ORIGINAL ARTICLE

Selfing potential in *Epipactis palustris*, *E. helleborine* and *E. atrorubens* (Orchidaceae)

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Abstract Factors enabling and limiting self-fertilization were examined in populations of *Epipactis palustris*, *E. helleborine* and *E. atrorubens* located in NE Poland. In these species we found self-compatibility within the same flower (even 100% of fruit set from induced autogamy) and the same inflorescence (reaching 90–100% of fruit set from induced geitonogamy). Facultative autogamy was found only in *E. palustris* (even 82.5% of fruit set in a bagged inflorescence), due to underdevelopment of the clinandrium and projection of pollinia over the rostellum. Allogamy was predominant in *E. helleborine* (maximum to 2.8% of fruits set spontaneously) and *E. atrorubens* (maximum to 3.4%), attributable to a well-developed rostellum and clinandrium and to the position of pollinia. Autonomous selfing takes place during flower wilting, when the viscidium becomes evanescent and the pollinia are fragile. Selfing is enabled mostly by pollinator behaviour, promoting both geitonogamy and autogamy, and influencing mixed-mating.

Keywords Autogamy · *Epipactis* · Flower morphology · Geitonogamy · Hand pollination · Orchidaceae · Pollinator behaviour

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Introduction

The breeding system, involving the anatomical, morphological and physiological aspects of generative reproduction of individuals and populations (Neal and Anderson 2005), is an important character used for identifying many problematic taxa, and this can facilitate conservation of properly distinguished species (Tyteca and Dufrêne 1994; Pedersen and Ehlers 2000; Squirrell et al. 2002; Bonatti et al. 2006). Some taxa may be accorded protection not justified by biological reality (Pillon and Chase 2007). This is more common in taxonomically complicated groups that include endangered species (Pilgrim et al. 2004). One of them is the genus *Epipactis* Zinn. (Orchidaceae), whose range fluctuates from 25 species (Richards 1982) to 36 (Delforge 1995), or even 56 species (Delforge 2001). The numbers of invalid, intraspecific and hybrid names of *Epipactis* genus are higher in Europe than in other areas; this may be the result of a regional bias towards the taxonomy and popularity of orchids in Europe (Pillon and Chase 2007). Generally, in *Epipactis* morphological adaptations to local environments are well described, and new species and subspecies of *Epipactis* are often the subject of much discussion (Bateman 2001). One such adaptation is connected with floral architecture and the possibility of a transition between cross- and self-pollination. It seems that in some cases new taxa are classified on the basis of misinterpretation of the breeding system found (Pedersen and Ehlers 2000). Claessens et al. (1998) prepared an interesting review of the relations between the breeding system and various factors of the natural environment. It shows that too much attention is devoted to the diversification of column morphology in relatively primitive groups such as *Epipactis* (primitiveness results from features including a poorly integrated gynostemium with

an erect anther, soft structure of the pollinia, a very primitive type of rostellum, the character of the viscidium, rhizomatous growth, and terrestrial habit; reviewed in Szlachetko 1995, and in Pridgeon et al. 2005), where such structural changes can be regarded as a minor adaptation to existing conditions (e.g. drought, poor nutrition, absence of pollinators). Their suggestion that *E. renzii* is a variety of *E. helleborine* ssp. *neerlandica* was later confirmed by Pedersen and Ehlers (2000), who applied isozyme analysis.

In our opinion, incomplete recognition of the full breeding potential of the genus *Epipactis* is one of the main reasons for the taxonomic difficulties in this group. This is clearly seen in *Epipactis helleborine*, a species widespread in Europe, and intensively studied in North America as an alien species. It is a progenitor of problematic allogamous and autogamous subgroups, which were distinguished in early systematic monographs (e.g. Keller and Schlechter 1940) and are still being modified (e.g. Tyteca and Dufrêne 1994; Harris and Abbott 1997; Squirrell et al. 2002). Discussion of its breeding is still open, despite the strong view that allogamy is predominant (e.g. Sundermann 1975; Richards 1982; Burns-Balogh et al. 1987; Müller 1988; Claessens and Kleynen 1995; Proctor et al. 1996; Ehlers and Pedersen 2000; Squirrell et al. 2001; Bonatti et al. 2006). Some reports have described *E. helleborine* as being capable of facultative autogamy, and this has raised the question of possible self-fertilization (e.g. Morong 1893; Mousley 1927; Hagerup 1952; Fredrikson 1992; Delforge 1996; Squirrell et al. 2001; Ehlers et al. 2002).

Two other *Epipactis* species form less problematic groups (Delforge 1995, 2001): *E. atrorubens*, located mainly in Europe but whose range extends from eastern Siberia to Central Asia and the Far East (Tuulik 1998), and *E. palustris*, locally common and very widely distributed throughout Europe, the Middle East and Japan (Pridgeon 2003). Their breeding systems are the subject of controversy, however. East (1940) stated that *E. atrorubens* is self-incompatible. This is inconsistent with self-pollination reports (Hagerup 1952; Proctor and Yeo 1973; Fredrikson 1992) and even those describing predominant but not obligatory allogamy (Sundermann 1975; Nilsson 1981; Richards 1982; Claessens and Kleynen 1995). Views about breeding in *E. palustris* are strongly divided into two groups. The first of them maintains that autogamy is present, regular and most important (Kirchner 1922; Hagerup 1952; Wiefelspütz 1970; Müller 1988; Delforge 1995). The second group describes *E. palustris* as a predominantly allogamous species (Sundermann 1975; Brantjes 1981; Nilsson 1981; Richards 1982; Scacchi et al. 1987). Additionally, Fredrikson (1992) reported that development of female gametophyte of *E. palustris* appears to be inconsistent with that of a self-fertilizing species.

Investigation of selfing in different *Epipactis* species can help resolve taxonomical problems and provide information useful in understanding the ecology and genetics of their populations on larger geographical scales. Knowledge about selfing is especially useful in explaining the colonization success which has been documented in the group (reviewed in Hollingsworth and Dickson 1997, and in Adamowski 2006).

In this study we evaluated selfing potential and the limits to it in *Epipactis helleborine*, *E. atrorubens* and *E. palustris* populations located in the compact geographical distribution and relatively natural habitats characteristic of Central Europe. We particularly wished to describe how floral architecture promotes the breeding types we found, as well as whether and how pollinator activity influences self-pollination in the three species. We also attempted to determine the breeding system model of a given *Epipactis* species using reports from other locations, with an emphasis on evolutionary strategies employed when insect-mediated pollination has failed.

Materials and methods

Flowers of studied species

Each *Epipactis* species produces an erect, multiflorous raceme. The labellum contains different amounts of nectar inside. The stigmatic surface slopes forwards over the hypochile. Wing-like staminodes usually form a distinct, well-developed clinandrium. The rostellum, if present, is shelf-like (Szlachetko 1995). The viscidium is semi-liquid, sticky and non-detachable, which is unique to the tribe Epipactideae according to Rasmussen (1985). Pollen, composed of many pollen grains arranged in isolated tetrads and united in two pollinia by unknown substances, can also be transferred as a moderate number of tetrads (Light and MacConaill 1998; Pacini and Hesse 2002).

In *Epipactis* a well-developed rostellum creates the most important physical barrier between the male and female parts of the flower, preventing self-fertilization (Richards 1982; Pedersen and Ehlers 2000; Bonatti et al. 2006). In most self-pollinated orchids this structure either does not develop, develops incompletely, or sometimes disintegrates during flowering (Catling 1990). Delforge (1996) observed both autogamous and allogamous flowers within the same *E. helleborine* plant. A well-developed clinandrium performs a function similar to that of the rostellum. This spacious hollow with the anther in the upper part of the column hides the pollinia inside and prevents the pollen from falling on the stigma. The presence of a large, viscous viscidium ensures that the pollinia are removed by pollinators, and hence the level of

allogamy is decreased. In self-pollinating species the viscidium withers as the flower opens (Richards 1982). More compact and less fragile pollinia protect against pollen falling on the active stigma zone (Johnson and Edwards 2000; Ehlers et al. 2002). When pollinia are less coherent and the viscidium is evanescent as the flower opens, the pollen grains disintegrate on the stigma surface, enabling rapid self-pollination (Richards and Porter 1982). Finally, the system that most effectively prevents self-fertilization is self-incompatibility.

Study sites

Populations (differing in size, origin, and location in different types of plant communities) were observed in two protected regions of North-East Poland c. 100 km apart: Biebrza National Park and Wigry National Park. The first includes the largest complex of peatbogs in Poland, with mineral islands formed by eolian processes. Wigry National Park protects natural and semi-natural forest communities surrounding lakes and peatbogs. The population names are given in Table 1.

Hand pollination

Numerous hand pollination treatments were performed during 2002–2006 (Tables 1, 2). Every inflorescence containing flower buds at an advanced stage of development was placed in a nylon mesh bag to exclude all potential pollinators. During anthesis, three pollination treatments described by Dafni (1992) were applied: (1) induced self-pollination to indicate the presence and level of self-compatibility; (2) spontaneous self-pollination (flowers untreated and bagged throughout blooming) to determine the probability of autogamy without the participation of pollinators; and (3) induced geitonogamy to evaluate the level of inflorescence self-compatibility. After treatments (1) and (3) the inflorescences were rebagged. The position of the flower on each inflorescence was numbered from the bottom up. The bracts were marked for the location of fruit via flowers, which is problematic in multiflorous stems with a high density of flowers. The pollinia were removed with a fresh wooden toothpick for each flower, and smeared over the whole surface of the stigma. Each flower

from treatment (3) was emasculated artificially to prevent self-pollination. Pollination was done quickly after emasculation to avoid the influence of pollinia removal on fertilization potential. Damaged and dried flowering ramets were excluded from the counts of fruits. Fruit set consistency is commonly used to estimate reproductive success in orchids (Chung and Chung 2005; Pellegrino et al. 2005, 2006; Ke-Wei et al. 2006).

Pollinator observations

Pollinator behaviour was observed under natural conditions in 2004–2006 (Table 1), during fine weather: 39.5 h of field observations of 112 ramets of *E. palustris*, 25.5 h of 27 ramets of *E. helleborine*, and 20 h of 50 ramets of *E. atrorubens*. An insect visitor was defined as a pollinator if pollinia were observed on its body; the insect went so far into the flower that it had a contact with the rostellum (many flowers no longer had pollinaria) and the insect took out the pollinia. Pollinator movement within inflorescences was recorded and classified. Return flights to the same inflorescence (=geitonogamy) and to the same flower (=autogamy) were also noted.

Flower properties

Flowers were analyzed in terms of whether their architecture might promote or protect against spontaneous autogamy. In each population, 20 inflorescences randomly chosen were checked for (1) the presence and development of the rostellum and clinandrium; (2) the strength of viscidium glue; and (3) the compactness of pollen grains. The last two characteristics were checked throughout flower development, from opening to wilting.

Results

Hand pollination

Fruit set was noted after non-induced self-pollination only in all the analyzed populations of *E. palustris* (Table 2A). In the other two species, fruits from spontaneously pollinated flowers were recorded sporadically (Table 2B, C).

Table 1 Name of population and time (years) when particular treatments and observations were conducted in each place

| | *E. palustris* | | | | *E. helleborine* | | | *E. atrorubens* | |
| | Biebrza | | Wigry | | Biebrza | | | Biebrza | Wigry |
	ZAB1	MG	NB	JR	ZAB2	OPA	DG	SOS	WAS
Hand pollination	3	1	4	–	5	5	1	2	4
Pollinator observation	1	–	3	3	2	2	1	1	2

Table 2 Results of hand-pollination treatments

A. *Epipactis palustris*

Year	Spontaneous autogamy			Induced autogamy			Induced geitonogamy		
	ZAB1	MG	NB	ZAB1	MG	NB	ZAB1	MG	NB
2003[a]	–	2/31 **11.1 ± 15.71**	10/72 **82.5 ± 14.74**	–	2/26 **85.4 ± 7.71**	–	–	3/33 **93.5 ± 5.78**	–
2004[b]	17/208 **29.1 ± 24.47**	–	5/18 52.8 ± 48.99	6/21 **6.7 ± 16.32** c*** d**	–	2/7 **100.0 ± 0.00**	3/9 **44.4 ± 57.73**	–	4/27 **90.6 ± 18.75**
2005[c]	14/80 **0.5 ± 1.67** b*** d***	–	19/83 43.2 ± 32.26 a*	4/36 **100.0 ± 0.00**	–	2/12 **65.0 ± 21.21**	3/25 **100.0 ± 0.00**	–	4/19 **61.9 ± 10.28**
2006[d]	6/54 **37.3 ± 34.01**	–	20/130 **60.0 ± 30.99**	3/18 **71.3 ± 7.50**	–	5/36 **85.0 ± 14.81**	3/45 **83.7 ± 5.77**	–	3/11 **50.0 ± 50.00**

B. *Epipactis helleborine*

Year	Spontaneous autogamy			Induced autogamy		Induced geitonogamy	
	ZAB2	OPA	DG	ZAB2	OPA	ZAB2	OPA
2002[a]	12/210 **0 ± 0.00**	15/311 **0 ± 0.00**	5/110 **2.7 ± 13.32**	–	–	–	–
2003[b]	7/90 **0 ± 0.00**	6/110 **0 ± 0.00**		–	–	–	–
2004[c]	11/206 **2.8 ± 5.67**	19/421 **0 ± 0.00**	–	8/42 **51.2 ± 52.49**	–	5/46 **84.4 ± 14.91**	–
2005[d]	11/120 **0 ± 0.00**	13/198 **0.3 ± 0.92**	–	3/20 **94.4 ± 7.86**	2/16 **78.6 ± 30.31**	4/43 **79.3 ± 17.71**	2/16 **81.2 ± 1.66**
2006[e]	7/84 **0 ± 0.00**	6/93 **0 ± 0.00**	–	3/29 **93.0 ± 11.27**	1/15 **100.0 ± 0.00**	3/31 **91.0 ± 15.59**	2/33 **63.5 ± 27.58**

C. *Epipactis atrorubens*

Year	Spontaneous autogamy		Induced autogamy		Induced geitonogamy	
	SOS	WAS	SOS	WAS	SOS	WAS
2003[a]	2/53 **0 ± 0.00**	10/234 **3.4 ± 3.75**	–	–	–	–
2004[b]	2/22 **0 ± 0.00**	9/53 **0.8 ± 2.57**	–	3/12 **13.3 ± 23.09** c* d*	1/16 **37.5 ± 0.00**	4/22 **51.2 ± 44.79**
2005[c]	–	14/110 **0 ± 0.00**	–	4/20 **100.0 ± 0.00**	–	3/36 **96.7 ± 5.77**
2006[d]	–	9/105 **0 ± 0.00**	–	1/20 **95.0 ± 0.00**	–	–

N/n, examined ramets/flowers; mean value of fruit set in percentage (in bold) ± standard deviation (SD). Fisher's exact probability test between years (*a–e*) in the particular population: $P < 0.05$*, $P < 0.01$**, $P < 0.001$***

High levels of fruiting from artificial autogamy were recorded in each *Epipactis* species, reaching even 100%, and fruit set from artificial geitonogamy was also confirmed (Table 2A–C). There were differences in fruit set between years within population and between populations within year for each treatment for a given species, except for accidental fruit set noted after autonomous selfing of *E. helleborine* and *E. atrorubens*, but almost none of the

observed differences were significant. The biggest exception was for spontaneous self-pollination in population ZAB1 of *E. palustris* (Table 2A). Fruit set from spontaneous pollination for the whole 2004–2006 period was significantly higher in population NB than in ZAB1 ($\chi^2 = 31.79$, *df* = 1, *P* = 0.00).

Pollinator observations

Pollinators of *E. palustris* presented similar percentages of both foraging movements: single-flower visits (52% of all visits) and visits to many flowers within the same inflorescence (48%). In *E. helleborine* populations, pollinator activity was slightly more frequent in many flowers within the same stem (59%), whereas in *E. atrorubens* populations it was the significantly dominant type of foraging (87%; $\chi^2 = 26.75$, *df* = 1, *P* = 0.00). In a given species, return flights to the same flower during many flower visits with a mixed pattern of movement, was noted. It was difficult to assess such activity precisely, due to the density of flowers on the inflorescences, especially in *E. helleborine* and *E. atrorubens*. Immediate returns to the same raceme [A] and the same flower [B] were documented at least in the following: *E. palustris* − [A] = 17% (of all noted visits), [B] = 40% (of all [A] visits); *E. helleborine* – [A] = 10% and [B] = 55%; *E. atrorubens* – [A] = 3% and [B] = 0%. Return flights to the same shoot were significantly lower in *E. atrorubens* than in *E. palustris* (Fisher's exact probability test, *P* = 0.0029).

Flower properties

A well-developed rostellum was found in each *Epipactis* species (Fig. 1a, c, e). Flower architecture differed in terms of clinandrium development. The clinandrium was perfectly formed in *E. helleborine*, where the pollinia were at the back of the rostellum and cradled inside the anther thecae (Fig. 1c, d). In *E. atrorubens* this structure was also well developed (Fig. 1e, f). In *E. palustris* the clinandrium was barely formed and the anther resembled a flat shelf (Fig. 1b). The pollinia were covered by well-developed staminodes, but from the front they were projected over the rostellum (Fig. 1a). This location was well observed after we had removed the pollinia. Adherence of the viscidium surface to both pollinia was moved slightly towards the wider pole (Fig. 1g), whereas in *E. helleborine* and *E. atrorubens* the viscidium was connected to the pollinaria at the end of their narrow part (Fig. 1h, i).

In *E. helleborine* and *E. atrorubens,* the pollinia were very compact during the entire flowering period, whereas in *E. palustris* they were more pulverulent. Afterwards, in a given *Epipactis*, most of them were crumbled into incoherent tetrads and became fragile when the flower withered. In some *E. helleborine* flowers, pollinia not taken by pollinators before the moment of flower wilting became visibly swollen; this was noted most frequently in population DG. Less than 10% of the pollinia of a given species became dry and hard in ageing flowers, except for population SOS of *E. atrorubens*, where it was noted in more than half of the analyzed flowers each year.

During anthesis the viscid matter was highly glutinous in almost all of the populations studied. In the flowers of one population of *E. atrorubens* (SOS), however, the viscidium was barely viscous from flower opening, and dried quickly during flowering. Such a viscidium was noted sporadically in populations ZAB1 and NB of *E. palustris* (1.2–4.7%). The viscidium dried during flower withering in each population of the *Epipactis* species.

Discussion

Selfing as a consequence of flower architecture

The very high level of fruit set in the artificial autogamy experiment, reaching even 100%, clearly shows that every analyzed *Epipactis* species is self-compatible. Proper classification of *E. atrorubens* as a self-compatible orchid is much needed. East (1940) ascribed self-incompatibility to it, and that characterization has been used in some analyses (Tremblay et al. 2005).

Of the species studied, only *E. palustris* may be characterized as a facultative spontaneous self-pollinator: fruit set after the pollinator exclusion treatment was noted every year in each population. In the two remaining species, very low fruit set recorded after such experiments suggest that spontaneous autogamy takes place accidentally. These differences are connected with gynostemium morphology and the properties of the pollinia. The well-developed rostellum in *E. palustris* does not seem to be a significant barrier preventing self-fertilization. That is because the pollinia are located terminally (as also observed by Godfery 1933) and even slightly projected over the rostellum. Moreover, the clinandrium in *E. palustris* is weakly developed. Thus, although the pollinia are covered by the staminodes, pollen grains are pushed forward, especially during flower senescence. In that period the pollinia become less compact and enlarge their volume. The structure of the pollinia is also rather fragile during flowering (as reported also by Godfery 1933). When the flower is in the typical position, pollen cannot fall straight onto the stigma zone, which is placed at the back of the projected rostellum with the viscidium and pollinia. However, this position enables the pollen tetrads to fall onto and surround the rostellum/viscidium structure and the upper edge of the stigma. This process becomes easier when during wilting

Fig. 1 **a**, **c**, **e** View of column of mature flowers. **b**, **d**, **f** Dorsal view showing clinandrium and rostellum. **g**, **h**, **i** Detail of the connection between pollinia and viscidium. *a* Fertile anther; *cc* clinandrium cavity; *p* pollinium; *r* rostellum; *rv* rostellum-viscidium; *s* staminode; *st* stigma; *v* viscidium
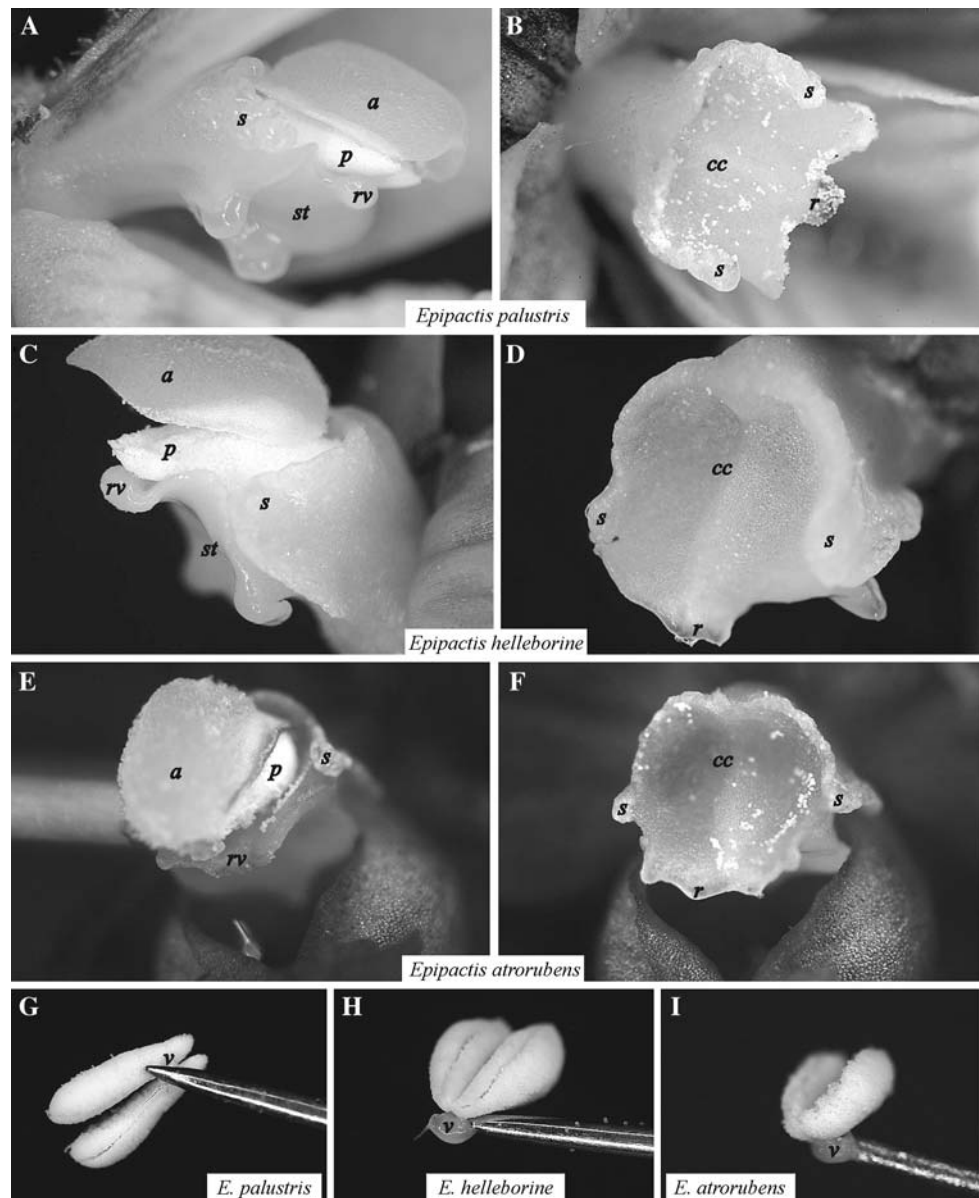
the viscidium dries, the rostellum/viscidium barrier is weaker, and the pollen tetrads are not trapped in the sticky glue. Pollen grains may spread out over the active stigma lobe from these localities, and this process can be promoted by stigma exudates. Our observations confirm reports of regular self-pollination in *E. palustris* when insect visits failed—a situation similar to a pollination exclusion experiment—and the pollen falls from the fragile pollinia onto the upper edges of the stigma (Kirchner 1922; Wiefelspütz 1970). Further observations (Müller 1988) showed that pollinator visitation does not lead to complete fertilization of inflorescences and that a small percentage of the flowers are fertilized by themselves. Our work indicates that self-pollination in this species may occur before the non-fertilized flower starts ageing. The elongated and

heavy ovary is situated on a long pedicle. This flexible and delicate pedicle/ovary structure is very sensitive to any externally generated movements such as wind or animal penetration, when the whole flower moves in all directions. Such movements can disintegrate the pulverulent pollinia mechanically; especially those placed most externally, and can accelerate the process described above. The influence of wind or rain on self-fertilization in this orchid was analyzed by Müller (1988), who noted only 8% autonomously pollinated flowers in greenhouse conditions (without the external factors mentioned above), in contrast to 23% in the field. Some reports do not support autonomous pollination in *E. palustris*. Bagged inflorescences in bud stage from a semi-wild population in the botanical garden in Haren (The Netherlands) formed no fruits

(Brantjes 1981). Such a result, combined with our findings (Table 2A), suggests that the level of self-pollination in this species varies between sites and years in the same population.

In *E. helleborine* and *E. atrorubens*, unlike in *E. palustris*, the position of the pollinia versus the well-developed rostellum limits self-pollination (Fig. 1c, e). Moreover, in the typical position of the flower of *E. helleborine*, the pollinia are sheltered in the well-developed clinandrium and effectively separated from the stigma. Bonatti et al. (2006) made the same observation, and stated that coherent masses of pollen in open flowers of *E. helleborine* were held firmly to the well-developed rostellar apophysis by adhesive material. Therefore, even when pollinia become incoherent during flower ageing, the pollen grains separate mainly inside the anther thecae. Apart from our results, none of 116 bagged plants in 12 Swedish populations of *E. helleborine* produced fruit (Ehlers et al. 2002). In *E. atrorubens*, clinandrium development seems to ensure pollen fall sufficiently. However, in both species some tetrads were regularly found on the rostellum and on the edge of the upper stigma lobe. The work of Ehlers et al. (2002) supports our suggestion that in drier years the pollinia of *E. helleborine* may become less coherent during anthesis and thereby cause facultative autogamy by letting pollen fall left and right of the rostellum. A similar process has been observed in *E. helleborine* ssp. *neerlandica* (Claessens et al. 1998) and *E. helleborine* ssp. *orbicularis* (Claessens and Kleynen 1997) growing in extremely hot and dry conditions. In our study, in drier conditions the pollinia usually became dry and hard in *E. helleborine* and *E. atrorubens*, especially in population SOS of *E. atrorubens*, which grows on a sandy and sunny dune. In both species, crumbling of pollinia was associated mostly with flower wilting and therefore with self-pollination as insurance against the failure of insect visitors; Proctor and Yeo (1973) came to the same conclusion. Moreover, in *E. helleborine* populations Mousley (1927) infrequently observed that when flowers failed to be visited by insects the pollinia become swollen and fragile, the anther cap lifted and pollen grains separated; the tetrads were pushed over the edge of the stigma, causing self-fertilization. We did not observe such a mechanism, but we noted swollen pollinia during flower wilting in *E. helleborine*. We found them mostly in population DG, where a few fruits from autonomous autogamy were set (Table 2B). Squirrell et al. (2001) reported an interesting observation (by M. Light) of spontaneous self-fertilization in *E. helleborine* in some introduced populations at Getineau Park (North America). In some plants that flower in dry conditions, when subjected to showers and relatively high humidity the pollinia have been observed to flip 180° longitudinally and come to rest on the stigmatic surface. Such a report merits careful analysis. Bending movements of pollinaria usually have been viewed as a mechanism for preventing geitonogamous self-fertilization, but sometimes it leads to autogamy among orchids in a weedy habitat (Dafni and Firmage 2000; Ke-Weu et al. 2006).

In the three species studied, it seems that the quality of pollen grains reaching the stigma surface is not always high enough to induce fertilization; the pollen loses its capacity to germinate and/or to fertilize during flower wilting. This was observed in our study in 2004, when hand-pollination in population WAS (*E. atrorubens*) and population ZAB1 (*E. palustris*) was done quite late: fruit set from each hand-pollination experiment was lower than in other years (Table 2A, C). Fruit set is a valid and accurate test of pollen viability (Dafni and Firmage 2000). Our results suggest that viable pollen grains are most likely to be found among the many tetrads reaching the stigma surface in *E. palustris*, but not in *E. helleborine* and *E. atrorubens*.

Selfing as the consequence of pollinator behaviour

Self-pollination in *Epipactis* species can be the result of insect activity within the same flower as well as in the same inflorescence. The examined orchids are not dependent on strictly specific pollinators, as many others are. There are other reports of a variety of pollinators from other geographical regions, giving the largest number of insect agents for *E. palustris* (we observed mainly *Vespidae*, *Colletidae*, *Eumenidae*, *Syrphidae*; other reports: Nilsson 1978; Brantjes 1981), fewer kinds of pollinators for *E. helleborine* (we noted mostly *Vespidae* and *Colletidae*; other data: Ehlers and Olesen 1997; Light and MacConaill 1998; Hartmann-Schröder and Brauckmann 2002; Jakubska et al. 2005) and the fewest for *E. atrorubens* (we observed *Bombus* species as the dominant visitors; see also: Godfery 1933; Claessens and Kleynen 1995). Lower diversity of insects means a narrower range of foraging behaviour. This is exemplified by *E. atrorubens*, having bumble-bees as the main pollinators: many visits to flowers within the same inflorescence were the dominant modality, whereas returns to the same flower were noted sporadically. *E. palustris* was visited by the most behaviourally diversified group of insects: visitation of a single flower only (promoting crossing) as well as visits to many flowers on a single inflorescence (promoting autogamy, geitonogamy and crossing) were noted in equal proportions; return flights to the same inflorescence (promoting geitonogamy) and even to the same flower left a moment ago (promoting autogamy) was observed most often. Finally, in *E. helleborine* the range of foraging behaviour could be considered intermediate between the other two species.

There are not many reports of pollinator activity promoting selfing in the three *Epipactis* species studied. Some

observations of *E. palustris* pollinator activity in a few flowers, in one inflorescence, as well as repeated visits to the same flower have been reported (Nilsson 1978; Brantjes 1981). Geitonogamy has been analyzed mainly in *E. helleborine*, due to the presence of special compounds in its nectar which influence pollinator behaviour (Ehlers and Olesen 1997; Jakubska et al. 2005). According to our observations, such an influence is habitat-dependent. Toxic nectar is effective in very shady habitats where the major pollinators are scarce and insects can be held in an inflorescence for a longer time. In sunny patches, visitors stay for few seconds in an inflorescence, and it is clear that visits to many flowers on a given inflorescence are the result of their natural foraging behaviour. Light and MacConaill (1998) observed in *E. helleborine* that pollinator movements were very active, not sluggish. They noted visits that lasted a few seconds and could promote geitonogamy and autogamy.

Geitonogamous pollination is made possible by self-compatibility within the inflorescence, as shown by the induced geitonogamy experiment. The absence of this barrier was also confirmed by the positive result of a germination experiment using seeds obtained from artificial geitonogamy (Light and MacConaill 1998). As we found, the chance for geitonogamy but also xenogamy in a given *Epipactis* species decreases during flower ageing as the viscidium dries. During this period, insects cannot take pollinia and autonomous autogamy is the only possible mode of fertilization.

Selfing strategies

Our studies showed that in undisturbed habitats and with an abundance of insects, selfing is the most probable mode of pollination in the three *Epipactis* species, as pollinator behaviour promotes geitonogamous pollination and influences mixed-mating. Autonomous selfing occurs as insurance when insect-mediated pollination fails. It can take place easily in *E. palustris*, which should be considered facultatively autogamous, though the intensity of such a process in this orchid varies between sites and even years in the same population. In typical conditions, spontaneous autogamy is accidental in *E. helleborine* and *E. atrorubens*, and both species should be classed as predominantly allogamous. As *Epipactis* species can rapidly evolve changes in column morphology as a minor adaptation to unfavourable conditions (Claessens et al. 1998), it is also possible to find facultative autogamy in both of those orchids. Although such an evolutionary strategy has been described only in *E. helleborine* (Delforge 1996), *E. atrorubens* column architecture merits attention because it often occupies harsh, dry habitats.

To investigate the full evolutionary potential of the breeding system of the genus *Epipactis*, studies covering a geographically wide habitat range are needed. The present data should be used cautiously in discussions of the ecology or genetics of populations from different sites. Experiments to verify the presence or absence of spontaneous autogamy in a particular population should continue for at least 1 or 2 years.

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