BIOLOGIA PLANTARUM 53 (1): 175-178, 2009

BRIEF COMMUNICATION

Different levels of inbreeding depression between outcrossing and selfing *Serapias* species

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Abstract

We quantified inbreeding depression for fruit production, embryo vitality and seed germination in three deceptive orchids, *Serapias vomeracea*, *S. cordigera* and *S. parviflora*, which do not provide any reward to their pollinators, and are predicted to experience high outcrossing. Of the three species examined only *S. parviflora* was autonomously selfing. Both *S. vomeracea* and *S. cordigera* showed highly significant differences in fitness between selfed and outcrossed progenies, resulting in high levels of inbreeding depression, which increased in magnitude from seed set to seed germination. Inbreeding depression may promote outcrossing in *Serapias* by acting as a post-pollination barrier to selfing. Cumulative inbreeding depression across three stages in *S. parviflora* was lower that in both outcrossing species. The large difference in germination between selfed and outcrossed seeds is an important issue in conservation biology.

Additional key words: autogamy, orchids, reproductive success.

Inbreeding depression, the reduction in fitness of progeny derived from inbreeding relative to those derived from outcrossing, is invoked to explain the maintenance of plant mating system (Charlesworth and Charlesworth 1987). Inbreeding depression varies from year to year within the same population and as to which life history stages are more susceptible to inbreeding depression within a population and across taxa (Charlesworth and Charlesworth 1987). Moreover, a significant correlation exists between inbreeding depression and selfing/outcrossing species, suggesting that repeated selfing reduces the magnitude of inbreeding depression (Husband and Schemske 1996, Pico *et al.* 2007). Selfing increases homozygosity and more homozygous genotypes may be susceptible to environmental harshness. In contrast, outcrossing usually increases or maintains heterozygosity that tends to produce genotypes better able to buffer themselves against environmental variability (Falconer 1981).

Inbreeding depression has been seen as a potential mechanism favouring the evolution and maintenance of deception in orchids (Johnson and Nilsson 1999). Pollinators visit fewer flowers per plant of a deceptive orchid, thus reducing geitonogamous selfing, compared to rewarding species (Johnson *et al.* 2004, Smithson 2006). In this sense, deceptive species should be more prone to inbreeding depression than nectar-producing species.

In this study we evaluated stage-specific measures (fruit production, embryo vitality and seed germination) from controlled crosses, to estimate *1*) the magnitude of inbreeding depression (ID) in *Serapias vomeracea*, *S. cordigera* and *S. parviflora*, *2*) the autogamy in *S. parviflora*, and *3*) the relationship between the magnitude of ID and selfing or outcrossing taxa.

The genus *Serapias* is distributed throughout the Mediterranean area and its number of species recognized may differ markedly among authors (Baumann and Künkele 1989, Delforge 2005). *S. parviflora* Parlatore was originally described from Sicily but is widely distributed in the Mediterranean region; *S. vomeracea* (N.L. Burman) Briquet is rather widerspread and often abundant in its stations; *S. cordigera* L. is a Mediterranean-Atlantic species, distributed from Crete and Anatolia to Portugal and northern France.

Among the Mediterranean orchids, an unusual type of deceptive pollination strategy has been observed in the genus *Serapias*. Here, the sepals, petals and lateral lobes of the hypochile form a tunnel-like corolla, varying in diameter and depth among taxa (Baumann and Künkele

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Received 23 February 2008, *accepted* 10 September 2008.

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1989), that insects utilize as a refuge under bad or rainy weather conditions (Van der Cingel 1995). The main pollinators of *S. vomeracea* and *S. cordigera* L. are *Eucera* bees, while for *S. vomeracea* a number of pollinators have been observed *Andrena*, *Osmia*, *Tetralonia* bees (Vöth 1980), and coleopteran insects of *Oedemeridae* and *Lymexylidae* (Pellegrino *et al.* 2005a).

Pollination treatments were conducted during the flowering period in May - June of 2006 in Calabria (Southern Italy). Just before flowering, 30 plants of *S. vomeracea*, *S. cordigera* and *S. parviflora* were bagged with a fine-meshed cloth to exclude pollinators. When all flowers were open, five randomly selected flowers on each plant were manually pollinated using two pollinia per stigma to saturate available ovules (Neiland and Wilcock 1995): one flower was covered without manipulation (to test for spontaneous autogamy), a second and third were self-pollinated with pollinia of the same flower (induced autogamy) and with pollinia of a different flower on the same plant (induced geitonogamy) and two flowers were cross-pollinated with the pollen of the same species (intraspecific crossing). After treatments, plants were bagged again to prevent any further natural pollination or predation. In June, the number of capsules was counted and the ratio between the number of fruit produced/flowers treated was determined. Ripe fruits, when produced, were collected and stored in silica gel in order to prevent their degradation. Capsules were surface-sterilized by immersion in 1:10 *Chlorox* solution (0.5 % available chlorine) for 10 min, drained, then opened longitudinally with a sterilized razor blade. Seeds were removed from the center of the capsule. For each capsule the presence or absence of embryos in 500 seeds was observed using an optical microscope to determine the ratio between seeds with embryo and seeds without embryo. 500 seeds were sown asymbiotically in triplicate Petri dishes filled BM1 medium (Van Waes and Debergh 1986) adding vitamins (*1000x* vitamin stock solution, *Duchefa Biochemie*, The Netherlands). Petri dishes were sealed with *Parafilm* tape and kept in dark growth chamber at 25 °C. Total proportion of seeds germinating was calculated when no more germination occurred or, at the latest, two months after sowing. Inbreeding depression coefficients were calculated for each species as $\delta = 1 - ws/wo$, where wo and ws were the fitness estimates of outcrossed and selfed progenies respectively (Ågren and Schemske 1993). The effects of pollination treatments on seed set, embryo vitality and seed germination between self and cross pollination were tested using *ANOVA* or pairwise *t*-test with *SPSS* software (version *10*, 1999).

Fruit set by spontaneous autogamy was 0 % for *S. vomeracea*, *S. cordigera*, while *S. parviflora* showed a high percentage (48 %) of spontaneous autogamy. Thus, among examined species just *S. parviflora* is autonomous selfing. In various angiosperm families (Proctor and Yeo 1973, Elle and Carney 2003) autogamous taxa possess flowers of reduced dimensions with respect to allogamous related taxa, and thus we argued that the strong reduction of flower dimensions might have prelude to the shifting from the out- to self-crossing reproductive strategy in *Serapias*. Facultative autogamy may present an opportunity for plants to receive the potential benefits from outcrossing while lessening the impact of pollinator limitation.

Fruit set by hand self-pollination (autogamy and geitonogamy) was 50.0 % in *S. vomeracea*, 57.1 % in *S. cordigera* and 70.5 % in *S. parviflora*, while outcrossing varied from 80.7 % (*S. parviflora*) to 85.7 % (*S. cordigera* and *S. vomeracea*) (Table 1). There was no significant difference in the capsule production rate (Table 2) between self and cross pollination, also if self-crosses showed lower value of fruit set than intraspecific allogamy (Table 1).

Significant differences (Table 2) have been detected among self and cross pollination in the percentage of living embryos in the seeds for both *S. vomeracea* and *S. cordigera*. Indeed, selfcrossing gave 22.5 % in *S. vomeracea* and 25.2 % in *S. cordigera* of seeds with embryo, while cross pollination gave 92.0 and 85.7 % for *S. vomeracea* and *S. cordigera*, respectively. *S. parviflora* showed percentage of embryos from 64.7 % (selfing) to 85.4 % (outcrossing) (Table 1).

Seed germination was significantly lower for selfed fruits for all three species. *S. vomeracea* and *S. cordigera* showed a percentage of germinability ranging from 5.0 % (selfcrossing) to 70.0 % (outcrossing) while *S. parviflora* showed 46.2 % (selfcrossing) and 74.0 % (outcrossing) (Table 1). The purely autogamous selfing seeds of *S. parviflora* germined (44.5 %) as well as those from induced selfing.

Hand pollination showed that *Serapias* species are self-compatible but *S. vomeracea* and *S. cordigera* depends on pollinators to produce fruits. Self-compatibility has been widely reported in terrestrial orchids of Mediterranean regions and is considered an adaptation to a poor insect fauna and/or low insect visitation rates (Van der Pijl and Dodson 1966,

Table 1. Comparison between self- and cross-pollination of fruit set, embryo presence and seed germination [%] of *S. cordigera* (C), *S. vomeracea* (V) and *S. parviflora* (P). Means ± SE, *n* = 30.

	Fruit set			Embryo presence			Seed germination		
	C	V	P	C	V	P	C	V	P
Self	57.14±0.65	50.00±0.55	70.50±1.05	25.25±0.15	22.50±0.13	64.70±0.52	5.00±0.05	5.00±0.05	46.20±0.55
Cross	85.70±0.55	85.70±0.85	79.90±1.12	87.50±0.87	92.00±0.95	85.40±1.02	70.00±0.58	70.00±1.11	74.05±0.72

Robertson and Wyatt 1990). All three *Serapias* species showed a highly significant differences in fitness between selfed and outcrossed progenies which increased with lateness of stage studied (percentage of fruit, seeds with embryo and seed germinability) (Table 1), indicating a significant strong inbreeding depression (δ), which increased in magnitude with lateness of stage recorded. Based on fruit production, embryo vitality and germination rates, δ were positive for all three fitness estimates and increased in magnitude with lateness of stage recorded for all three *Serapias* species. Interestingly, cumulative inbreeding depression across three stages in predominantly selfing (*S. parviflora*) (mean δ = 0.25, SE = 0.03) was 35 % of that in both outcrossing species (*S. cordigera*, mean δ = 0.66, SE = 0.04; *S. vomeracea*, mean δ = 0.70, SE = 0.05).

Table 2. Results of *ANOVA* to test the effects of pollination treatments on fruit set (A), embryo presence (B) and seed germination (C) between self and cross pollination in *S. cordigera*, *S. vomeracea* and *S. parviflora* (*** - $P < 0.001$; ** - $P < 0.01$; * - $P < 0.05$; ns - not significant).

		MS	d.f.	*F*	*P*
Selfing *vs*	A	0.38	1	1.69	0.210ns
outcrossing	B	3875.06	1	23.80	0.039*
S. cordigera	C	4456.25	1	142.80	0.001***
Selfing *vs*	A	0.59	1	2.59	0.120ns
outcrossing	B	4830.25	1	30.14	0.031*
S. vomeracea	C	4556.25	1	145.80	0.001***
Selfing *vs*	A	0.26	1	1.55	0.198ns
outcrossing	B	2568.21	1	25.21	0.022*
S. parviflora	C	3865.10	1	140.60	0.003**
Selfing	A	0.25	2	0.20	0.698ns
	B	756.25	2	20.02	0.042*
	C	808.01	2	30.09	0.043*
Outcrossing	A	0.32	2	0.12	0.892ns
	B	0.04	2	21.00	0.015*
	C	0.05	2	0.71	0.430ns

The quality of seeds (embryo presence or seed germinability) is expected to reflect differences in reproductive success, and all three *Serapias* species showed the highest levels of inbreeding depression for these stages. δ increased from fruit mass to seed germinability has been reported for two nectarless orchid *Barlia robertiana* and *Anacamptis morio* (Smithson 2006) with values ranging from 0.2 - 0.3 to 0.7 - 0.8. Similar results were obtained from approx. 80 orchid species in which fruit set from outcross pollinations was slightly higher (average 81 %) than from self pollinations (average 72 %) and embryo formation ranged from 24 to 99 % (average 64.8 %) from cross pollination and from 0 to 99 % (average 40.8 %) from self pollination. However, in few cases (6 out 76) fitness from self pollination was higher than cross pollination (Tremblay *et al.* 2005 and references therein).

The magnitude of inbreeding depression is a crucial parameter in the evolution of the mating system and pollination strategies (Charlesworth and Charlesworth 1987). Also if between nectarless and rewarding orchids there was no significant difference in fitness comparing selfed and outcrossed treatments, as well as in inbreeding coefficient (Smithson 2006), highly significant differences in fitness between selfed and outcrossed progenies and highly positive values of δ for *Serapias* species could be put in relation to their pollination strategies.

Orchids are widely known for their floral specialization and use of animal pollinators. Most species are self-compatible with pre-pollination barriers to self-fertilization (Van der Pijl and Dodson 1966). As in many other outcrossing angiosperm species, though, inbreeding depression may also promote outcrossing in orchids by acting as a post zygotic barrier to selfing. Currently, different opinions exist regarding the exact insect-plant interactions occurring in *Serapias*. Pollination strategy acting in the *Serapias* species might potentially promote outcrossing at the population level, because insects rarely insist on a given flower or plant, but rather only occasionally utilize them as a refuge (Van der Cingel 1995). *Serapias* species utilize several insects as pollinators, and thus its mating system could be affected by different composition and behaviour of pollinators which are available in each locality. There is experimental evidence that pollinators visit fewer flowers per inflorescence on nectarless plants, although the evidence that this causes reduced geitonogamy is contradictory (Johnson and Nilsson 1999, Johnson *et al.* 2004). Van der Cingel (1995) proposed that insects concentrate on only one or few plants (house plants) of a *S. vomeracea* population, thus promoting self-pollination (geitonogamy) in this species. Hence, *Serapias* species, similarly to other plants that use a broad array of generalist visitors, may experience spatio-temporal variation in pollinator faunas that may create fluctuating selective regimes (Ollerton 1996).

In our study, cumulative inbreeding depression in self-fertilizing species (*S. parviflora*) was 35 % of that in outcrossing species. This estimate is consistent with the results of studies reviewed by Husband and Schemske (1996) in which mean cumulative inbreeding depression in self-fertilizing species was found to be approx. 40 % of that in primarily outcrossing taxa. A similar pattern has been reported for experimental crosses of genera such as *Epilobium* (Parker *et al.* 1995), *Lupinus* (Karoly 1992) and *Mimulus* (Carr and Dudash 1996). These results support the hypothesis that recessive lethal and highly deleterious alleles are an important source of inbreeding depression in natural populations, which may be purged upon selfing (Charlesworth and Charlesworth 1987). Our observation of lower levels of inbreeding depression in selfing *S. parviflora* is expected under the hypothesis that genetic load is due to the balance between selection and recurrent mutations to deleterious recessive alleles. Furthermore, lower levels of inbreeding depression could be simply a consequence of the reduction in levels of genetic variation in *S. parviflora* observed in molecular survey (Pellegrino *et al.* 2005b).

Inbreeding depression plays a central role for several evolutionary topics (*e.g*. mating system evolution and local adaptation), and the consequences of inbreeding depression have also become an important issue in conservation biology and is currently considered as one of the most important threats to the persistence of rare taxa (Hedrick and Kalinowski 2000). From a conservation perspective, the great difference in germination rate between selfed and outcrossed seeds is an important parameter to take into account before propagating orchids from seeds, and before introducing new plants in a population.

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