

Severe outbreeding and inbreeding depression maintain mating system differentiation in *Epipactis* (Orchidaceae)

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Abstract

In hermaphroditic plants, theory for mating system evolution predicts that populations will evolve to either complete autonomous selfing (AS) or complete outcrossing, depending on the balance between automatic selection favouring self-fertilization and costs resulting from inbreeding depression (ID). Theory also predicts that selection for selfing can occur rapidly and is driven by purging of genetic load and the loss of ID. Therefore, selfing species are predicted to have low levels of ID or even to suffer from outbreeding depression (OD), whereas predominantly outcrossing species are expected to have high levels of ID. To test these predictions, we related the capacity of AS to the magnitude of early-acting inbreeding or OD in both allogamous and autogamous species of the orchid genus *Epipactis*. For each species, the level of AS was assessed under controlled greenhouse conditions, whereas hand-pollinations were performed to quantify early costs of inbreeding or OD acting at the level of fruit and seed production. In the autogamous species, the capacity of AS was high (> 0.72), whereas in the allogamous species AS was virtually absent (< 0.10). Consistent with our hypothesis, allogamous *Epipactis* species had significantly higher total ID (average: 0.46) than autogamous species, which showed severe costs of OD (average: -0.45). Overall, our findings indicate that strong early-acting ID represents an important mechanism that contributes to allogamy in *Epipactis*, whereas OD may maintain selfing in species that have evolved to complete selfing.

Introduction

The Orchidaceae is well known for its spectacular variation in flower morphology and mating systems that were most likely generated from rather trivial phenotypic divergences (Alcock, 2005). This wide variation has largely been attributed to specific adaptations to pollinators, most likely to increase cross-pollination and to avoid the deleterious effects of selfing (Darwin, 1877; Tremblay *et al.*, 2005). Nonetheless, facultative and obligate autonomous self-pollination has been repeatedly observed within the orchid family (Hagerup, 1952; Catling, 1983, 1990; Van der Cingel, 1995, 2001; Duffy *et al.*, 2009; Bateman *et al.*, 2005). These

observations agree to some extent with the general theory for mating system evolution, which predicts that species will evolve to either complete selfing or complete outcrossing, depending on the balance between automatic selection favouring self-fertilization and costs resulting from inbreeding depression (ID) (Lande & Schemske, 1985; Charlesworth & Charlesworth, 1987; Goodwillie *et al.*, 2005).

Starting from the classical work of Stebbins (1950, 1957), two major selective forces have been proposed to promote a shift from outcrossing to selfing. The first one is the genetic transmission advantage: a selfing genotype transmits twice as many copies of its genes to offspring as does an outcrossing genotype. Therefore, selfing is selectively favoured when ID is less than the transmission advantage (Lande & Schemske, 1985; Goodwillie *et al.*, 2005). A second alternative ecological force favouring selfing is reproductive assurance under

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gamete limitation (Charlesworth, 2006). These conditions may arise either due to reduced gamete exchange, reduced mate availability or a combination of both (Busch & Delph, 2012; Griffin & Willi, 2014).

Inbreeding depression, the higher performance of outbred individuals compared to inbred individuals, is, however, assumed to counterbalance selection for selfing (Lande & Schemske, 1985). It is generally believed that ID arises when the gene pool of a population or species carries genetic load and mating between relatives increases the incidence of homozygosity. Under such conditions, the chance that homozygous deleterious recessive or partially recessive alleles come to expression may increase (i.e. the dominance hypothesis) (Charlesworth & Charlesworth, 1987; Charlesworth & Willis, 2009). The impact of ID depends on the amount of segregating recessive genetic load and its expression, that is the extent to which a given load reduces the fitness of selfed progeny. A second hypothesis states that ID results from overdominance giving heterozygous genotypes a superior fitness over homozygous genotypes at a single locus (Carr & Dudash, 2003). Whatever the genetic basis of ID, they both predict the same qualitative outcome of inbreeding from one generation to the next. However, theory also predicts that the evolution to selfing can occur rapidly because genetic load can be purged (Crnokrak & Barrett, 2002). Purging occurs when successive generations of inbreeding may result in a rebound in fitness due to the selective decrease in frequency of deleterious alleles, which in turn can lead to rapid evolution of selfing (Bodbyl Roels & Kelly, 2011; Brys & Jacquemyn, 2012).

In species or populations that are predominantly selfing and where the costs of ID have been purged, the opposite shift from selfing towards outcrossing can theoretically occur (Wright *et al.*, 2013). However, this evolutionary transition is unlikely to take place because there is no advantage that can overcome a selfer's two-fold transmission advantage (Takebayashi & Morrell, 2001). Furthermore, outbreeding depression (OD) can be expected to select against outcrossing (Takebayashi & Morrell, 2001). However, at present there is very little empirical data that have convincingly demonstrated that OD limits the possibilities of mating system transitions in selfing populations (Lynch, 1991; Wright *et al.*, 2013).

Notwithstanding the fact that the effects of outcrossing and selfing on fruit and seed production can be easily determined in orchid species by hand-pollinating flowers (Tremblay *et al.*, 2005; Jersakova *et al.*, 2006; Smithson, 2006), only a limited number of studies have investigated the relative differences in fitness resulting from selfing and outcrossing on early life-stages. In these studies, the costs of ID varied between very high (e.g. *Brownleea galpinii* ($\delta = 0.982$; Johnson *et al.*, 2003), over no costs (*Ionopsis utricularoides* ($\delta = 0.000$; Montalvo & Ackerman, 1987), towards severe costs of

OD (e.g. *Bulbophyllum weddellii* ($\delta = -0.414$; Borba *et al.*, 1999). These results open the possibility that both ID and OD are involved in driving mating system evolution in orchids. However, there are only a few studies that have related the magnitude of ID or OD to mating system variation in orchids (Johnson *et al.*, 2009; Peter & Johnson, 2009).

In this study, we related the capacity of autonomous selfing (AS) to early costs of inbreeding or outbreeding in five species of the genus *Epipactis*. Although most species in this genus are nectar rewarding (Claessens & Kleynen, 2011), there are notable differences in mating system, with a large number of allogamous species relying on pollinators for successful fruit and seed set and a series of species that are predominantly autogamous and are no longer dependent on pollinators for fruit set (Van der Cingel, 1995; Claessens & Kleynen, 2011). Morphological (Richards, 1982) and molecular studies (Squirrell *et al.*, 2002; Hollingsworth *et al.*, 2006; Bateman *et al.*, 2015) have shown that autogamy has iterative, but recent origins from allogamous ancestor populations or species in *Epipactis*. The shift towards selfing is frequently associated with small distribution areas, low plant densities and limited pollinator availability, all of which may have created gamete limitation and therefore enhanced rapid evolution towards selfing (Van der Cingel, 1995; Squirrell *et al.*, 2002; Tranchida-Lombardo *et al.*, 2011). Nonetheless, there are some autogamous species that are fairly widespread across Europe and form extensive populations (e.g. *E. leptochila* and *E. phyllantes*). In this study, we combined pollinator exclusion experiments with controlled pollination treatments to test the hypothesis that the capacity of AS is inversely related to costs of inbreeding or outbreeding.

Materials and methods

Study species and populations

Five species of the genus *Epipactis* showing pronounced differences in mating system were selected for the experiments: two putatively autogamous species (*E. muelleri* and *E. neglecta*) and three putatively allogamous species (*E. atrorubens*, *E. helleborine* and *E. palustris*) (Van der Cingel, 1995; Claessens & Kleynen, 2011; Jacquemyn & Brys, 2015). The three allogamous species belong to different phylogenetic clades within the genus *Epipactis* (Hollingsworth *et al.*, 2006; Tranchida-Lombardo *et al.*, 2011; Bateman *et al.*, 2015), and the two autogamous species also show significant genetic differentiation and are expected to have independent origins (Squirrell *et al.*, 2002; Hollingsworth *et al.*, 2006; Tranchida-Lombardo *et al.*, 2011; Bateman *et al.*, 2015). The autogamous *E. muelleri* and *E. neglecta* mostly grow in closed and dark deciduous forest sites (Delforge, 2006), whereas the allogamous species generally prefer

somewhat more open habitats. *E. atrorubens* and *E. palustris* are both restricted to nutrient-poor, calcareous soils, but they occur in habitats with pronounced differences in soil moisture content (Delforge, 2006). *E. palustris* generally prefers seasonally inundated areas (such as wet dune slacks and calcareous fens) (Jacquemyn *et al.*, 2014), whereas *E. atrorubens* mostly grows in dry calcareous grasslands or forest edges on rocky soils. *E. helleborine* can be found in a much broader variety of habitats, ranging from open grasslands, ruderal communities along road verges, streamside areas, to forest edges (Delforge, 2006).

In 2013, eight *Epipactis* populations were selected in Belgium. Two populations were sampled of each of the three allogamous species (*E. atrorubens*, *E. helleborine* and *E. palustris*). Due to their extreme rarity, only one population was selected for the two autogamous species (*E. muelleri* and *E. neglecta*). The studied populations of *E. palustris* were located in dune slacks at the Belgian coast, whereas *E. atrorubens* was sampled at calcareous grasslands in the eastern part of Belgium. The forest species *E. muelleri* and *E. neglecta* were sampled in the eastern part of the Ardennes, whereas *E. helleborine* was sampled in the centre of Belgium near Leuven.

Capacity of autonomous selfing and costs of early inbreeding depression

For each population, ten flowering individuals were selected that were located at least 5 m from each other. Two months before flowering, all selected plants were transferred to a pollinator-free greenhouse. Plants were excavated with the surrounding soil and put in large plastic containers (30 cm diameter). To minimize the impact of disturbance and to avoid desiccation, plants were regularly watered during the whole experiment. During flowering, three pollination treatments were randomly assigned to flowers of each plant with a target of five replicates per treatment and plant. Pollination treatments were as follows: (1) flowers left intact and unmanipulated, (2) supplemental self- and (3) supplemental outcross-pollination during anthesis after flowers were emasculated immediately after opening. Outcross pollen was obtained from individuals growing in the same population. Two pollinia from two different individuals were used for the outcross-pollination treatment and from two different flowers for the self-pollination treatment to saturate available ovules. When fruits were ripe, fruit set following the different pollination treatments was determined per plant, and a maximum of three mature fruits were harvested per plant and pollination treatment to manually count all seeds per fruit in the laboratory using a dissecting microscope (10×). Afterwards, average seed production was determined for each plant and pollination treatment. For each individual plant, the results of pollination treatments (1) and (2) were then used to determine the

capacity of AS following (Lloyd & Schoen, 1992):

$$\left[\frac{\text{Fruit set of unmanipulated flowers}}{\text{Fruit set following self-pollination}} \times \frac{\text{Seed set of unmanipulated flowers}}{\text{Seed set following self-pollination}} \right] \quad (1)$$

A tetrazolium treatment (protocol identical to that reported in Van Waes & Debergh, 1986) was used to determine seed viability of three supplemental and autonomous selfed fruits and three outcrossed fruits per plant. Three batches of seeds were inspected under a dissecting microscope per fruit by counting up to 50 seeds per batch. Seeds were classified into viable seeds (seeds with a red coloured embryo) and nonviable seeds (seeds lacking an embryo or having an uncoloured and/or abnormal embryo). Finally, we calculated for each maternal plant and pollination treatment the mean proportion of viable seeds and mean total female fitness at fruit level as: (average number of seeds) × (average proportion of viable seeds).

For each maternal plant, ID or OD was then estimated as:

$$\delta = \frac{\omega_o - \omega_s}{\max(\omega_o, \omega_s)} \quad (2)$$

where ω_o and ω_s represent average total female fitness following supplemental outcrossing and selfing, respectively (Ågren & Schemske, 1993). This measure varies between -1 and 1 , with positive values indicating that outcrossing outperformed selfing and vice versa.

Statistical analyses

General and generalized linear mixed models were used to test whether the capacity of AS (i.e. fruit and seed set following AS, and the resulting AS) differed significantly between the five studied *Epipactis* species. To test whether proportional fruit set, seed production per fruit and seed viability differed between species and pollination treatment, a two-level hierarchical model was constructed with species, pollination treatment and their interaction as independent variables. To investigate whether the extent of ID or OD differed significantly between each of the five studied species, a similar model was constructed with species as the main factor. In case species and/or pollination treatment had a significant effect in the above mentioned analyses, *post hoc* Tukey–Kramer adjustments were used to compare treatment means. All analyses were conducted in SAS 9.1, with population included as a random effect (SAS Institute, 2005). The MIXED procedure was used to analyse normal continuously distributed data, whereas the GLIMMIX procedure with logit link function was used for the dependent variables with a binomial distribution (values of proportional fruit set and AS and ID indices) (SAS Institute, 2005).

Results

Capacity of autonomous selfing

Spontaneous fruit set following AS differed significantly between the species studied ($F_{4,58} = 96.29$; $P < 0.0001$), with significantly lower fruit set in allogamous than in autogamous species (on average 8% and 94.5% fruit set at plant level, respectively). Seed production also differed significantly between species ($F_{4,55} = 20.65$; $P < 0.0001$), with allogamous species containing significantly fewer seeds per fruit after spontaneous selfing (on average 864 ± 121 seeds per fruit) than autogamous species (on average 1937 ± 354 seeds per fruit) (Fig. 1a). The latter represented a respective difference of 8.9% and 64.5% compared to supplemental selfing. As a result, the capacity of AS also differed significantly between the study species ($F_{4,55} = 179.41$; $P < 0.0001$), and was significantly higher in the autogamous species (range: 0.73–0.97) than in the allogamous species (range: 0.00–0.10) (Fig. 2).

Costs of early inbreeding depression

There were no significant differences in fruit set between species and pollination treatments (selfing vs. outcrossing), nor was there a significant interaction between both factors. Fruit set was in all cases very high (average fruit production: 98.1%) (Table 1). Seed set following hand pollination, on the other hand, depended significantly on species, pollination treatment

Table 1 Results of general and generalized linear mixed models to test for effects of species and pollination treatment (supplementary outcrossing and selfing) on fruit set, seed number and quality in *Epipactis atrorubens*, *E. helleborine*, *E. palustris*, *E. neglecta* and *E. muelleri*.

Variable	d.f.	F	P
(a) Fruits per flower			
Species	4, 116	0.15	0.9640
Pollination treatment	1, 116	0.20	0.6558
Species × pollination treatment	4, 116	0.52	0.7224
(b) Seeds per fruit			
Species	4, 115	4.20	0.0033
Pollination treatment	1, 115	5.46	0.0212
Species × pollination treatment	4, 115	1.61	0.1766
(c) Seed viability			
Species	4, 115	10.36	<0.0001
Pollination treatment	1, 115	3.37	0.0069
Species × pollination treatment	4, 115	4.79	<0.0013

and the interaction between both factors (Table 1). In allogamous species, average seed production of outcrossed flowers was higher than in selfed flowers (average seed production: 3036 ± 459 and 2188 ± 199 , respectively), whereas the opposite pattern was observed in the autogamous species. These species showed higher seed production in selfed flowers (2121 ± 169 seeds) than in outcrossed flowers (1546 ± 270 seeds) (Fig. 1a). Seed viability was also significantly affected by pollination treatment, species and the interaction between both factors (Table 1).

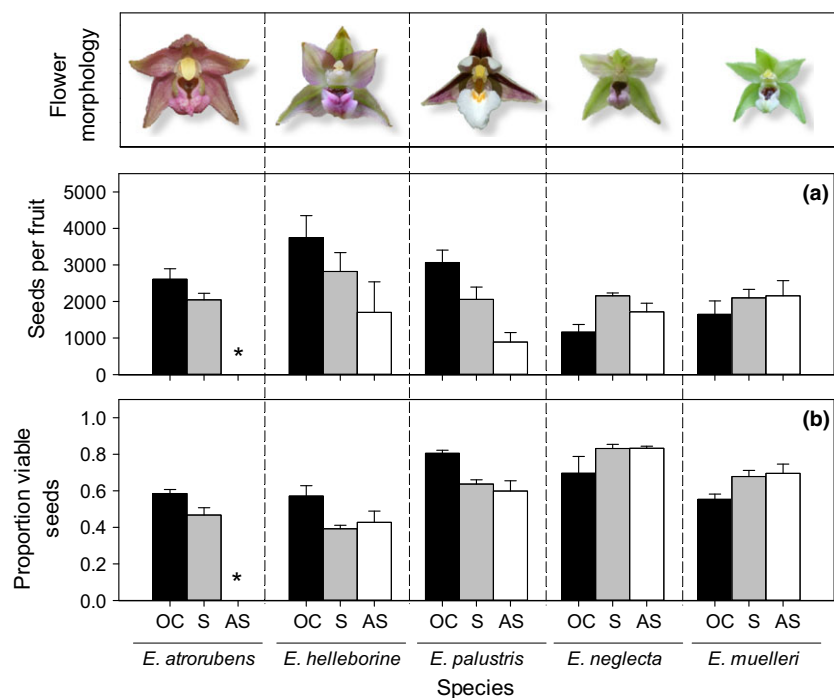


Fig. 1 Flower morphology and mean (\pm SE) seed production (a) and proportion of viable seeds (b), from flowers that received supplementary outcrossing (OC), selfing (S) or autonomous selfing (AS) in *Epipactis atrorubens*, *E. helleborine*, *E. palustris*, *E. neglecta* and *E. muelleri*. *Indicates zero values. Data are pooled from the different populations.

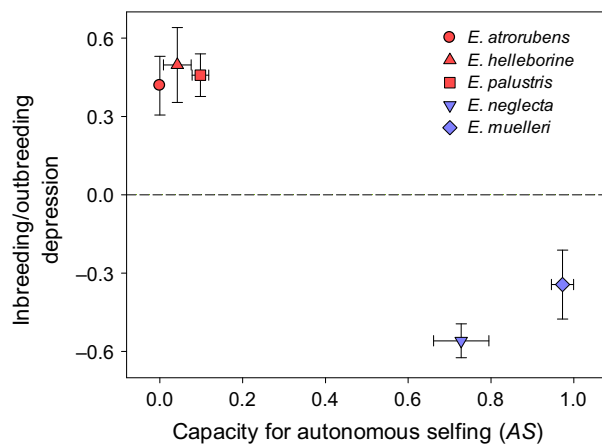


Fig. 2 The association between the capacity of autonomous selfing (AS) and early-acting inbreeding/outbreeding depression based on seed number and quality in *Epipactis atrorubens*, *E. helleborine*, *E. palustris*, *E. neglecta* and *E. muelleri*. Positive values in the Y-axis indicate inbreeding costs, whereas negative values indicate outbreeding costs. Data are pooled from the different populations.

Seed viability in the allogamous species was significantly higher after outcrossing than after selfing (average seed viability: 68.8% and 53.9%, respectively, Fig. 1b), whereas the opposite pattern was found in the autogamous species (average seed viability: 59.3% and 71.5% following outcrossing and selfing, respectively; Fig. 1b). As a result, early ID/OD differed also significantly between species ($F_{4,55} = 9.20$; $P < 0.0001$), with the allogamous species showing high and positive values of ID (mean $\delta = 0.46 \pm 0.14$), whereas the autogamous species had negative values (mean $\delta = -0.45 \pm 0.09$), indicating severe OD (Fig. 2).

Discussion

Fruit set in orchids often tends to be low (Neiland & Wilcock, 1998; Tremblay *et al.*, 2005), suggesting that many orchid species are prone to pollen limitation. In the case that plants are exposed to chronic pollinator limitation, recent studies have shown that they can evolve rapidly towards an AS mating system (Bodbyl Roels & Kelly, 2011; Bryn & Jacquemyn, 2012). In *Epipactis*, the transition from outcrossing to selfing may be facilitated by mutations that cause a plant to lose much of its rostellum and clinandrium. When the seeds of such mutant individuals manage to encounter a suitable fungal partner, selfing variants may establish within populations and slowly replace outcrossing variants, especially when pollinators are limited. However, the transition from outcrossing towards selfing also depends on the interplay between factors that select for self-fertilization (such as reproductive assurance and/or transmission advantage) and costs that arise from ID (Charlesworth & Charlesworth, 1987; Goodwillie *et al.*,

2005; Charlesworth, 2006). When selfing is the predominant means of mating, ID caused by deleterious recessive alleles can be purged by selection (Crnokrak & Barrett, 2002), and therefore, only two mating system extremes – selfing with low ID and obligate outcrossing with high ID – are theoretically expected to be evolutionary stable (Lande & Schemske, 1985). Our study is in line with these expectations and showed that the capacity to self autonomously was either very low ($AF < 0.1$) or very high ($AF > 0.7$), indicating that the studied *Epipactis* species were either largely outcrossing or predominantly selfing. In addition, the allogamous species studied showed high ID (on average 0.46), whereas autogamous species were characterized by severe outbreeding costs (on average -0.45). Notwithstanding there is no formal proof that shifts in mating system from selfing towards outcrossing have not taken place in *Epipactis* (Bateman, 2012), our results suggest that due to strong OD and the twofold transmission advantage of selfing the reversal from autogamy to allogamy is unlikely to take place in nature.

Allogamy in *Epipactis* is generally associated with the production of large, open flowers that possess a well-developed rostellum and clinandrium (Richards, 1982; Tałaj & Brzosko, 2008). Both features function as a physical barrier between the male and female organs, thereby preventing self-fertilization. In addition, allogamous species in this genus are also frequently characterized by ingenious strategies that allow efficient attraction of pollinators, most often social wasps of the genera *Vespula* and *Dolichovespula* (Richards, 1982; Jakubská-Busse & Kadej, 2011) and ensure successful pollination (Müller, 1873; Darwin, 1877; Van der Cingel, 1995; Claessens & Kleynen, 2011; Jakubská-Busse & Kadej, 2011). In particular, the emission of floral scents plays an important role in attracting specific insects. For example, *E. helleborine* and *E. purpurata* have been shown to attract social wasps of the genus *Vespula* (*Vespula germanica* and *V. vulgaris*) (Brodmann *et al.*, 2008), whereas *E. atrorubens* and *E. veratrifolia* mainly attract *Bombus* (Brodmann *et al.*, 2008; Jakubská-Busse & Kadej, 2011) and specific hoverflies (Suet-sugu, 2013; Jinn *et al.*, 2014), respectively. The use of specific scent profiles and lack of showy colours makes these *Epipactis* species often entirely ignored by other potential pollinators, despite their large nectar reward (Claessens & Kleynen, 2011). *E. palustris* represents a notable exception, however, as this species is visited and probably pollinated by a large number of insect species (Jacquemyn *et al.*, 2014; Jacquemyn & Bryn, 2015).

In the three allogamous *Epipactis* species, selfing strongly reduced seed set and seed viability compared to outcrossed seeds. Our observations agree with earlier findings that have shown reductions in seed quality (measured as percentage of seeds with an embryo) after

selfing in a number of other allogamous orchids (Tremblay *et al.*, 2005). High costs of early ID were also documented in the allogamous *Gymnadenia conopsea* (Sletvold *et al.*, 2012), *B. galpinii* ssp. *major* (Johnson *et al.*, 2003) and *Acrolophia cochlearis* (Peter & Johnson, 2009). Although ID has often been shown to act intensely at early life cycle stages, such as seed set and seed viability (Husband & Schemske, 1996; Melser *et al.*, 1999; Harder *et al.*, 2012; Kolb & Durka, 2013), it is likely that it also reduces plant fitness at later acting life cycle stages (e.g. Husband & Schemske, 1996), suggesting that the measured levels of ID represent an underestimation of the real levels of ID. The percentage of seeds that successfully germinates and establishes in natural populations is often much lower than estimates of seed viability, suggesting that the costs of inbreeding will increase once seed germination and seedling establishment have been taken into account and may exceed the critical 0.5 level.

The two autogamous *E. muelleri* and *E. neglecta*, on the other hand, showed negative values of ID ($\delta = -0.55$ and -0.34), resulting in opposite reductions of total female fitness after selfing. Spontaneous selfing within the genus *Epipactis* is not unusual and probably evolved as a strategy to ensure reproductive success after colonization of new habitats from small founding populations or to deal with severe pollinator limitation and/or harsh environmental conditions (Pedersen & Ehlers, 2000; Squirrell *et al.*, 2002; Levin, 2012). Given that both *E. muelleri* and *E. neglecta* typically grow under heavy shade in closed forest sites where pollinators are scarce, reproductive assurance was also most likely the initial driver towards autogamy in these species. There are, however, also allogamous species, such as *E. purpurata*, that generally prefer closed woodlands. It has probably arisen due to specific adaptations in flower morphology or a mutation that increase the capacity of AS and prevent the chance of outcrossing (Richards, 1982; Claessens *et al.*, 1998; Pedersen & Ehlers, 2000; Ehlers *et al.*, 2002; Bonatti *et al.*, 2006; Delforge, 2006). First, the viscidium, a small sticky exudate from the tip of the rostellum that acts as a glue to bind the pollinia to visiting pollinators, is either largely degenerated (such as in *E. neglecta*) or completely missing (such as in *E. muelleri*) (Richards, 1982; Delforge, 2006). Second, the flowers of autogamous *Epipactis* species tend to be smaller and more pendulous, and both the rostellum and clinandrium are often reduced in size (Richards, 1982). Furthermore, in both study species, but most evidently in *E. muelleri*, the pollinia are pushed forward during flower ageing which easily results in spontaneous self-fertilization when the fragile pollinia disintegrate and pollen tetrads or aggregates of pollen tetrads fall on the stigmatic surface (Richards, 1982; Delforge, 2006; Claessens & Kleynen, 2011). Under such conditions of predominant selfing, homozygosity

can be expected to increase with each generation and the potential for severe OD to arise within populations. High levels or complete homozygosity have indeed been shown in a number of autogamous *Epipactis* species, including *E. muelleri*, whereas allogamous species, such as *E. helleborine*, usually show much more genetic variation (Ehlers & Pedersen, 2000; Squirrell *et al.*, 2002).

Our results are in line with these expectations and indicate that within selfing populations of *Epipactis* any outcrossing will lead to severe reductions in progeny vigour, limiting the chance of outbred individuals to establish in selfing populations and in this way contributing to the maintenance of selfing (Takebayashi & Morrell, 2001). Outbreeding depression is normally found at much larger geographical scales (Ferdyn *et al.*, 2001; Sletvold *et al.*, 2012) and is generally associated with the disruption of adaptation to local environmental conditions (the so-called extrinsic, genotype-by-environment factors). Outbreeding depression acting within populations has only rarely been described (Waser *et al.*, 2000; Bailey & McCauley, 2006). In this case, high incidence of homozygosity, due to a long history of selfing, may result into intrinsic genetic incompatibilities and can therefore cause significant outbreeding costs, even among individuals within the same population (Ritland & Ganders, 1987; Schierup & Christiansen, 1996; Wright *et al.*, 2013).

Overall, our results support previous studies that have documented the occurrence of facultative and obligate autonomous self-pollination within the orchid family (Catling, 1990; Van der Cingel, 1995; Peter & Johnson, 2009). Our data further demonstrate that in the studied *Epipactis* species severe and early-acting ID and OD may represent important mechanisms that contribute to the observed differences in mating system. In multiflowered species, such as the studied *Epipactis* species, it is also likely that selfing can accidentally occur via geitonogamy as an unavoidable by-product of outcrossing. However, even if mating in the field is neither strictly outcrossing nor strictly selfing, our results indicate that offspring resulting from self-fertilization in allogamous species, and cross-fertilization in autogamous species, may be gradually replaced by offspring resulting from the opposite cross-type, thus maintaining the observed mating system differentiation.

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