

## ENANTIOSTYLY IN *WACHENDORFIA* (HAEMODORACEAE): THE INFLUENCE OF REPRODUCTIVE SYSTEMS ON THE MAINTENANCE OF THE POLYMORPHISM<sup>1</sup>

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Enantiostyly is a form of directional asymmetry in plants in which the style is deflected away from the main axis of the flower, either to the left or right side. In *Wachendorfia* (Haemodoraceae), a small genus of insect-pollinated geophytes restricted to the Cape Province of South Africa, populations are usually polymorphic for asymmetry. Here we investigate dimorphic enantiostyly in the four species of *Wachendorfia* to determine whether variation in their reproductive systems influences the maintenance of this genetic polymorphism. Experimental field pollinations of *W. paniculata* and *W. thyrsoiflora* indicated higher fertility for cross- than for self-pollinations, whereas in *W. brachyandra*, these types of pollination produced similar levels of fertility. Outcrossing rates were highest in *W. paniculata* ( $t = 0.78$ – $0.98$ ), with *W. brachyandra* ( $t = 0.39$ – $0.79$ ) and *W. thyrsoiflora* ( $t = 0.76$ ) exhibiting mixed mating systems. Outcrossing rates in two populations of *W. parviflora* varied from mixed mating ( $t = 0.61$ ) to predominant selfing ( $t = 0.07$ ). Population style-morph ratios ranged from 1 : 1 in outcrossing *W. paniculata* to monomorphism in selfing *W. parviflora* and clonal *W. thyrsoiflora*. In *W. brachyandra*, a species with delayed selfing, morph ratios were usually biased. The maintenance of enantiostyly in *Wachendorfia* appears to be strongly influenced by levels of disassortative mating and the balance between sexual and clonal recruitment.

**Key words:** delayed selfing; enantiostyly; experimental pollinations; floral design; Haemodoraceae; outcrossing rates; spatial analysis; style-morph ratios; *Wachendorfia*.

Bilateral symmetry is a fundamental aspect of the morphology of many organisms and has probably evolved a limited number of times. Indeed, breaking the symmetry of morphological structures is very difficult, as indicated by experiments that have attempted to select for the direction of asymmetry (Maynard Smith and Sondhi, 1960; Coyne, 1987; Tuinstra, de Jong, and Scharloo, 1990). Despite this, conspicuous directional asymmetries in morphology are known from a wide range of organisms (reviewed in Palmer, 1996). However, the co-occurrence of both asymmetric forms in a population is much less common, although such variation has been reported from various animals, including snails (Johnson, 1982; Asami et al., 1998), cichlid fish (Hori, 1993), and birds (Benkman, 1996). Directional asymmetry polymorphisms can be used as model systems for asking evolutionary questions concerning their origin and maintenance within populations. The phenotypes (hereafter morphs) are easily recognizable in populations, are often under simple genetic control, and equilibrium morph ratios implicate frequency-dependent selection as the primary mechanism maintaining these asymmetries (Johnson, 1982; Hori, 1993; Benkman, 1996; Asami et al., 1998). Determining the reproductive systems of species with asymmetric polymorphisms is critical for understanding their evolution and maintenance in nature. This is particularly important in plants in which hermaphroditism and clonal propagation can interfere with outcrossing opportunities (Barrett

and Harder, 1996), thus reducing the intensity of frequency-dependent selection.

Enantiostyly is a plant sexual polymorphism in which the style is positioned to the left (left-styled) or to the right (right-styled) side of the flower. The polymorphism is widely distributed occurring in at least a dozen animal-pollinated angiosperm families in both monocotyledons and dicotyledons (Barrett, Baker, and Jesson, 2000; Barrett, Jesson, and Baker, 2000). Enantiostyly has usually been interpreted as a floral design that promotes insect-mediated cross-pollination in a manner functionally analogous to heterostyly (Todd, 1882; Wilson, 1887; Robertson, 1890; Iyengar, 1923; Ornduff, 1974; Ornduff and Dulberger, 1978; Fenster, 1995). However, the hypothesis that the floral polymorphism promotes proficient cross-pollination between flower types has not been examined experimentally in any detail (although see Bowers, 1975). Moreover, the occurrence of two distinct forms of the polymorphism (monomorphic and dimorphic enantiostyly—Barrett, Baker, and Jesson, 2000; Barrett, Jesson, and Baker, 2000) complicates this simple adaptive explanation.

In monomorphic enantiostyly, both left- and right-styled flowers occur on the same plant, resulting in a form of within-organism antisymmetry (see Palmer, 1996). In contrast, dimorphic enantiostyly is a genetic polymorphism analogous to directional asymmetry, with populations composed of left- and right-styled plants. The two forms of enantiostyly differ markedly in their abundance and phylogenetic distribution among angiosperm families. Monomorphic enantiostyly is widely distributed, whereas dimorphic enantiostyly is only known from a few species in three monocotyledonous families (for Haemodoraceae; Wilson, 1887; Ornduff and Dulberger, 1978; for Pontederiaceae; Barrett, Baker, and Jesson, 2000; Barrett, Jesson, and Baker, 2000; and unpublished data; for Tecophilaeaceae; Dulberger and Ornduff, 1980). Phylogenetic evidence suggests that dimorphic enantiostyly is most likely derived from monomorphic enantiostyly (reviewed in Barrett, Baker,

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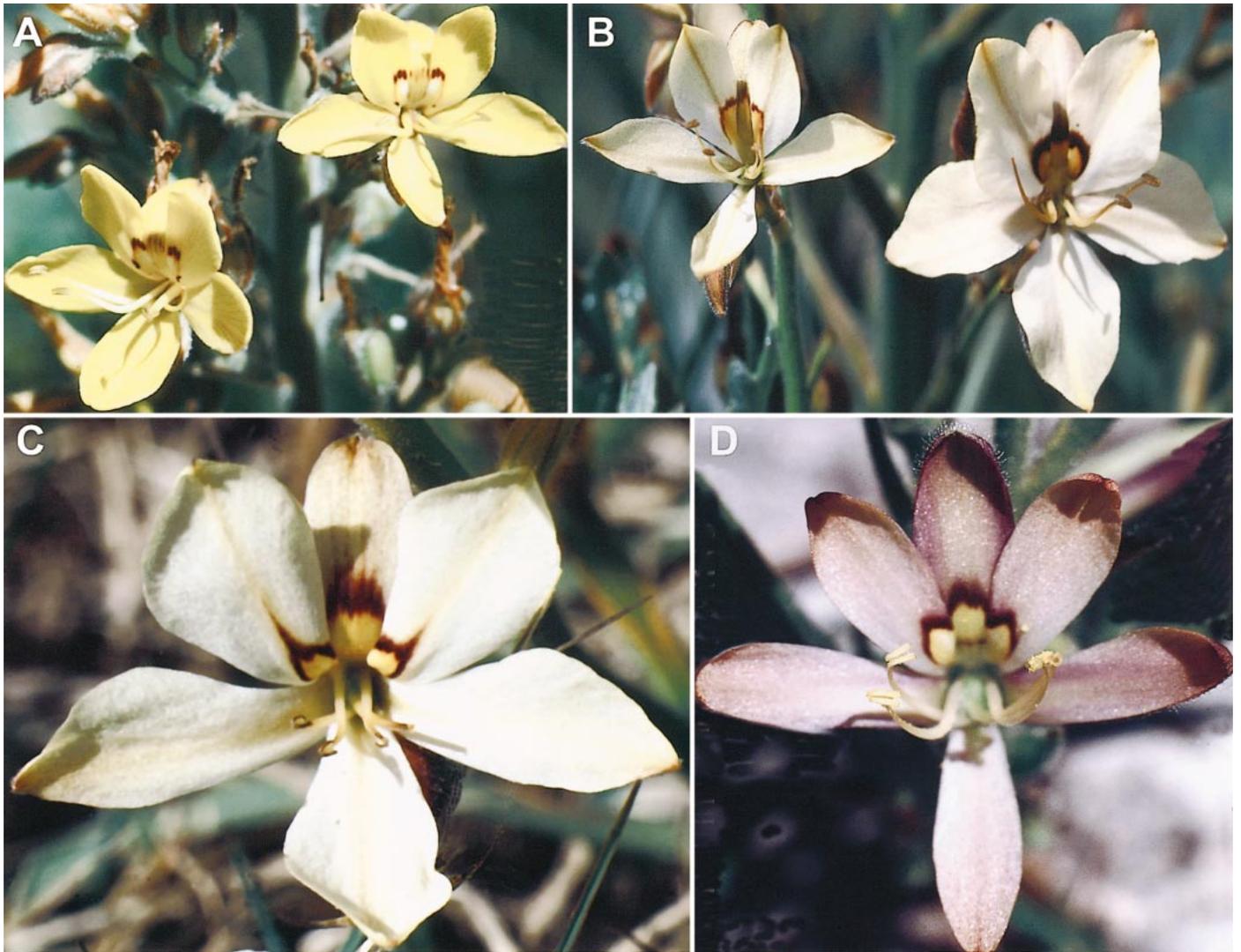


Fig. 1. Flowers of *Wachendorfia* species. (A) *W. thyrsoiflora*. (B) *W. paniculata*. Forms 1 (right) and 3 (left). (C) *W. brachyandra*. (D) *W. parviflora*. See Table 1 for mean flower sizes and numbers.

and Jesson, 2000; Barrett, Jesson, and Baker, 2000). The rarity of dimorphic enantiostyly implies that there must be strong constraints on its evolutionary origins.

*Wachendorfia* Burm. (Haemodoraceae) is a small genus of four species of insect-pollinated geophytes native to the Cape Province of South Africa (Helme and Linder, 1992; Fig. 1). The first report of enantiostyly in *Wachendorfia* involved *W. paniculata* (Wilson, 1887), a common species of open sites in fynbos vegetation. Subsequent research on this species reported 1 : 1 morph ratios from four populations (Ornduff, 1974) and weakly developed self-incompatibility in which intermorph crosses produced more seeds than self- or intramorph pollinations (Ornduff and Dulberger, 1978). There have been no detailed studies of the reproductive ecology of the remaining species of *Wachendorfia*, although both Ornduff (1974) and Helme and Linder (1992) mentioned that all species in the genus possess enantiostyly and that the floral morphology of *W. brachyandra* suggests some degree of autogamy.

As part of a broader study of the evolution and functional significance of enantiostyly we were interested in further in-

vestigating the polymorphism in *Wachendorfia*. Our preliminary field observations indicated that the four species differed in floral traits likely to influence selfing, as well in the extent of clonal propagation. Equal morph ratios are predicted in an enantiostylous population with equivalent levels of disassortative (between morph) mating (Ornduff, 1974; Barrett, Baker, and Jesson, 2000; Barrett, Jesson, and Baker, 2000). However, reductions in disassortative mating through either selfing or a reliance on clonal propagation are likely to result in deviations from 1 : 1, potentially leading to floral monomorphism and destabilization of the polymorphism. These processes are well known in heterostylous plants, resulting in biased morph ratios and in some cases the breakdown of heterostyly (reviewed in Barrett, 1992). The major goal of this study was to determine whether similar evolutionary processes might occur in populations of *Wachendorfia* species.

Here we report the first comparative data on the reproductive ecology of an enantiostylous plant group. In this study we employed field surveys, experimental pollinations, and electrophoretic analyses of mating patterns in *Wachendorfia* spe-

cies to address the following specific questions: (1) Do the four species differ in aspects of floral biology that are likely to influence mating patterns and hence morph frequencies? (2) What is the relation between floral features predicted to influence selfing and the outcrossing rates of populations? (3) What are the ratios of left- and right-styled plants in natural populations of each species and are differences in their frequency and distribution associated with the reproductive systems of populations? We conclude by discussing the various reproductive factors likely to influence the maintenance of enantiostyly and consider evidence for the evolutionary breakdown of the polymorphism.

## MATERIALS AND METHODS

**Natural history of *Wachendorfia***—All *Wachendorfia* species produce paniculate inflorescences composed of varying numbers of yellow to apricot zygomorphic flowers. Flowers of all species last 1 d, with the exception of *W. thyrsiflora*, which has flowers that can last 2 d under rainy conditions. Flowering occurs from late August to December with the exception of *W. parviflora*, which flowers for a shorter duration from early August to September. Peak flowering usually occurs in September for *W. brachyandra*, *W. paniculata*, and *W. thyrsiflora*. Habitat specialization varies among species, with *W. paniculata* and *W. parviflora* occurring in dry, open sites, *W. brachyandra* in damp soil, and *W. thyrsiflora* usually in habitats with standing water. Populations of *W. thyrsiflora* in aquatic habitats reproduce primarily by vegetative propagation with clones often many meters in diameter as a result of the lateral growth of rhizomes (Helme and Linder, 1992). In the remaining largely terrestrial species, only limited clonal propagation occurs through corm splitting. *Wachendorfia paniculata* is the most abundant of the four species, ranging from Nieuwoudtville to Port Elizabeth in the Cape Province. *Wachendorfia thyrsiflora* has a similar range, whereas *W. parviflora* is confined to the western Cape, and the rarest species, *W. brachyandra*, is restricted to the extreme southwestern Cape (Helme and Linder, 1992).

*Wachendorfia paniculata* is a highly variable taxon previously divided into two species and three varieties (Barker, 1950). More recently, Helme and Linder (1992) distinguished three distinct forms of *W. paniculata*. Form 1 is the most common type occurring throughout the species' range. Form 2 is restricted to mountains in northern areas of the western Cape. Form 3 is found along the coast of the Cape Peninsula and southern Cape, especially in exposed, often windy areas. These three forms differ morphologically from one another: Form 1 is the most variable, Form 2 is frequently very tall, with lax panicles and long-thin leaves, and in Form 3, plants are of dwarf stature with very narrow hairy leaves. Forms 1 and 2 both exhibit large flowers with significant spatial separation between anthers and stigma (herkogamy), whereas Form 3 has smaller flowers with less herkogamy.

To determine reproductive attributes of the four species, we measured flower diameter, stigma-anther separation, daily flower number, total flower number, fruit set, and seed set in several populations of each species. Sample sizes and the populations sampled are presented in RESULTS. Pollinators were also recorded whenever they were observed, and their foraging behavior noted.

**Compatibility relationships**—We performed controlled pollinations in natural populations of *W. brachyandra* (Oranjekloof) and *W. paniculata* Form 1 (Vredehoek and Camps Bay) in 1997, and *W. thyrsiflora* (Oranjekloof) in 1998 to determine the compatibility status of species. Sample sizes for plants and flowers are presented in RESULTS. Three treatments were applied to each style morph: self-pollination, intramorph pollination, and intermorph pollination. All three treatments were applied to flowers on a given plant each day. Cross-pollinations used a single pollen donor for each flower that was pollinated. Prior to pollination, we bagged plants with fine curtain mesh to exclude pollinators and emasculated flowers early each morning, prior to anther dehiscence. Following pollination, flowers were rebagged for a 3–5 d period. Fruits were collected 4 wk later, and while the seeds had not completely matured, it was possible to record whether a capsule was formed (fruit set) and the number of seeds present in each capsule (seed set).

We performed general linear models on seed and fruit set data in S-Plus (Mathsoft, 1997); capsules that produced no seeds were excluded from the analysis of seed set. Due to violation of assumptions of normality, we analyzed data for *W. paniculata* seed set using general linear models (GLM) with an inverse link function (Venables and Ripley, 1994). In *W. brachyandra*, we performed a logistic GLM, coding seed set as a binary response; capsules with either one or two seeds were coded as 0, and capsules with three seeds were coded as 1. For *W. thyrsiflora*, we log-transformed the number of seeds and analyzed the data using a Gaussian distribution. Transformation of data did not influence the qualitative results. For all species except *W. brachyandra*, which exhibited near maximal fruit set, we analyzed fruit set data using logistic regression. We performed pairwise contrasts comparing self- and outcross- and comparing intramorph and intermorph pollinations to examine the effect of treatment on mean seed and fruit set.

**Daily variation in sex-organ position**—Preliminary observations indicated that stigma-anther separation in *Wachendorfia* flowers changed during anthesis, which has potential implications for self-pollination. To quantify these morphological changes, we measured the distance from the stigma to all anthers (three) in a flower in *W. brachyandra* (Oranjekloof), *W. paniculata* Form 1 (Silvermine), and *W. thyrsiflora* (Oranjekloof). During the course of a day, we measured one flower on each of eight plants per species at 2-h intervals. We present data for stigma-anther separation for a single anther only. The anther chosen was the closest anther to the stigma. Changes in the distance between the anther and the stigma were analyzed using linear mixed-effects models (Mathsoft, 1997). Time was treated as a random effect and species as a fixed effect. We used log-likelihood ratios to test for the importance of the fixed effects in the final model.

**Measurement of outcrossing rates**—To determine multilocus outcrossing rates ( $t_m$ ) in the four species, we performed horizontal starch gel electrophoresis on open-pollinated seeds from two populations each of *Wachendorfia brachyandra* [Oranjekloof (OK) and Kommetjie (Kom)], *W. paniculata* Form 1 [Camps Bay (CB) and Kirstenbosch (Kir)], *W. paniculata* Form 3 [Red Hill (RH), Scarborough (Scar)] and *W. parviflora* (Red Hill and Kirstenbosch), and one population of *W. thyrsiflora* (Oranjekloof). We scored 20–28 families sampled from each population for allozyme variability. Eighteen enzyme systems were originally screened for polymorphism, following the methods of Glover and Barrett (1987). Three polymorphic enzyme systems (*Pgi*, *Pgm-1*, and *Pgm-2*) were reliably resolved in *W. brachyandra*, *W. parviflora*, and *W. thyrsiflora*, and five enzyme systems were resolved in *W. paniculata* (*Pgi*, *Pgm-1*, *Pgm-2*, *Pgd*, and *Adh*) on a histidine-citrate buffer system (pH = 6.2). Ritland's (1990) MLTR program was used to estimate  $t_m$  for each population. Standard errors were calculated as the standard deviation of 1000 bootstraps with the family as the unit of resampling. Differences in outcrossing rate between populations were assessed using pairwise comparisons of bootstrap estimates (Eckert and Barrett, 1994). Two populations were considered to differ significantly in outcrossing rate if  $100(1 - \alpha_{PC}/2)$  of the differences between randomly paired bootstrap estimates lay either all above or all below zero (where  $\alpha_{PC}$  is the Type I error rate). When examining differences in the estimate of outcrossing rate between the four populations of *W. paniculata*, the experimental error rate ( $\alpha_{EW}$ ) was held at  $\alpha_{EW} = 0.05$  using Sidak's correction for nonorthogonal contrasts.

**Population surveys**—To determine the relative frequencies of left- and right-styled plants in populations of each species of *Wachendorfia*, we surveyed 31, 14, 5, and 4 populations of *W. paniculata*, *W. thyrsiflora*, *W. brachyandra*, and *W. parviflora*. These sample sizes reflect the differing abundance of the four species. Only populations of Forms 1 and 3 of *W. paniculata* were sampled. Pooled goodness-of-fit *G* tests were conducted to determine whether morph ratios of all populations in a species differed from 1 : 1. *G* tests of heterogeneity were also performed to examine heterogeneity in morph ratios between populations. Data on style-morph ratios, population sizes, and localities are available on request from L. K. Jesson.

We investigated spatial structuring of morphs in *W. brachyandra*, *W. paniculata*, and *W. thyrsiflora* in either  $3 \times 3$  m or  $6 \times 6$  m plots by mapping

TABLE 1. Variation in reproductive traits and female fertility of *Wachendorfia* species.

Reproductive traits	<i>W. brachyandra</i>	<i>W. paniculata</i>	<i>W. thyrsoiflora</i>	<i>W. parviflora</i>
Mean stigma-anther separation in mm (range)	1.12 (0–2.77) <sup>1</sup>	4.47 (2.00–7.48) <sup>3</sup>	4.50 (2.16–7.52) <sup>1</sup>	1.22 (0–4.25) <sup>9</sup>
Mean daily flower number (range)	3.02 (1–11) <sup>1</sup> 2.70 (1–7) <sup>2</sup>	2.57 (1–6) <sup>4</sup> 2.14 (1–5) <sup>5</sup> 4.68 (1–11) <sup>6</sup>	11.51 (1–57) <sup>1</sup> 34.94 (3–90) <sup>7</sup>	3.16 (1–8) <sup>9</sup> 1.49 (1–4) <sup>10</sup>
Mean total flower number (range)	27.69 (5–152) <sup>1</sup> 41.36 (4–89) <sup>2</sup>	49.26 (13–89) <sup>4</sup> 44.04 (14–102) <sup>5</sup> 65.50 (17–186) <sup>6</sup>	478.90 (372–638) <sup>1</sup> 237.20 (131–458) <sup>8</sup>	11.31 (3–26) <sup>9</sup> 33.95 (10–80) <sup>10</sup>
Percent natural fruit set (SE)	0.70 (0.02) <sup>1</sup> 0.78 (0.03) <sup>2</sup>	0.52 (0.03) <sup>4</sup> 0.45 (0.03) <sup>5</sup> 0.52 (0.03) <sup>6</sup>	0.84 (0.02) <sup>1</sup> 0.52 (0.07) <sup>8</sup>	0.77 (0.03) <sup>5</sup>
Mean natural seed set (SE)	2.50 (0.04) <sup>1</sup>	2.28 (0.04) <sup>6</sup>	2.48 (0.09) <sup>8</sup>	2.60 (0.04) <sup>5</sup>

Note: Populations are <sup>1</sup>Oranjekloof, <sup>2</sup>Kommetjie, <sup>3</sup>Silvermine, <sup>4</sup>Cape Point (*Wachendorfia paniculata* Form 3), <sup>5</sup>Kirstenbosch, <sup>6</sup>Camps Bay, <sup>7</sup>Paarl Nature Reserve, <sup>8</sup>Tokai Forest, <sup>9</sup>Red Hill, and <sup>10</sup>Pipeline Track, Table Mountain.

the location and morph of each flowering plant. Plot size was dependent on the density of plants in a population. Two populations of *W. paniculata* Form 1 were mapped: one in the Silvermine Reserve on the Cape Peninsula (6 × 6 m plot) and the other in natural vegetation at Kirstenbosch Botanic Gardens in Capetown (3 × 3 m). Plots for *W. brachyandra* (6 × 6 m) and *W. thyrsoiflora* (3 × 3 m) were located at Kommetjie and Oranjekloof, respectively. We performed a log-linear analysis to test for independence between the morph of each focal plant and the morph of its nearest neighbor, as well as the distance to the nearest neighbor (Pielou, 1961).

## RESULTS

**Natural history**—Our observations of visitors to flowers of *Wachendorfia* species revealed that honey bees (*Apis mellifera*) were the most common visitors to *W. brachyandra*, *W. paniculata*, and *W. thyrsoiflora*. We did not observe any visits to *W. parviflora* flowers. Other visitors observed on *W. paniculata* included tabinid flies (especially *Philolich* spp.) and anthophorid bees. Hopliid beetles also visited flowers regularly, although this often involved predation of floral organs. Carpenter bees (*Xylocopa* spp.) were occasionally observed visiting flowers of *W. thyrsoiflora*. After heavy rain, butterflies visited flowers of *W. thyrsoiflora* in great numbers. Nectar was the main reward for most visitors, although honey bees also collected pollen, and hopliid beetles consumed pollen and anthers.

TABLE 2. Summary of general linear models of seed set and fruit set from controlled pollinations in three species of *Wachendorfia*. Fruit set of *W. brachyandra* was not analyzed due to near maximal fruit set. \*0.05 > P > 0.01; \*\*0.05 > P > 0.001; \*\*\*P < 0.001.

Species	Effects	df	Seed set deviance	Fruit set deviance
<i>W. brachyandra</i>	Morph	1	1.63	
	Plant	28	56.18***	
	Treatment	2	13.21***	
	Error	215	190.94	
<i>W. paniculata</i> (Form 1)	Population	1	0.13	49.29***
	Morph	1	0.01	15.29***
	Plant	27	3.83**	66.37***
	Treatment	2	0.576*	17.21***
	Error	202/382 <sup>a</sup>	18.55	371.69
<i>W. thyrsoiflora</i>	Morph	1	0.01	1.18
	Plant	19	22.34***	81.57
	Treatment	2	2.18*	3.45
	Error	161/160 <sup>a</sup>	32.27	168.33

<sup>a</sup> Error degrees of freedom are presented for seed set and fruit set, respectively.

There was striking variation among the four species in mean flower diameter. Two species were characterized by large flowers (*W. paniculata* Form 1 = 38.08 mm, SE = 0.49, N = 88; *W. thyrsoiflora* = 32.02 mm, SE = 0.35, N = 40), while the other two possessed much smaller flowers (*W. brachyandra* = 19.68 mm, SE = 1.02, N = 40; *W. parviflora* = 18.31 mm, SE = 0.86, N = 57). Comparisons of mean daily and total flower number per inflorescence also revealed large differences among the four species (Table 1). *Wachendorfia brachyandra*, *W. paniculata*, and *W. parviflora* all had daily flower numbers ranging between 1 and 11 flowers, whereas *W. thyrsoiflora* had a much higher daily flower number, ranging between 1 and 90 flowers. Total flower number of *W. thyrsoiflora* was also considerably higher than the other three species. There was considerable variation between species in natural fruit set, ranging from 45% in *W. paniculata* at Kirstenbosch to 84% in *W. thyrsoiflora* at Oranjekloof. Ovule number in *Wachendorfia* species is fixed at three (Helme and Linder, 1994). The mean number of seeds per fruit was similar among the four species, ranging from 2.28 in *W. paniculata* to 2.6 in *W. parviflora* (Table 1).

**Compatibility relationships**—Controlled self- and cross-pollinations in the field revealed that *W. brachyandra* is strongly self-compatible, whereas *W. paniculata* and *W. thyrsoiflora* exhibit weak self-sterility. While there was a significant difference in seed set between pollination treatments for *W. brachyandra* (Table 2), self-pollinations produced slightly more seeds than intra- or intermorph pollinations (Fig. 2). Fruit set of *W. brachyandra* was near maximal for all classes of pollination (mean fruit set per plant, self = 98.8%, SE = 1.0; intramorph = 95.6%, SE = 2.1; intermorph = 97.8%, SE = 1.5, N = 30 plants for all treatments). In both populations of *W. paniculata*, self-pollinations resulted in statistically lower seed set than intramorph or intermorph pollinations (Fig. 3; Table 3). There were no significant differences in seed set between intra- and intermorph pollinations (Table 3). Fruit set was significantly lower after self-pollination than outcross pollination at Vredehoek, but not at Camps Bay (mean fruit set per plant at Vredehoek, self = 72% SE = 5.1; intramorph = 78.9%, SE = 4.6; intermorph = 94.1%, SE = 2.6; N = 28 plants for all treatments; at Camps Bay, self = 41.5%, SE = 5.6; intramorph = 54.4%, SE = 5.4; intermorph = 55.1%, SE = 3.2; N = 23 plants for all treatments; Table 3). Pairwise contrasts also revealed a statistically significant reduction in fruit set following intramorph pollination compared with in-

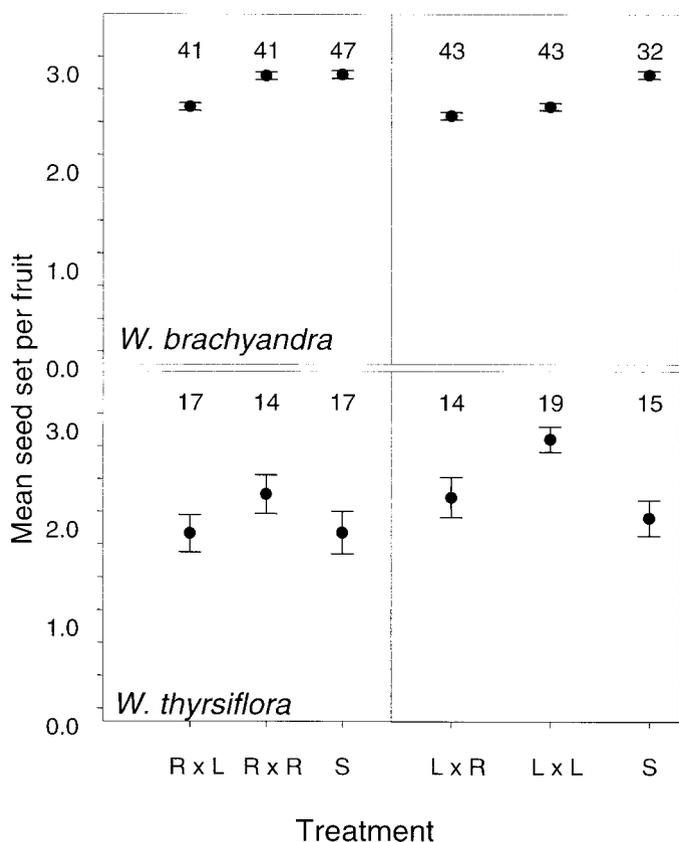


Fig. 2. Mean seed set ( $\pm$ SE) of the left- and right-styled morphs in one population each of *Wachendorfia brachyandra* and *W. thyrsoflora* following intramorph (R  $\times$  R or L  $\times$  L), intermorph (R  $\times$  L, L  $\times$  R), and self-pollinations (S) in the field. Samples sizes are listed above means.

termorph pollination at Vredehoek, but not at Camps Bay (Table 3). Self-pollinations of *W. thyrsoflora* similarly resulted in reduced seed set compared with outcross pollinations (Fig. 2), and contrasts revealed these differences were statistically significant (Table 3). There was no difference in fruit set from self-, intramorph, or intermorph pollinations (mean fruit set per plant for self = 48%, SE = 7.9; for intramorph = 46%, SE = 10.1; for intermorph = 58%, SE = 4.6;  $N = 20$  plants for all treatments; Table 2).

**Daily variation in sex-organ position**—Measurements of the distance separating the stigma and the closest anther indicated a decrease in stigma-anther separation for *W. brach-*

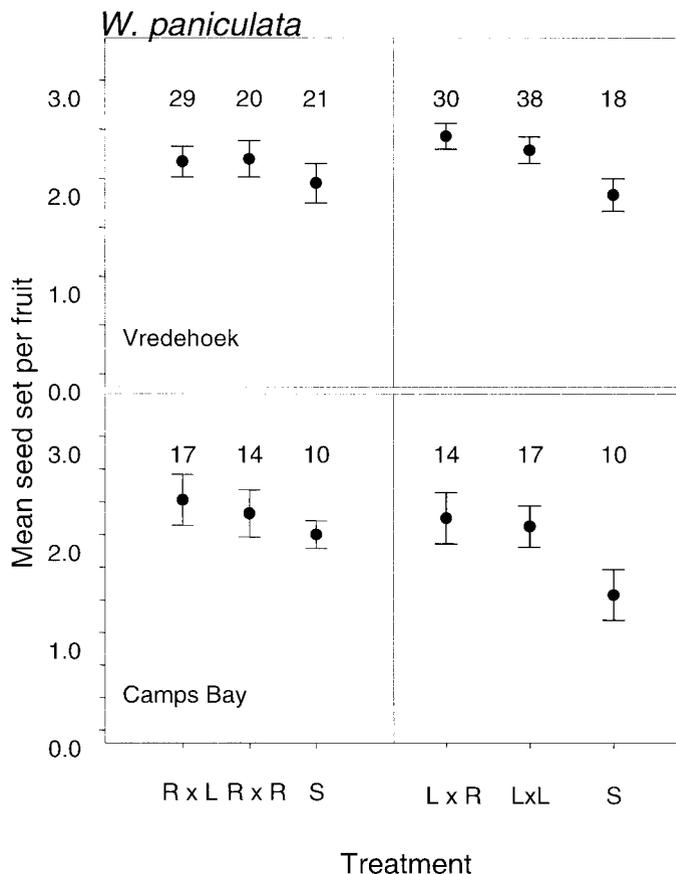


Fig. 3. Mean seed set ( $\pm$ SE) of the left- and right-styled morphs in two populations of *Wachendorfia paniculata* (Form 1) following intramorph (R  $\times$  R or L  $\times$  L), intermorph (R  $\times$  L, L  $\times$  R), and self-pollinations (S) in the field. Samples sizes are listed above means.

*yandra*, *W. paniculata*, and *W. thyrsoflora* over the course of a day (Fig. 4). This decrease in distance was also observed for the other two anthers in a flower (results not shown). There was a significant difference between species in the distance between the stigma and the nearest anther (species effect: likelihood ratio = 9.84,  $df = 1$ ,  $P = 0.007$ ). Stigma-anther separation was greatest in *W. thyrsoflora*, while *W. brachyandra* had the smallest separation between the sex organs, and by the end of the day, the anther and stigma were often in contact (Fig. 4). Linear mixed-effects models revealed no difference between the slope of regression lines (species  $\times$  time interaction: likelihood ratio = 2.02,  $df = 2$ ,  $P = 0.363$ ), showing

TABLE 3. Summary of contrasts of treatment on significant effects only (from Table 2) on seed set and fruit set from controlled pollinations in three species of *Wachendorfia*. Due to a significant effect of population on fruit set of *W. paniculata*, contrasts are presented for both populations. \* $0.05 > P > 0.01$ ; \*\* $0.05 > P > 0.001$ ; \*\*\* $P < 0.001$ .

Species	Contrast	Seed set estimate (SE)	Fruit set population	Estimate (SE)	
<i>W. brachyandra</i>	Self vs. outcross	1.88 (0.74)***			
	Intra vs. intermorph	0.43 (0.36)			
<i>W. paniculata</i> (Form 1)	Self vs. outcross	2.81 (0.78)***	Vredehoek	3.71 (1.39)***	
	Intra vs. intermorph	0.08 (0.44)	Vredehoek	$5.44 \times 10^{-6}$ (0.00)***	
	Self vs. outcross		Camps Bay	2.13 (1.26)	
	Intra vs. intermorph		Camps Bay	$6.26 \times 10^{-7}$ ( $4.5 \times 10^{-7}$ )	
	<i>W. thyrsoflora</i>	Self vs. outcross	0.46 (0.18)***		
		Intra vs. intermorph	0.18 (0.11)		

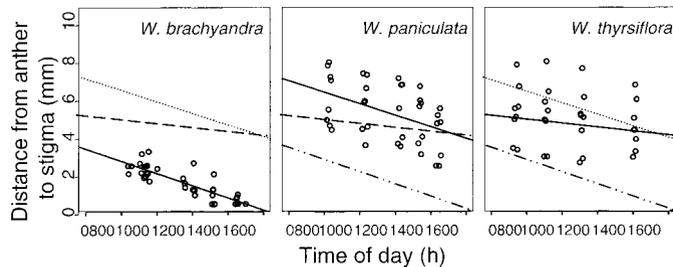


Fig. 4. The distance between the stigma and nearest anther in *Wachendorfia brachyandra*, *W. paniculata* (Form 1), and *W. thyrsoiflora* at ~2-h intervals over the course of 1 d. The slope of the regression of the species is shown by the solid line. For comparison, the slopes of the other species are shown by the dotted (*W. paniculata*), dotted and dashed (*W. brachyandra*), or dashed line (*W. thyrsoiflora*).

that the rate of decrease in stigma-anther separation did not differ between the three species. While we did not measure changes in stigma-anther separation during the course of a day in *W. parviflora*, mean stigma-anther separation at mid-afternoon at the Red Hill population was 1.22 mm (SE = 0.16), and contact between the sex organs was observed in 11 of the 42 flowers measured.

**Outcrossing rates**—Populations of *Wachendorfia* species exhibited striking variation in their mating systems, ranging from high outcrossing to predominant selfing. Multilocus outcrossing rates among the four species ranged from  $t_m = 0.98$  for *W. paniculata* at Camps Bay to  $t_m = 0.07$  for *W. parviflora* at Red Hill (Fig. 5). *Wachendorfia brachyandra* had a mixed mating system in the two populations surveyed (Kommetjie  $t_m = 0.39$ ; Oranjekloof  $t_m = 0.59$ ). These estimates were not significantly different from each other (proportion of pairwise differences lying above zero = 0.48). Populations of both Form 1 and Form 3 of *W. paniculata* were largely outcrossing, ranging from  $t_m = 0.78$  to  $t_m = 0.98$ . Form 3 of *W. paniculata* had lower outcrossing estimates than Form 1, although there was no significant difference between the populations (e.g., proportion of pairwise differences lying above zero for  $t_{\text{oudekraal}} - t_{\text{scarborough}} = 0.024$ ; with an experimental Type-I error rate held at  $\alpha_{\text{EW}} = 0.05$ ,  $\alpha_{\text{PC}} = 0.017$ ). The two populations of *W. parviflora* had significantly different outcrossing estimates (proportion of pairwise differences in bootstrap estimates lying above zero = 1), from mixed mating ( $t_m = 0.60$ ) to predominant selfing ( $t_m = 0.07$ ). The single population of *W. thyrsoiflora* examined also exhibited a mixed mating system ( $t_m = 0.76$ ).

**Frequency and distribution of style morphs**—Populations of *Wachendorfia* spp. displayed a wide range of style-morph frequencies from stylar dimorphism with equal frequencies to monomorphism (Fig. 6). All 31 populations of outcrossing *W. paniculata* that we sampled were dimorphic for style morph. In all populations of Form 1, the two morphs were not significantly different from 1 : 1 in frequency. In contrast, five of the seven populations of Form 3 that we surveyed for morph ratios were significantly biased. When populations of Forms 1 and 3 were pooled, morph ratios were not significantly different from 1 : 1 ( $G_{\text{pooled}} = 0.47$ ,  $df = 1$ ,  $P = 0.49$ ). There was, however, significant heterogeneity among populations ( $G_{\text{het}} = 341.17$ ,  $df = 1$ ,  $P < 0.001$ ). All populations of *W. brachyandra* were dimorphic for style morph; however, there was con-

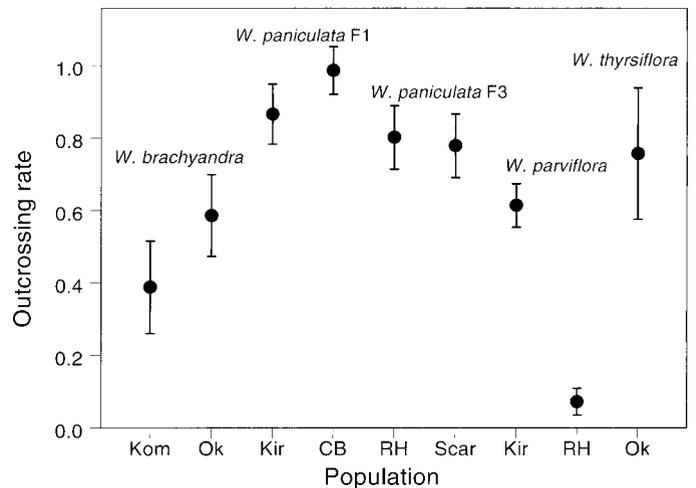


Fig. 5. Estimated multilocus outcrossing rates ( $\pm$ SE) in populations of *Wachendorfia brachyandra*, *W. paniculata*, Forms 1 and 3, *W. parviflora*, and *W. thyrsoiflora*. Populations are KOM, Kommetjie; OK, Oranjekloof; KIR, Kirstenbosch; CB, Camps Bay; RH, Red Hill; and SCAR, Scarborough.

siderable variation in morph ratios. In mixed-mating *W. parviflora* and clonal *W. thyrsoiflora*, we observed both monomorphic and dimorphic populations (Fig. 6). Pooled populations within *W. brachyandra*, *W. parviflora*, and *W. thyrsoiflora* all differed significantly from 1 : 1 ( $G_{\text{pooled}} = 15.62$ , 66.69, and 13.34, respectively;  $df = 1$ ;  $P < 0.001$  for all species). There was also significant heterogeneity among populations for all three species ( $G_{\text{het}} = 129.95$ , 102.41, and 708.62, respectively;  $df = 1$ ;  $P < 0.001$  for all species). In all four species, population morph ratios ranged from a significant excess of left-styled plants to an excess of right-styled plants.

Mapping of the spatial distribution of morphs within plots revealed obvious differences between *W. brachyandra*, *W.*

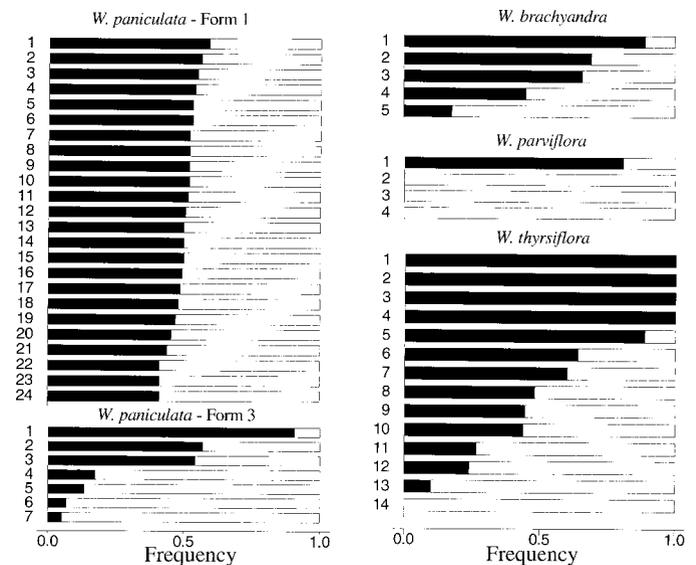


Fig. 6. The frequency of left- (white) and right-styled plants (black) in populations of *Wachendorfia* species. Numbers represent each separate population. Mean (and range) number of individuals sampled per population was 127 (45–231), 85 (28–100), 97 (45–100), and 36 (31–69) in *W. paniculata*, *W. thyrsoiflora*, *W. brachyandra*, and *W. parviflora*, respectively.

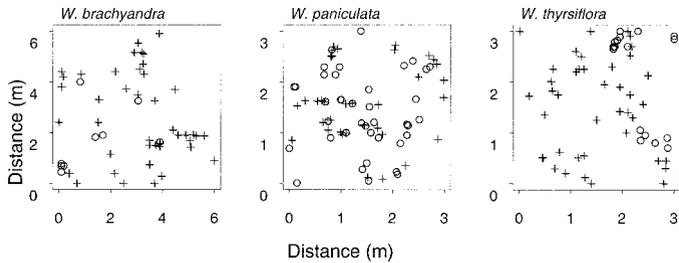


Fig. 7. The mapped spatial distribution of left- (+) and right-styled plants (o) in plots of *Wachendorfia paniculata* (Form 1) (Kirstenbosch), *W. thyrsoiflora* (Oranjekloof), and *W. brachyandra* (Kommetjie). Note the difference in scale for *W. brachyandra*.

*paniculata*, and *W. thyrsoiflora* (Fig. 7). In mixed-mating *W. brachyandra* and clonal *W. thyrsoiflora*, nearest-neighbor analyses revealed a significant positive relation between the style morph of a plant and the style morph of its nearest neighbor. In contrast, style morphs in two populations of outcrossing *W. paniculata* were randomly distributed (Table 4). There was also a significant effect of the distance to the nearest neighbor of style morph for *W. brachyandra* and *W. thyrsoiflora*.

#### DISCUSSION

Mirror image flowers have been known for over a century, although virtually nothing is known about their evolution and adaptive significance. While many workers have considered the function of enantiostyly to be analogous to the heterostylous polymorphisms distyly and tristly, this hypothesis has not been examined in detail. There has only been one other study apart from our own on the reproductive ecology of enantiostyly in natural populations. Bowers (1975) examined pollination in monomorphically enantiostylous *Solanum rostratum* and found that pollen from flowers of one style type was more likely to be deposited on flowers of the opposite form. However, because flowers with right- and left-bending styles occur on the same plant in *S. rostratum*, this condition is not a true genetic polymorphism. The results of our study demonstrate that all four species of *Wachendorfia* exhibit dimorphic enantiostyly; however, the expression of the polymorphism varies significantly both among species and among populations. This variation appears to be largely governed by differences in the reproductive systems of populations. We now discuss how variation in the mating patterns and degree of sexuality of populations can influence morph ratios and the implications that this variation has for the functioning and evolutionary maintenance of the polymorphism.

**Variation in reproductive systems**—Our field observations, as well as earlier taxonomic descriptions of *Wachendorfia* species, suggested that their mating systems might range from largely outcrossing in *W. paniculata* and *W. thyrsoiflora* to at least partially selfing in *W. brachyandra* and *W. parviflora*. Measurements of floral traits tended to support these inferences. Both *W. paniculata* and *W. thyrsoiflora* have large, showy flowers and inflorescences with large floral displays. In contrast, *W. brachyandra* and *W. parviflora* have considerably smaller, less colorful flowers with much reduced daily and total floral displays. Fruit set tended to be higher in populations of *W. brachyandra* and *W. parviflora*, a frequent condition in species with a high degree of self-pollination. Lower

TABLE 4. Log-linear analysis of style-morph and distance of the closest neighbor to all plants in a plot in three species of *Wachendorfia*. \*0.05 >  $P$  > 0.01; \*\*0.05 >  $P$  > 0.001; \*\*\* $P$  < 0.001.

Species	Effects	df	Deviance
<i>W. brachyandra</i>	Distance	1	5.30*
	Neighbor	1	4.56*
	Error	49	38.06
<i>W. paniculata</i> (Form 1)	Population	1	1.74
	Distance	1	0.15
	Neighbor	1	3.50
<i>W. thyrsoiflora</i>	Error	151	208.71
	Distance	1	2.84
	Neighbor	2	46.11***
	Error	55	22.47

investment in floral structures that attract pollinators and a greater investment in seeds are common in selfing species (Cruden and Lyon, 1985; Lloyd, 1987). These findings suggested at least a partial reliance on selfing in *W. brachyandra* and *W. parviflora*.

Our compatibility data supported the prediction that *W. brachyandra* was at least partially selfing. Controlled crosses revealed that *W. brachyandra* is completely self-compatible, whereas *W. paniculata* and *W. thyrsoiflora* display some self-sterility. Unfortunately, because of the early and limited duration of flowering in *W. parviflora*, we were not able to conduct controlled pollinations on this species, but its small flower size and high selfing rates indicate that, like *W. brachyandra*, the species is almost certainly highly self-compatible. While there were no differences in fruit set between the different types of pollinations in *W. paniculata* and *W. thyrsoiflora*, selfing reduced the number of seeds set per fruit. Earlier pollination studies of *W. paniculata* (Ornduff and Dulberger, 1978), as well as other members of the Haemodoraceae (Hopper, 1980), have also shown reduced seed set after selfing. It is not known whether reduced fertility following self-pollination results from self-incompatibility and/or from the selective abortion of selfed embryos due to inbreeding depression (Barrett, 1988; Charlesworth, 1989).

Delayed selfing is a floral strategy that optimizes outcrossing in situations with high pollinator visitation but gives reproductive assurance in environments with infrequent pollinator visitation (Lloyd, 1992). Our measurements of changes in stigma-anther separation during anthesis demonstrated that *W. brachyandra* exhibits delayed selfing. At the end of the day, the closest anther was in contact with the stigma, resulting in the autonomous self-pollination of flowers. Estimates of outcrossing rate in the two populations of *W. brachyandra* examined were  $t_m = 0.39$  and  $t_m = 0.58$ , values consistent with a partial reliance on selfing. Since the longevity of *Wachendorfia* flowers is normally 1 d, populations are vulnerable to pollen limitation if weather conditions in early spring restrict pollinator activity. Indeed, there is good evidence for widespread pollen limitation among insect-pollinated plants in the Cape region (Johnson and Bond, 1997). Under these circumstances, delayed selfing may be particularly important in overcoming the constraints imposed by short floral longevity.

Contact between stigmas and anthers was also observed in populations of *W. parviflora*, and outcrossing rates ranged from  $t_m = 0.61$  at Kirstenbosch to  $t_m = 0.07$  at Red Hill. Flowers of *W. parviflora* at Red Hill were often not open by mid-afternoon, and the nearest anther was observed to touch

the stigma in 25% of the flowers measured. It is possible that the very high selfing rate of this population may be due to poor pollinator service (no pollinators were observed) and the sparse distribution of plants at this particular site. Clearly, given the large difference in outcrossing rate between the two populations of *W. parviflora* investigated, more work on this species is required. *Wachendorfia paniculata* and *W. thyrsoiflora* also exhibited a decrease in stigma-anther separation during anthesis; however, the large initial distance between the sex organs (because of their larger flower sizes) prevents contact between stigmas and anthers. It seems likely that in these species, any selfing that does occur results from geitonogamy enhanced by their larger display sizes.

Reproductive systems are influenced both directly and indirectly by the amount of clonal reproduction in populations (Handel, 1985). In sexually polymorphic species, clonality affects the spatial patterning of morphs with implications for both mating patterns and fertility (Wyatt and Hellwig, 1979; Barrett and Thomson, 1982; Eckert, 2000; Weller, Keeler, and Thomson, 2000). Large clone size, especially with an abundance of inflorescences, may impede cross-pollination resulting in high levels of geitonogamous selfing (Eckert, 2000). Among the four *Wachendorfia* species, only *W. thyrsoiflora* exhibits large clone sizes, and the moderate levels of selfing (24%) estimated at Oranjekloof probably result mainly from geitonogamous pollen transfers both within and between inflorescences of clones in this population. Analysis of the spatial distribution of inflorescences in this population illustrates the considerable opportunities that likely exist for inter-inflorescence geitonogamy in *W. thyrsoiflora* (Fig. 7).

**Patterns of morph-ratio variation**—Equal morph ratios in plant species with polymorphic sexual systems are usually interpreted as resulting from negative frequency-dependent selection operating during the mating cycle (Fisher, 1941; Charlesworth and Charlesworth, 1979a, b; Charnov, 1982; Lloyd and Webb, 1992). For example, in distylous and tristylous populations, equal morph ratios can be explained by disassortative mating due to the higher proficiency of pollen transfer between rather than within morphs and/or the presence of heteromorphic incompatibility preventing selfing and intramorph mating (reviewed in Barrett, 1992). However, unequal morph ratios and stylar monomorphism in heterostylous populations can arise for a variety of reasons, including founder effects and drift, clonal propagation, and, in species without heteromorphic incompatibility, morph-specific differences in selfing or assortative mating (e.g., Eckert and Barrett, 1992; Husband and Barrett, 1992; Barrett et al., 1997). By analogy with heterostylous species, two features of the reproductive systems of *Wachendorfia* species are likely to be particularly important in influencing population morph ratios. These are the absence of a strong self-incompatibility system that prevents selfing and intramorph mating and, in *W. thyrsoiflora*, the occurrence of extensive clonal propagation.

*Wachendorfia* species exhibit population morph ratios ranging from 1 : 1 to those that are strongly biased or monomorphic for a single style morph (Fig. 6). In all 31 populations of Form 1 of *W. paniculata*, the two style morphs were not significantly different from the expected 1 : 1 ratio. This stability in morph ratios suggests that most matings in these populations result from outcrossing, with equivalent levels of disassortative mating in the style morphs. Outcrossing rates in the two populations of Form 1 of *W. paniculata* were 0.86 and

0.98 (Fig. 5) but we have no information on the actual levels of disassortative mating in these populations. Morph-specific markers would enable estimation of this particular mating parameter.

The precise mechanisms maintaining 1 : 1 morph ratios in *W. paniculata* remain to be determined. While there is some evidence that intramorph crosses have lower fertility than intermorph crosses (Ornduff and Dulberger, 1978; Table 3 [Fruitset, Vredehoek population]) this effect is relatively weak and not consistent among populations. Thus, it seems unlikely that intramorph incompatibility is the primary mechanism maintaining disassortative mating in populations of *W. paniculata*. A more attractive hypothesis is that the morphology of enantiostylous flowers promotes more proficient pollen transfer between rather than within the style morphs because of segregated pollen deposition on different sides of the bodies of pollinators (Bowers, 1975). Field experiments with pollen dyes have provided some evidence that intermorph pollen transfer is more frequent than intramorph transfer in *Wachendorfia paniculata* (L. K. Jesson and S. C. H. Barrett, unpublished data). However, further field studies on the mechanics of the pollination process, as have been conducted in heterostylous species (reviewed in Lloyd and Webb, 1992), are needed to confirm that pollinators promote disassortative pollen transfer.

Morph ratios in Form 3 of *W. paniculata* were strikingly different from Form 1, with biased ratios evident in five of the seven populations sampled (Fig. 6). Flowers of Form 3 are significantly smaller with less stigma-anther separation than Form 1 (Fig. 1; L. K. Jesson and S. C. H. Barrett, personal observation) and it seems probable that the biased morph ratios in Form 3 are associated with increased opportunities for selfing. Selfing is likely to be associated with unequal morph frequencies because it reduces the intensity of disassortative mating. Mean population sizes in Form 3 were significantly smaller than Form 1 and thus the joint effects of stochastic forces and selfing may explain the biased morph ratios. The smaller flowers associated with Form 3 may also lead to a less precise placement of pollen on a pollinator's body, lowering the amount of disassortative mating. However, it is important to note that while outcrossing rates in the two populations of Form 3 that we examined were lower than Form 1, they were not significantly different.

There seems little doubt that the unequal morph ratios and stylar monomorphism in *W. brachyandra* and *W. parviflora*, respectively, are associated with the higher selfing rates that characterize these taxa. Species capable of selfing are especially prone to founder events, as single individuals have the capacity to found colonies. These processes probably account for the occurrence of monomorphic populations in *W. parviflora* (Fig. 6), as well as the strong spatial structuring of morphs within populations of *W. brachyandra*. Founder events are also important in species that rely heavily on clonal propagation, and the biased morph ratios and stylar monomorphism typical of *W. thyrsoiflora* populations in the Cape Peninsula and southern Cape have most likely arisen in a similar manner. Restrictions on seedling establishment in the aquatic conditions that often characterize clonal populations can result in striking founder events and very long delays before polymorphic equilibria are reached (Eckert and Barrett, 1995).

The two disjunct populations of *W. thyrsoiflora* that we sampled in the eastern Cape exhibited equal morph ratios. These populations were some 800 km from the remaining popula-

tions and the ecological conditions in which they occurred were quite different. Unlike populations from the Cape Peninsula and southern Cape, which grow in river valleys in standing water, the populations from the eastern Cape are more terrestrial in nature occurring along roadsides on damp soil. Under these conditions, it seems probable that sexual recruitment occurs regularly, allowing equilibrium morph ratios to be reached more rapidly. Similar patterns of style-morph frequency variation associated with differences in sexual recruitment among geographical areas also occur in several heterostylous aquatics (Barrett, 1980; Eckert and Barrett, 1992, 1993). These cases provide strong evidence for the important role that ecology can play in governing morph ratios in species with polymorphic sexual systems.

**Evolutionary maintenance of enantiostyly**—The range of variation in style-morph ratios in *Wachendorfia* suggests that the polymorphism may be difficult to maintain under certain conditions. In monomorphic populations or those with strongly biased morph ratios, selection to maintain the morphology associated with enantiostyly is likely to be relaxed, particularly under conditions that favor increased selfing. Reduced stigma-anther separation, flower size, and floral display in *W. brachyandra* and *W. parviflora* may represent a derived condition associated with the evolution of selfing in these species. A phylogenetic analysis of *Wachendorfia* based on morphological traits found that these taxa were the most derived in the genus (Helme and Linder, 1992), although molecular data would be necessary to confirm this interpretation because of the difficulty that the selfing syndrome presents for phylogenetic reconstruction (see Graham and Barrett, 1995).

Breakdown of enantiostyly in *Wachendorfia* may represent a parallel condition to the well-known evolution of homostyly from heterostyly in many heterostylous families (reviewed in Charlesworth and Charlesworth, 1979a; Barrett, 1992). In both cases, outcrossing is more likely to be replaced by selfing under conditions in which disassortative pollen transfer is no longer mediated by pollinators. Elsewhere, among dimorphically enantiostylous species, populations of *Heteranthera multiflora* (Pontederiaceae) are commonly monomorphic (L. K. Jesson, unpublished data), as are populations of *Barbaretta aura* (Haemodoraceae, Ornduff, 1974; S. Johnson, University of Natal, personal communication), and it may be that the loss of effective pollinator service makes dimorphic enantiostyly a difficult floral polymorphism to maintain. Nevertheless, 1 : 1 morph ratios are a general feature of *W. paniculata* Form 1, indicating that stable morph ratios can be maintained under some circumstances. This stability is likely enhanced by the large population sizes and widespread distribution of this form, which reduce the influence of stochastic forces. However, regular pollinator service, frequent sexual recruitment, and the predominantly outcrossing mating system of this form appear to be the key ingredients in maintaining dimorphic enantiostyly.

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