perhaps reflecting a role of incomplete protogyny in promoting delayed selfing. This mechanism incurs no seed discounting because it does not detract from maternal fitness through outcrossing; similarly, it does not incur pollen discounting or diminish male outcross success. Consequently, delayed selfing is favored because increases fertilization whenever a flower has been insufficiently cross-pollinated.

Figure 3. Inflorescences showing the potential consequences of different pollen transfers: "legitimate" (potentially fertile) pollen transfer in yellow; "illegitimate" (sterile) pollen transfer in pink. A, *Polygonatum odoratum*; B, *P. verticillatum*.

Self-compatible *Polygonatum verticillatum* does not show male specialization in top flowers. Unlike *P. odoratum*, female inversion in top flowers of the inflorescence can act as a reproductive insurance in the adverse climatic conditions of the western Cantabrian mountains.

In conclusion, the genus *Polygonatum* presents a wide range of breeding systems ranging from self-incompatibility to different levels of auto-compatibility. These variations are consistent with differences in floral inversion ranging from the presence of unisexual flowers to complete cosexuality, with important consequences on the reproductive ecology of the species (Figure 3).

We propose that differences in allocation to male and female functions in sequential blooming inflorescences of *Polygonatum* species with different breeding systems can be modulate by pollen-transfer probabilities between different flowers. Comparative studies of the variation in floral sex allocation between taxonomically related species should contribute to a better interpretation of the relationships between sexual investment, the breeding system, and the robustness of the predictions discussed here.

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