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TRIMORPHIC INCOMPATIBILITY IN *PONTEDERIA SUBOVATA* (PONTEDERIACEAE): AN AQUATIC MACROPHYTE FROM LOWLAND SOUTH AMERICA

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Premise of research. Tristyly is a floral polymorphism that is reliably reported from six flowering plant families. Populations of tristylous species often contain three floral morphs that differ reciprocally in stigma and anther height. Trimorphic incompatibility and a suite of ancillary pollen and stigma polymorphisms are commonly associated with sex organ trimorphism. Here, we investigate the tristylous syndrome of *Pontederia subovata* (Pontederiaceae), a little-known aquatic from southwest Brazil.

Methodology. We conducted measurements of sex organ position, pollen size, pollen production, and stigmatic papillae length on the three floral morphs under glasshouse conditions and carried out a controlled pollination program to investigate whether *P. subovata* possesses a trimorphic incompatibility system and whether its expression varies among the floral morphs.

Pivotal results. *Pontederia subovata* displays reciprocal stigma and anther heights and trimorphism in the size of pollen grains and the length of stigmatic papillae. Controlled pollinations provided evidence of trimorphic incompatibility: self- and intramorph crosses yielded significantly less seed than do intermorph crosses when pollen from anthers and stigmas of equivalent height were employed. However, trimorphic incompatibility was weakly expressed in the mid-styled morph when pollinations involved long-level anthers. The occurrence of stem-borne tubers is reported for the first time from Pontederiaceae.

Conclusions. Our results confirm the presence of tristyly in *P. subovata*. The species has several features in common with other tristylous members of *Pontederia*, including striking variation in the strength of incompatibility among the floral morphs. We suggest that weak incompatibility in the mid-styled morph may provide reproductive assurance during colonization. Tuber formation in *P. subovata* probably evolved as an adaptation enabling persistence of genets during the marked dry season that characterizes the aquatic habitats in which the species occurs.

Keywords: aquatic macrophyte, Neotropics, pollen trimorphism, *Pontederia subovata*, stem tubers, tristyly, trimorphic incompatibility.

Introduction

Self-incompatibility systems in flowering plants can be conveniently subdivided into two major classes, depending on whether the mating groups can be distinguished morphologically (reviewed in Williams et al. 1994; Franklin-Tong 2008). In plant species with homomorphic self-incompatibility, populations contain numerous morphologically indistinguishable mating groups. In contrast, species with heteromorphic self-incompatibility contain two (distyly) or three (tristyly) mating groups that differ reciprocally in style length and stamen height (Darwin 1877; Ganders 1979; Barrett and Shore 2008). The floral morphs are usually self- and intramorph incompatible, and only pollinations between anthers and stigmas of equivalent height produce abundant seed. Flowers of each of the three floral morphs (long-, mid-, and short-styled; hereafter L-, M-, and S-morphs) produce two distinct stamen levels containing pollen that differs in size and incompatibility behavior (Barrett and Cruzan 1994). As a consequence, in tristylous populations there are 18 incompatible and six compatible pollen-pistil combinations.

Tristyly is a relatively uncommon floral polymorphism that is reliably reported from six flowering plant families (Amaryllidaceae, Connaraceae, Linaceae, Lythraceae, Oxalidaceae, and Pontederiaceae; Darwin 1877; Ganders 1979; Barrett 1993; Thompson et al. 1996). Experimental studies of taxa in the three well-known families (Lythraceae, Oxalidaceae, and Pontederiaceae) have established that the polymorphism is usually composed of a suite of morphological and physiological traits—"the tristylous syndrome"—including reciprocal herkogamy, trimorphic incompatibility, and various pollen and stigma polymorphisms (reviewed in Barrett 1993). In these families, tristyly is controlled by a similar genetic system involving two diallelic gene loci (*S* and *M*) with epistasis between the *S* and *M* locus (e.g., *Decodon*-Lythraceae [Eckert and Barrett 1993], *Oxalis*-Oxalidaceae [Weller 1976], *Pontederia*-Pontederiaceae [Gettys and Wofford 2008]; reviewed in Lewis and Jones 1992). Despite similar patterns of inheritance, there is considerable variation in the expression of the tristylous syndrome both within and among families. This variation includes incomplete sex organ reciprocity (Eckert and Barrett 1994), differences between morphs in the strength of trimorphic incompatibility (Barrett

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and Anderson 1985), and variation in the degree of pollen trimorphism (Barrett 1988). In some species, the atypical trait expression is a stable feature of tristylous populations (Barrett et al. 1983, 2004), whereas in others, modifications to tristyly are associated with the breakdown of the polymorphism and transitions to alternative mating systems (reviewed in Weller 1992). Thus, investigating the characteristics of the tristylous syndrome in particular taxa is of importance for understanding the maintenance and breakdown of this complex polymorphism.

Tristyly occurs in two genera—*Eichhornia* and *Pontederia*—of the primarily Neotropical aquatic family Pontederiaceae. They differ in the expression of the tristylous syndrome, particularly in the strength of trimorphic incompatibility and the degree of pollen size heteromorphism (Barrett and Anderson 1985; Barrett 1988). Among the six species of *Pontederia*, trimorphic incompatibility is reliably reported from three, all of which have been investigated in some detail (*Pontederia cordata* [Ornduff 1966; Price and Barrett 1982; Barrett and Anderson 1985; Richards and Barrett 1987], *Pontederia rotundifolia* [Barrett 1977], and *Pontederia sagittata* [Glover and Barrett 1983; Scribailo and Barrett 1991*a*, 1991*b*]). Of the remaining three species, *Pontederia subovata* is reported as tristylous, based on observations of herbarium specimens (Lowden 1973), but the species is poorly known and has not been examined experimentally; *Pontederia parviflora* is reported as monomorphic for style length (Lowden 1973); and the heterostylous status of *Pontederia triflora* is unknown. The main goal of this study was to confirm tristyly in *P. subovata* and to compare its features with those of other tristylous taxa, particularly of *Pontederia*.

We examined the morphological and physiological components of the tristylous syndrome of *P. subovata* plants sampled from a population in southwest Brazil. We addressed the following three main questions: (1) Are stigmas and anthers reciprocally positioned in the three floral morphs? (2) Is there evidence of ancillary polymorphisms involving the pollen and stigmatic papillae of the floral morphs? (3) Does *P. subovata* exhibit trimorphic self-incompatibility, and, if so, are there differences among the floral morphs in the compatibility of crosses between various pollen-pistil combinations? An additional finding of our study was the discovery that *P. subovata* produces stem tubers. We therefore also document this new clonal strategy for Pontederiaceae.

Material and Methods

Study Species and Field Sampling

Pontederia subovata (Seub.) Lowden (=*Reussia subovata*), known as Camalotinho, is a trailing, prostrate, perennial aquatic with subovate leaves and showy pale lilac to purple inflorescences of eight to 16 flowers with prominent yellow nectar guides (fig. 1). The species inhabits swamps, low-lying pastures, temporary lagoons, and river edges in low-lying areas of Brazil, Argentina, Bolivia, and Paraguay, with scattered localities in Venezuela and the Guyanas (Lowden 1973). The species is especially common in the Pantanal region of lowland South America.

We obtained material for this study (15 plants, five of each floral morph) from a population growing at the edge of a seasonal marsh at Finca Sta. Catarina, 150 km northeast of Corumbá, Mato Grosso do Sul state, southwest Brazil (lat. 18.107°S, long. 56.239°W). Preliminary observations of the population indicated that it was tristylous. An effort was made to sample intact shoots from different morphs separated by 5–10 m along a 100-m stretch of shoreline; however, because *P. subovata* has the capacity for clonal propagation (see "Results"), the number of genets in our sample of 15 plants could not be determined with any certainty. The shoots were returned to a glasshouse at the University of Toronto, where they were propagated clonally to produce 30 clonal replicates. All measurements of floral traits and controlled crosses described below included flowers sampled from clones descended from each of the original five plants per morph. We grew plants in large plastic pots submersed in water-filled containers under uniform conditions on two benches until experiments commenced. The glasshouse was maintained at 25°–30°C, and flowering occurred at intervals during the year, particularly from September to April.

Morphological Measurements

To investigate the relative positions of sex organs in *P. subovata* and confirm that the species is tristylous, we measured the stigma and anther heights of three to 12 flowers from 11–14 inflorescences per floral morph (total flowers measured: L-, M-, and S-morphs, $n = 114$, 100, and 90, respectively). Measurements were made with digital calipers and a stereoscopic microscope (Zeiss 47+50+57 binocular microscope). Flower length, stigma height, and the midpoint of the upper and lower anther levels were measured from the base of the ovary. We compared stigma and anther heights within a level using a generalized linear mixed model (SAS GLIMMIX; SAS 2011), with flower length as a covariate, floral morph as a fixed effect, and flowers measured from the same inflorescence as repeated measures. We used multiple comparisons to test for significant differences between stigma and anther height within a level. We also compared flower length among the floral morphs using the GLIMMIX procedure, with flowers within inflorescences treated as a repeated measure.

To examine whether there were size differences of pollen originating from different anther levels of the floral morphs, we measured the equatorial and polar axes of five to 40 dry pollen grains per anther level from five, six, and six flowers from the L-, M-, and S-morphs, respectively (total pollen grains measured per anther level: $n = 265$, 280, and 277 for long-, mid-, and short-level anthers, respectively). Measurements were made using a Zeiss Axioplan Universal compound light microscope at a magnification of 400× using oil immersion. Because pollen size did not differ between the same anther levels of different floral morphs (data not shown), we pooled data by anther level for illustrative purposes (see figs. 3, 4).

We estimated pollen production per anther level using an ELZONE (Micrometric, Atlanta, GA) particle counter. Pollen was collected just before anthesis from each anther level and morph ($n = 42$ long-level anthers from seven mid-styled flowers and seven short-styled flowers; $n = 45$ mid-level anthers from nine long-styled flowers and six short-styled flowers; $n = 42$ short-level anthers from 10 long-styled flowers and four mid-styled flowers). We used the mode rather than the arithmetic mean to compare the size of pollen grains. The mode is not

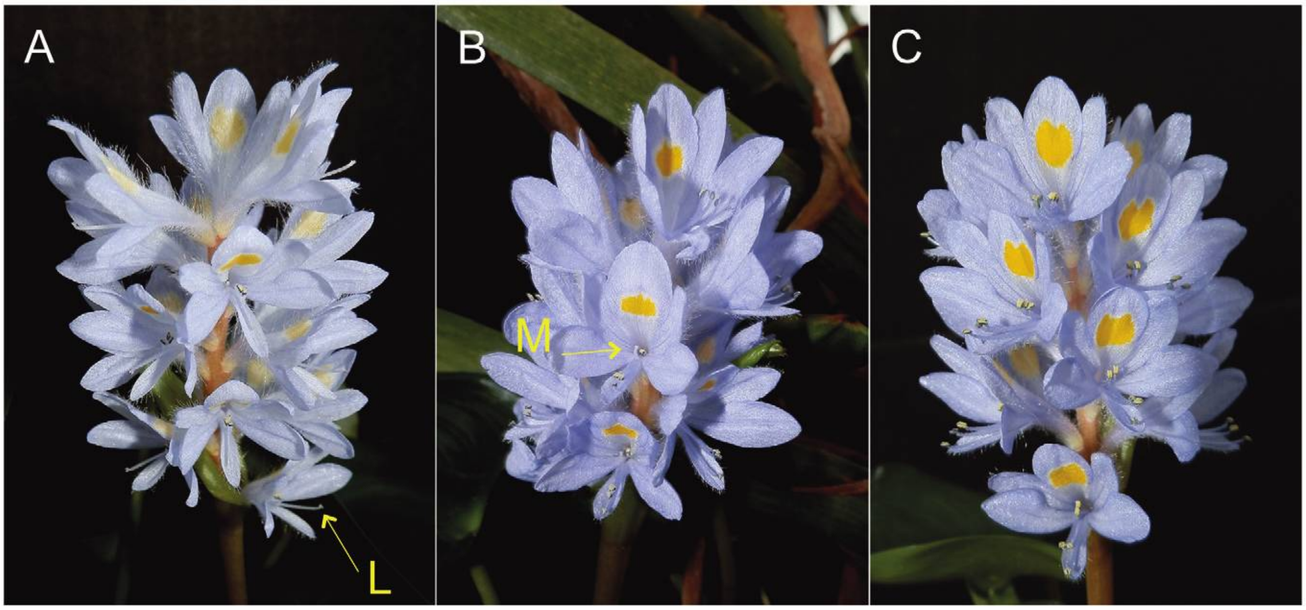

Fig. 1 Inflorescences of the long-styled (*A*), mid-styled (*B*), and short-styled (*C*) morphs of *Pontederia subovata*. The arrows in *A* and *B* indicate the long style (L) and mid style (M); the short style in *C* is not visible because it is located deep in the floral tube.

affected by the tails of the distribution, which often contain shriveled pollen grains (for details see Harder 1990; Harder and Barrett 1993). We examined the relation between pollen size and the natural logarithm of pollen production per anther level. Pollen grains are spherical in the salt solution used to estimate pollen production, so the size estimate we obtained is smaller than for the dry pollen diameter reported in figure 3.

To determine whether the stigmatic papillae of the floral morphs differed in length, we measured 50 stigmatic papillae from 10 stigmas per floral morph using a Zeiss Axioplan Universal light microscope at 400×. The papillae were placed on a small drop of distilled water on a microscope slide. Differences in mean stigmatic papillae length among the floral morphs were examined by repeated-measures ANOVA using JMP, version 8.02 (SAS 2009), and the differences between morphs were tested with specific contrasts.

Controlled Crossing Program

We conducted a controlled crossing program to determine whether *P. subovata* possesses a trimorphic incompatibility system. Flowers are short lived and senesce within 6–8 h of opening, and pollinations were therefore performed each morning before noon. Flowers to be cross-pollinated were emasculated, and pollen was transferred to stigmas using fine forceps. All flowers on a given inflorescence received the same pollination treatment, and the number of flowers pollinated was recorded. We harvested and counted fruits 15–19 d later when they were mature. Because flowers of *P. subovata* are uniovulate, fruit set is equivalent to seed set. We performed the following eight controlled hand pollinations on each floral morph: (1) self-pollinations with each anther level, (2) intramorph cross-pollinations with each anther level, (3) intermorph cross-pollinations with anthers and stigmas of dissimilar height, and (4) intermorph cross-pollinations with anthers and stigmas of equivalent height. Following Darwin (1877), pollinations 1–3 are termed "illegitimate pollinations," and those in pollination category 4 are referred to as "legitimate pollinations." If *P. subovata* exhibits trimorphic incompatibility, the amount of seed produced by illegitimate pollinations should be significantly less than in legitimate pollinations. Five inflorescences per morph received each pollination treatment, with the number of flowers pollinated ranging from 50 to 81. We analyzed the results of the crossing program using a generalized linear mixed model (SAS GLIMMIX; SAS 2011) that considered seed set a binary response (logit link function; Fitzmaurice et al. 2004). Multiple flowers received the same treatment on a given inflorescence, and the possible correlation within subject was accounted for by repeated measures in the statistical analysis. Specific contrasts were used to test for significant differences between treatments.

Results

Morphological Differences among the Floral Morphs

Measurements of sex organ height confirm that *Pontederia subovata* is tristylous. The three floral morphs differed in stigma and anther height, and these organs were positioned at three distinct levels corresponding to one another (fig. 2). The L-morph possesses a long style with mid- and short-level anthers, the M-morph a mid-length style with long- and short-level anthers, and the S-morph a short style with mid- and long-level anthers. The organ levels are reciprocally positioned, although there is considerable variation among flowers. Long- and mid-level organs are less separated from one another than short-level organs are from mid-level organs (fig. 2). Statistical analysis using repeated measures revealed differences in the

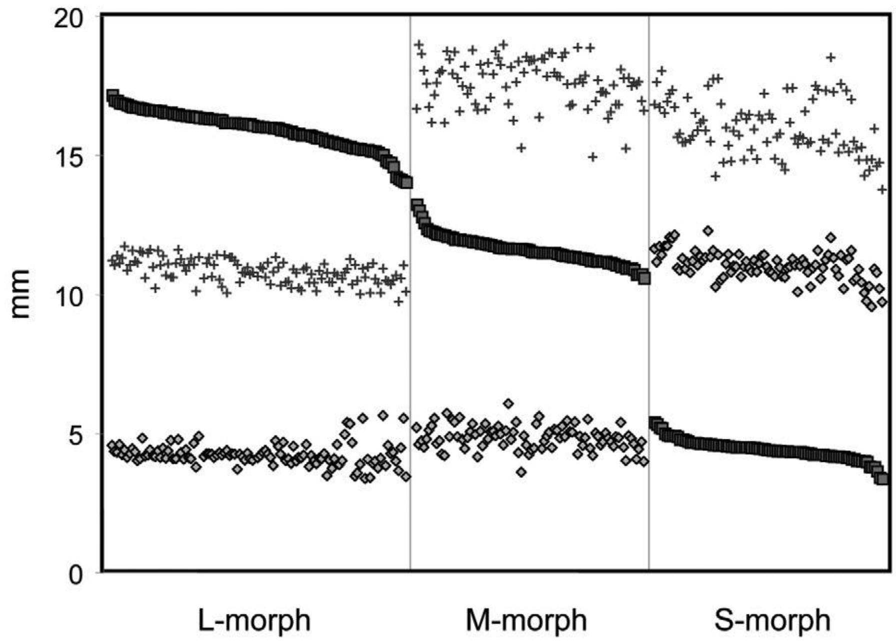

Fig. 2 Variation in stigma and anther height in the floral morphs of tristylous *Pontederia subovata* illustrating three sex organ heights and reciprocity between stigmas and anthers. Squares indicate stigma height, plus signs indicate heights of upper anther levels, and diamonds indicate heights of lower anther levels within each flower. Flowers are ranked by stigma height within each floral morph.

degree of variation among the three organ levels. Short-level organs were not significantly different in height ($F_{2,34} = 3.07$, $P = 0.06$), but stigmas and anthers of mid- and long-level organs exhibited weaker reciprocity (mid level, $F_{2,34} = 5.56$, $P = 0.008$; long level, $F_{2,34} = 10.96$, $P = 0.0002$).

There was significant variation in flower length among the floral morphs (mean flower length ± SD [mm]: L-morph, 19.5 ± 0.08; M-morph, 22.3 ± 0.09; S-morph, 20.7 ± 0.15; $F_{2,34} = 30.31$, $P < 0.0001$). The flower length of the M-morph was significantly longer than that of the L- and S-morphs, and the flower length of the L-morph was significantly shorter than that of the M- and S-morphs (all contrasts significant from $P < 0.02$ to <0.0001).

Pollen originating from the three anther levels of *P. subovata* differed significantly in size ($F_{5,2} = 75.68$, $P = 0.013$), and the species exhibits pollen size trimorphism (fig. 3). There was a greater overlap in the size of pollen from long- and mid-level anthers in comparison with pollen from mid- and short-level anthers. As expected in a tristylous species, pollen size declined with anther height, with the largest pollen produced by long-level anthers and the smallest by short-level anthers. As a result, there were significant differences between the size of pollen originating from alternate anther levels within each morph ($F_{3,2} = 95.17$, $P = 0.010$). There was no significant difference in the size of pollen originating from the same anther levels of different morphs (long-level anthers, $F_{1,2} = 0.685$, $P = 0.495$; mid-level anthers, $F_{1,2} = 0.853$, $P = 0.453$; short-level anthers, $F_{1,2} = 0.42$, $P = 0.584$).

Associated with pollen size trimorphism were corresponding differences in the amount of pollen produced by the three anther levels. Repeated-measures ANOVA indicated that short-level anthers produced significantly more pollen than did either mid- or long-level anthers ($F_{1,12} = 12.62$, $P = 0.004$), which were not significantly different from each other. Overall, there was a significant relation between pollen size and pollen production per anther level ($F_{1,13} = 4.45$, $P = 0.05$; fig. 4).

Measurements of stigmatic papillae length revealed significant differences among the floral morphs ($F_{2,27} = 137.7$, $P < 0.0001$). The stigmatic papillae of the L-morph were longest in length, whereas the shortest papillae were evident on stigmas of the S-morph, with intermediate-length papillae in the M-morph (mean stigmatic papillae length ± SD [μm]: L-morph, 50.44 ± 5.42; M-morph, 38.74 ± 2.45; S-morph, 33.07 ± 2.12; specific contrasts: L-morph vs. M-morph, $F_{1,27} = 120.2$, $P < 0.0001$; M-morph vs. S-morph, $F_{1,27} = 28.2$, $P < 0.0001$).

Controlled Pollinations of the Floral Morphs

Controlled hand pollinations demonstrated that *P. subovata* exhibits a trimorphic incompatibility system. On average, self-, intra-, and intermorph illegitimate pollinations produced only 25.5%, 25.8%, and 23%, respectively, of the seed produced from legitimate crosses. However, the overall reduction in seed set was strongest in the L-morph and weakest in the M-morph, indicating differences among the floral morphs in the overall strength of trimorphic incompatibility (fig. 5). This was reflected in the highly significant interaction of pollination treatment by floral morph in the statistical analysis ($F_{24,96} = 15.02$, $P < 0.0001$).

There were also striking differences among morphs in the expression of incompatibility between anther levels within a flower. For example, self-pollinations of the L-morph using pollen from mid- or short-level anthers resulted in almost no

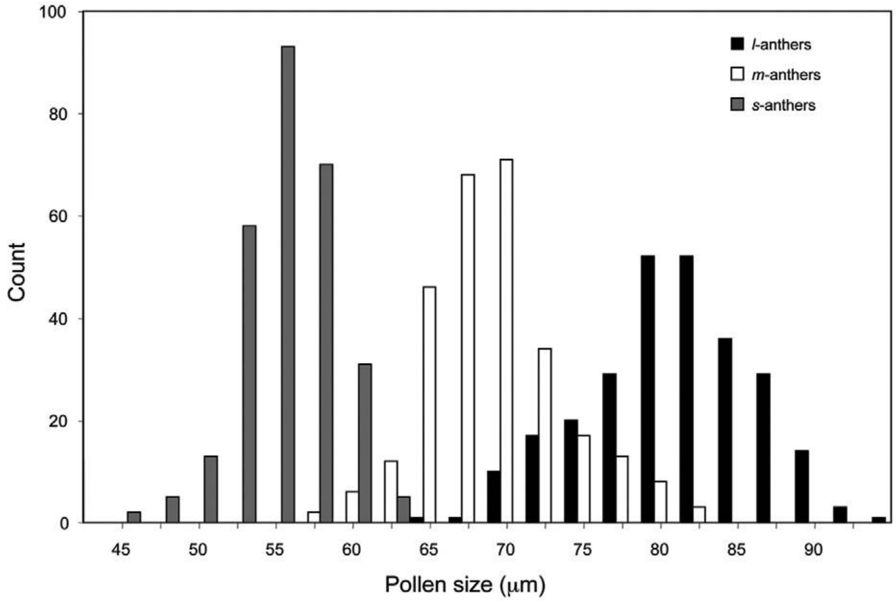

Fig. 3 Distribution of the size of pollen originating from the three anther levels in tristylous *Pontederia subovata*. The diameter of dry pollen was measured.

seed set. In contrast, self-pollinations of the M-morph using pollen from long-level anthers resulted in 86% seed set, whereas pollen from short-level anthers resulted in only one fruit. Similarly, in the S-morph incompatibility expression also depended on anther level, with 35% and 0% seed set when pollen from mid- and long-level anthers, respectively, was used in self-pollinations. Intramorph and intermorph illegitimate cross-pollinations resulted in patterns of seed set similar to those obtained from self-pollinations, as would be predicted in a species with trimorphic incompatibility. For example, the morph-specific differences in incompatibility expression revealed by self-pollination in the M- and S-morph also occurred in illegitimate cross-pollinations (fig. 5).

With the exception of illegitimate self- and cross-pollinations of the M-morph employing pollen from long-level anthers, legitimate pollinations of the floral morphs generally resulted in much higher seed set than did illegitimate pollinations, although the productivity of crosses varied among morphs (L-morph average seed set ± SE, 82.1% ± 4.4%; M-morph, 93.5% ± 3.6%; S-morph, 56.7% ± 3.8%). The seed set of the S-morph was significantly lower than that of the L- and M-morphs following legitimate pollination ($F_{1,96} = 45.94$, $P < 0.001$), perhaps because of technical difficulties in pollinating stigmas of the concealed short style of this morph. There were no significant differences in seed set between the two legitimate pollen donors within each morph (L-morph, $F_{1,96} = 0.23$, $P = 0.63$; M-morph, $F_{1,96} = 0.17$, $P = 0.68$; S-morph, $F_{1,96} = 1.09$, $P = 0.30$).

Clonal Regeneration

Pontederia subovata has spreading rhizomes that produce foliage leaves, inflorescences, and axillary elongated stolons with scale leaves and occasional adventitious roots. The distal nodes and internodes of stolons are swollen and produce elongated spherical stem tubers (fig. 6*A*). The tubers vary in size and when mature become brown in color and detached from the parent plant as a result of the disintegration of stolons. The tubers have conspicuous nodes and internodes and at their apical ends produce terminal scale leaves (fig. 6*B*, 6*C*). Individual plants produced dozens of tubers under glasshouse conditions.

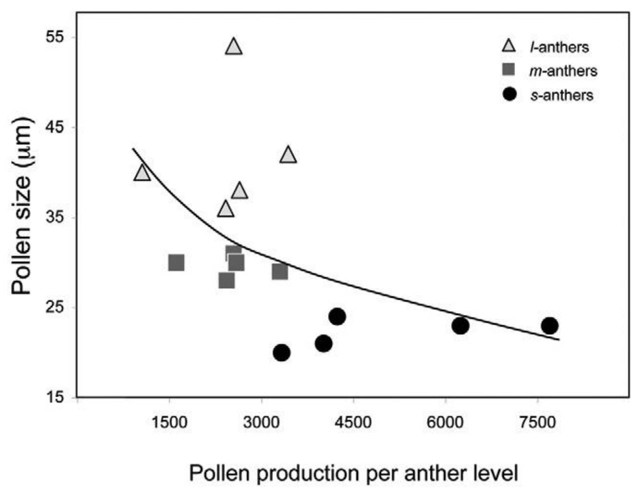

Fig. 4 Relation between the modal pollen size and pollen production per anther level for the three anther heights in tristylous *Pontederia subovata*. The diameter of pollen grains was measured in solution, and hence the measurements are different from those indicated in figure 3.

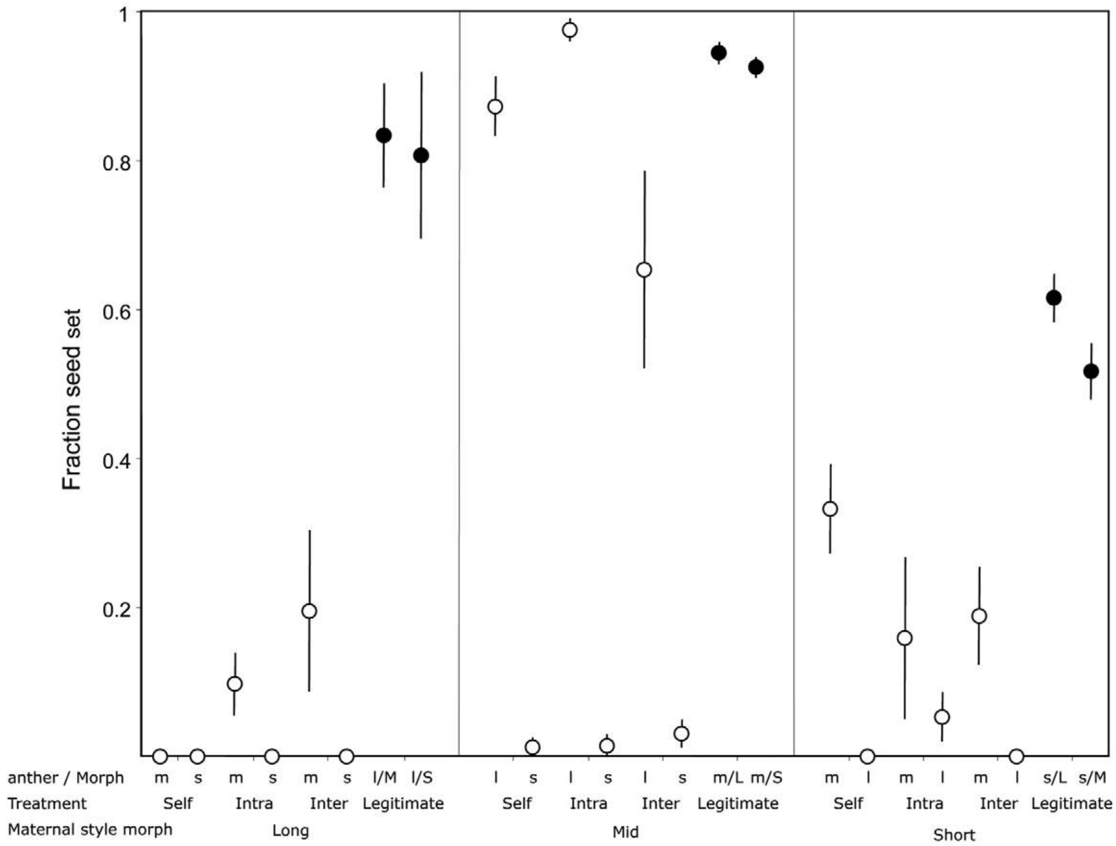

Fig. 5 Results of a controlled pollination program conducted on the three floral morphs of tristylous *Pontederia subovata* under glasshouse conditions. Fraction seed set ± SE from each of eight different pollinations conducted on the L-, M-, and S-morphs are illustrated. Filled circles indicate legitimate pollinations, and open circles indicate illegitimate pollinations (self-, intra-, and intermorph).

Discussion

Our study confirms earlier observations of herbarium specimens that *Pontederia subovata* exhibits tristyly. The species also possesses several features commonly associated with this complex floral polymorphism, including trimorphic incompatibility, pollen size trimorphism, variation in pollen production among anther levels, and differences in stigmatic papillae length among morphs. We begin by reviewing how the patterns of variation we observed compare with other tristylous taxa, particularly of *Pontederia*, and consider the possible ecological and evolutionary consequences of variation in the expression of self-incompatibility. We conclude by discussing the significance of our discovery of a new clonal strategy for Pontederiaceae and its probable ecological functions.

Morphological Polymorphisms

Our study was based on a relatively small sample of clones from a single location in southwest Brazil. Ideally, characterization of the tristylous syndrome of a species should be based on more extensive sampling of populations over a broader geographical range. Nevertheless, despite this limitation, flowers of *P. subovata* exhibit three distinct organ heights, and, more important, the anthers and stigmas are arranged in a reciprocal manner typical of a tristylous species. The degree of reciprocity exhibited by heterostylous species can vary widely (Sanchez et al. 2008), as a result of both developmental and genetic factors, and the degree of variation in sex organ height evident in figure 2 is not atypical for a tristylous species (reviewed in Eckert and Barrett 1994; see their fig. 12). Part of the variation we observed in *P. subovata* was associated with differences in the size of flowers sampled for measurement.

The most notable feature of the relative positions of organ heights in the floral morphs of *P. subovata* is the greater separation of short-level organs from mid-level organs compared to the difference in height of mid- and long-level organs (fig. 2). Why this occurs is unclear, but the differences in degree of separation among anther levels are also reflected in the patterns of pollen size differentiation (fig. 3). Investigations of the pollination process in tristylous *Pontederia cordata* indicate that short-level organs experience the most unpredictable patterns of pollen deposition on stigmas (Wolfe and Barrett 1989) and pollen removal from anthers (Harder and Barrett 1993). This stochasticity probably reflects the deeply recessed location of short-level organs within the floral tube and the extent to which they successfully contact the proboscis of pollinators. No published records of pollinators are reported for *P. subovata*, but in common with other *Pontederia* species (Barrett 1977; Glover and Barrett 1983; Wolfe and Barrett 1987, 1988), it

Fig. 6 Stem tubers in *Pontederia subovata*. *A*, Flowering plant illustrating creeping stem with foliage leaves, rhizome, and stem tubers at the swollen distal end of slender stolons. *B*, Stem tubers with terminal scale leaves and adventitious roots. *C*, Stem tubers of different age, size, and color with conspicuous nodes and internodes.

is likely that long-tongued bees, butterflies, and flies are the main pollinators of the species.

Measurements of pollen size in *P. subovata* indicate that in common with most tristylous species, the size of pollen varies with anther height, with the largest pollen produced by long-level anthers and the smallest by short-level anthers (fig. 3). There was considerable overlap in the size of pollen from long- and mid-level anthers and less overlap between pollen from mid- and short-level anthers. In this respect, *P. subovata* differs from other tristylous *Pontederia* species in which pollen from the three anther levels is generally more distinct in size (Price and Barrett 1982; Glover and Barrett 1983). Associated with the differences in pollen size among anther levels in *P. subovata* were differences in pollen production, as occurs in other *Pontederia* species (Price and Barrett 1982; Glover and Barrett 1983; Harder and Barrett 1993). Short-level anthers produced significantly more pollen than did mid- and long-level anthers, which were not significantly different in pollen production. Thus, there was only weak evidence for a pollen size–number trade-off among anther levels in *P. subovata* (fig. 4).

The other ancillary morphological polymorphism that we observed was differences in the length of stigmatic papillae in the floral morphs similar to those reported in other tristylous species, including *Pontederia sagittata* (Scribailo and Barrett 1991*a*). The longest papillae were produced by long styles, the shortest papillae were produced by short styles, and the papillae of mid styles were intermediate in length. It has been suggested that polymorphism in stigmatic papillae length, in conjunction with pollen size heteromorphism, may serve to promote morphological complementarity between legitimate pollen and stigmas during the pollination process and thus favors compatible crosses (see Dulberger 1992). This hypothesis would be worth investigating in *Pontederia* species where pollen size trimorphism is well developed in comparison with other tristylous families.

Variation in Trimorphic Incompatibility

Controlled pollination studies of tristylous species frequently reveal that trimorphic incompatibility is not as strongly expressed as is often depicted in idealized diagrams of the polymorphism (e.g., fig. 1 in Barrett 1992). Although a few taxa display this pattern (e.g., *Lythrum junceum* [Dulberger 1970], *Oxalis* spp. section *Ionoxalis* [Weller 1980]), more commonly there is evidence of variation among the floral morphs in the expression of incompatibility (e.g., *Eichhornia azurea* [Bianchi et al. 2000], *Lythrum salicaria* [Colautti et al. 2010], *Oxalis* spp. [Ornduff 1972]), including several tristylous species that exhibit cryptic self-incompatibility (e.g., *Eichhornia paniculata* [Cruzan and Barrett 1993], *Decodon verticillatus* [Eckert and Allen 1997]). Our controlled crosses indicated that legitimate pollinations in most cases produced significantly more seed than did illegitimate pollinations, demonstrating the occurrence of trimorphic incompatibility in *P. subovata* (fig. 5). However, our results resemble those previously reported in four other tristylous taxa of *Pontederia* (see fig. 4 in Barrett and Anderson 1985), all of which reported significant morph-specific variation in the strength of trimorphic incompatibility, especially involving the M-morph.

The most notable feature of the patterns of seed set we obtained were the striking differences in compatibility evident between the two anther levels within a flower in illegitimate pollinations. For example, self-, intra-, and intermorph pollinations of the M-morph with short-level pollen produced very little seed and were thus strongly incompatible. In contrast, the same classes of pollination conducted with pollen from long-level anthers produced abundant seed (fig. 5). Indeed, in some cases seed set was equivalent to that produced from legitimate pollinations. Similar, although weaker, effects were evident in the S- and L-morphs, with the latter being the most strongly incompatible floral morph. Intraplant differentiation in pollen behavior is nearly unique to tristylous species but has been reported from *Collomia grandiflora* (Lord and Eckard 1984).

Our results demonstrating high levels of self-compatibility following illegitimate pollinations of the M-morph appear to be a general feature of trimorphic incompatibility in Pontederiaceae. This pattern has now been reported from five taxa (*E. azurea* [Bianchi et al. 2000], *P. cordata* var. *cordata* and *lancifolia* [Barrett and Anderson 1985], *Pontederia rotundifolia* [Barrett 1977], *P. sagittata* [Glover and Barrett 1983], and *P. subovata* [this study]) but may not be restricted to the family (see Charlesworth 1979). In *L. salicaria* (Lythraceae) trimorphic incompatibility is also weakly expressed in the M-morph when flowers are pollinated with pollen from long-level anthers (Darwin 1877; Colautti et al. 2010). Understanding of the proximate molecular, developmental, and physiological mechanisms governing variation in trimorphic incompatibility is rudimentary at best, and why this particular pattern of compatibility occurs remains a mystery, although it may have ecological significance.

Self-pollination providing reproductive assurance is of importance for establishment following dispersal or under low-density conditions when pollinators or mates are in short supply (Baker 1955; Pannell and Barrett 1998; Cheptou 2012). Heterostylous species may be especially prone to selection for reproductive assurance because they have only two or three mating types and often require long-tongued pollinators to maintain fertility. Aquatics such as *P. subovata* are especially adept at dispersal (Ridley 1930; Sculthorpe 1967), and this is reflected by the disjunct distribution of many species, including *P. subovata* (Lowden 1973). Recurrent colonizing episodes could therefore play a role in maintaining the weak expression of self-incompatibility in the M-morph, especially since the floral architecture of this morph makes it more vulnerable to self-pollination than the other morphs (Charlesworth 1979; Kohn and Barrett 1992). If the M-morph is better at establishing colonies following dispersal than are the L- and S-morphs, populations monomorphic for the M-morph, or dimorphic (L- and M-morphs) populations, would be expected. This latter condition would arise following genetic segregation in colonists heterozygous at the *M*-locus, assuming that in *P. subovata* the inheritance of tristyly is the same as in *P. cordata* (Gettys and Wofford 2008). Field surveys of population morph structure in *P. subovata* and studies of inbreeding depression in the M-morph would be valuable to assess this hypothesis.

A New Clonal Strategy for Pontederiaceae

Aquatic plants are well known for their reliance on asexual methods of propagation. A wide diversity of mechanisms for achieving vegetative reproduction, perennation, and dispersal are documented (Arber 1920; Sculthorpe 1967; Grace 1993). Pontederiaceae is no exception, and most perennials in this family possess some form of clonal propagation (reviewed in Barrett and Graham 1997). These include rhizomes (e.g., *P. cordata*, *Monochoria hastata*); fragmentation of creeping or floating stems in procumbent, amphibious (e.g., *E. azurea*, *P. rotundifolia*), or submersed taxa (*Eichhornia natans*, *Heteranthera dubia*); and the formation of brittle stolons and floating daughter rosettes (*Eichhornia crassipes*). An unexpected finding from our study was the discovery of a new clonal strategy not reported previously in Pontederiaceae. Plants grown in the glasshouse produced abundant tubers on slender stolons and also had elongated creeping stems and rhizomes (fig. 6).

Pontederia subovata is particularly abundant in the Pantanal of lowland South America, a region with a marked dry season from April to September. During this period the flooded habitats that the species normally occupies experience falling water levels and desiccation. In these environments, stem tubers likely function to enable the survival of genets during the unfavorable dry period. Indeed, we have regenerated plants from dormant tubers in the glasshouse by simply placing them in flooded soil and allowing them to sprout leaves and roots. Why this clonal strategy does not apparently occur in other Pontederiaceae is unclear. Most species in the family that occur in seasonal tropical environments with a dry season possess the annual habit (e.g., *E. paniculata* and *Heteranthera* spp.), presumably as a means of avoiding the desiccation of vegetative structures (Barrett and Graham 1997). Ecological and developmental constraints may limit the evolution of the annual life form in *Pontederia* where it is absent.

Stem tubers in *P. subovata* also have other ecological functions aside from perennation. They are easily detached from the parent plant and therefore serve as a mechanism for numerical increase through vegetative reproduction. Tubers that accumulate close to the maternal parent will increase clone size and during subsequent flowering would promote geitonogamous pollination. Under these circumstances tristyly may help to limit intraclonal pollen dispersal (Vallejo-Marín et al. 2010). On the other hand, stem tubers may also function as means of genet dispersal in water currents during flooding and would thus promote the mixing of genets and enhance cross-pollination. Future investigations of the reproductive consequences of clonal propagation and population morph structure for the functioning of tristyly in *P. subovata* would be of interest.

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