

Male and female reproductive success in natural and anthropogenic populations of *Malaxis monophyllos* (L.) Sw. (Orchidaceae)

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Abstract: In the presented study, male and female reproductive success was analyzed in relation to the population size, floral display and pollinators' availability in natural and anthropogenic populations of the orchid *Malaxis monophyllos* (L.) Sw. Our results indicated significant differences between all investigated populations in parameters of floral display, including heights and number of flowers per inflorescence, as well as the number of flowering individuals and their spatial structure. Additionally, populations differed both in male (pollinia removal) and female (fruit set) reproductive success, but only the fruit set clearly differentiated anthropogenic and natural populations. Despite the average flower number per plant being significantly higher in two of the anthropogenic populations, it was not related to the fruits set, which was significantly lower there. Moreover, our preliminary study concerning the potential pollinators of *M. monophyllos* showed a higher contribution of flies in natural habitats than in anthropogenic ones. Thus, we can suspect that the main factors influencing the level of female reproductive success in *M. monophyllos* populations are abundance of effective pollinators, as well as flower visitors, which may have resulted in a different level of pollen discounting in populations. Therefore, further studies concerning breeding system and pollination as important forces that shape demographic processes in *M. monophyllos* populations are necessary. Our results also indicate that suitable conservation methods in *M. monophyllos* should always include the preservation of potential pollinators, especially in these new, secondary habitats.

Key words: pollinators, pollen discounting, fruit set, red-listed species

1. Introduction

In animal-pollinated plants, male and female reproductive success is influenced by a number of factors, including the size of the population, floral display and environmental conditions. These factors affect the attractiveness to pollinators and the number of compatible mates (Ashman *et al.* 2004). Several studies have shown that pollinators spend longer time and visit more flowers in large populations than in small ones (Aguilar *et al.* 2006). Many authors have also suggested that reproductive success may decrease with increasing population size, when pollinators are scarce (Brody & Mitchell 1997; Ohashi & Yahara 1998; Brzosko 2002a). The foraging

behaviour of pollinators also largely depends on the density and spatial structure of flowering plants. In general, lower density reduces the attractiveness to pollinators (Bosch & Waser 2001), whereas clumped distributions result in higher visitation rates (Firmage & Cole 1988; Aragón & Ackerman 2001). Floral display measured by individual features (i.e. the number of flowers per plant), often varies tremendously between populations and also within a single population (Brzosko 2002b, 2003). Individuals exposing a large floral display experience higher visitation rates because they are more efficient in attracting pollinators, but it is also strictly dependent on whether it is a rewarding or non-rewarding species (Mitchell *et al.* 2004; Jacquemyn *et al.* 2008).

Apart from the impact of population and individual plant on the pollinator's foraging behaviour, pollinator availability seems to be crucial for the level of reproductive success. This is most visible in orchids, which have been shown to be severely pollinator limited (Tremblay *et al.* 2005). The rate of the pollinators' visits to orchid individuals may be affected by the degree of synchronous phenology of the plant and its pollinators (Zimmerman *et al.* 1989; Sabat & Ackerman 1996) and is strictly dependent on the microhabitat, including the plant community (O'Connell & Johnston 1998).

Simultaneously, the ongoing environmental changes both, natural and man-made, can significantly affect the reproductive success of plants, especially those dependent on pollinators. On the one hand, human activities lead to changes in habitat conditions, but on the other, they lead to the creation of new, secondary environments, which are, at least partially, alternative for some species, including the rarest ones (Nowak & Nowak 2006; Lundholm & Richardson 2010). The colonization of anthropogenic habitats by orchids is relatively frequent all over the world (Jakubska *et al.* 2006; Esfeld *et al.* 2008; Schefferson *et al.* 2008; Błońska 2010; Scheffknecht *et al.* 2010). In consequence, knowledge about the properties of these secondary populations is of particular importance, especially in the face of the ongoing declining of its natural locations. Moreover, there is little empirical evidence available on pollination efficiency and reproduction in anthropogenic orchid populations (Parra-Tabla *et al.* 2000; Pellegrino & Belusci 2014).

In the present study, male and female reproductive success was analysed in relation to floral display and pollinator availability in natural and anthropogenic populations of the orchid *Malaxis monophyllos* (L.) Sw. This boreal-mountain species is naturally linked with peaty environments, but populations in anthropogenic habitats (ditches, roadsides and railway embankments, excavations in old sand quarries and post-mining areas) have been quite well documented (Bernacki & Błońska 2006; Czylok *et al.* 2008; Gajewski 2010; Nowak *et al.* 2011). Suitable humidity and low competition in habitats transformed and created by humans have contributed to the growth of *M. monophyllos* populations, but have also made these populations unstable in the long term (Bernacki & Błońska 2006; Czylok & Szymczyk 2009). Furthermore, our previous studies revealed distinct demographic properties of anthropogenic populations in comparison to natural ones, in terms of larger densities and abundance (Jermakowicz & Brzosko 2011).

To explain the differences in reproductive success in contrasting environmental conditions, the following questions were investigated: (1) How do populations in natural and anthropogenic habitats differ in terms of

floral display? (2) Does male and female reproductive success differ between natural and anthropogenic populations of *M. monophyllos*? and, finally, (3) Is the reproductive success of natural and anthropogenic populations related to floral display and pollinator abundance?

2. Material and methods

2.1. Study species

Malaxis monophyllos var. *monophyllos* (L.) Sw. is an orchid with a fragmented distribution area covering the boreal part of Eurasia, part of North America, and Central-European mountain ranges, with surrounding foothills and uplands. Although it has a wide distribution range, it is a rare species, and has been entered in the Red Data Book of Plants in many countries, including Poland (Bernacki 2014). Its rarity is mostly caused by low availability of natural habitats as well by the small size and decline of natural populations (they rarely exceed 50 individuals).

The inflorescences bear several to tens of small, greenish flowers (up to 100, authors' observations), which start to open from the beginning of June to the middle of July. Each flower bears four pollinia attached to each other, slightly diverging at the base. Pollinia are always removed from the flower as a pair (Claessens & Kleynen 2011). Additionally, *M. monophyllos* flowers give off a faint, probably mushroom-like scent, but the data about nectar production are vague (Vöth 1999, Claessens & Kleynen 2011). The specific structure of *M. monophyllos* flowers involving the twisting of the pedicel by 360° results in returning the lip to the uppermost position in the flower ("hyperresupinate", according to Cameron 2005). All these features may determine the specific group of effective pollinators which, in alpine populations of this species, proved to be flies from the *Mycetophilidae* family. Moreover, it is presumably a self-incompatible species, pollinator-dependent for the fruit set and seed production (Vakhrameeva *et al.* 2008, Claessens & Kleynen 2011).

2.2. Study sites and populations

In 2014, we selected 6 populations of *M. monophyllos* located in contrasting conditions of natural, wet calcareous peatlands (N1, N2, N3), and in anthropogenic habitats of the Silesian Upland (A1, A2, A3) (Table 1, Fig. 1). All natural populations were located in North-East Poland in the East Baltic Lake District, in the boreal part of the range. Anthropogenic populations of *M. monophyllos* were included in the southern, mountainous-upland part of the range. In the case of anthropogenic populations, they were associated with afforested post-mining areas of zinc and lead ore (A1), railway embankments (A2) and with the excavation of sand (A3 population)

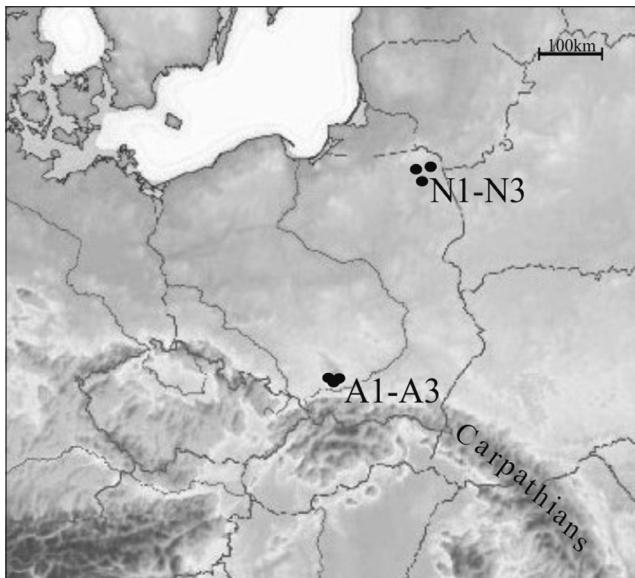


Fig. 1. Location of investigated *Malaxis monophyllos* populations

(Wójcik & Chmura 2005; Nowak *et al.* 2011). Habitats occupied by *M. monophyllos* differed in terms of type of community and in plant cover (Table 1).

2.3. Methods

Fieldwork took place from June to August 2014. At the beginning of anthesis, in each population, we determined the number of flowering plants. We also measured the distance between three nearest flowering plants or groups of flowering individuals (a group is an aggregation of flowering individuals equal to or exceeding two shoots), and we calculated the average distance between flowering individuals and groups of flowering individuals (D_F). Additionally, we assessed the mean density of flowering individuals in each group (d_F). These measurements were used to characterise the floral display in a given population. Depending on the population size, we marked from 11 to 20 flowering individuals. The height of labelled shoots and the total number of flowers per spike were used as other measures of the floral display. At the optimum of the flowering period, we recorded the frequency of pollinia removal from the examined plants (male reproductive success). At the end of the flowering period (beginning of August), we examined the fruiting level as the measure of female reproductive success. The fruit position on the inflorescence was also recorded in the context

Table 1. Codes, habitats and properties of 6 investigated *Malaxis monophyllos* populations

Population	Population code	Altitude (m.a.s.l.)	Habitat			Population area	N_F	Dispersion patterns	
			Type of plant community	Trees and shrubs layer cover (%)	Herbs layer cover (%)			D_F (m) (min.-max.)	d_F (min.-max.)
Rospuda River Valley	N	120	Alder-willow thickets on the edge of mineral island (<i>Alnetea-glutinosae</i>)	80	40-50	360	17	4.8 (2-10)	4 (2-5)
Kunis Lake	N2	124	Alder-willow thickets on the peat layer (<i>Alnetea-glutinosae</i>)	60	80-100	192	28	3.6 (1-6)	3.8 (2-6)
Czarna Hańcza River Valley	N3	138	Boreal spruce bog (<i>Vaccinio-Piceenion</i>)	50	50-70	420	27	10.2 (2.5-30)	4 (2-6)
Olkusz	A1	316	<i>Pinus sylvestris</i> cultivation with succession of coniferous forest species in undergrowth (<i>Vaccinio-Piceetea</i>)	40	20-50	15000	173	12.9 (2-40)	6.9 (3-25)
Sławków	A2	289	<i>Prunus spinosa</i> thickets on a railway embankment (<i>Rhamno-Prunetea</i>)	50-60	30-50	180*	21	6.2 (1-40)	2.3 (2-3)
Bukowno	A3	284	<i>Pinus sylvestris</i> and <i>Betula pendula</i> cultivation on a reclaimed heap with succession of coniferous forest species in undergrowth (<i>Vaccinio-Piceetea</i>)	50	30-50	200	7	9.2 (2-22)	2

Explanations: N_F – number of flowering individuals, D_F – average distance between single flowering shoots and group of shoots (minimal and maximal), d_F – mean density of flowering individuals in groups (minimal and maximal), * individual shoots within a distance > 20 m from the main concentration of shoots have been omitted

of the indirect assessment of the synchronization of flowering and pollinators' abundance. Simultaneously, in the optimum of the flowering period, sweep net sampling was used for estimating potential pollinators of *M. monophyllos*. Each sample was taken by 20 swings in a 180° arc. A sweep was taken from right to left, a step was taken, and then another sweep, left to right. To get a good representation of insects in Olkusz, the largest population (A1), the sweep net sample was doubled. All individuals of insects in each population were divided by the number of swings to get the number of insects per sweep (about 3 m²).

We used analysis of variance (ANOVA) to analyse differences between populations in terms of floral display measured by heights of shoots and number of flowers per inflorescence. We used the χ^2 test to examine differences between the level of pollinia removal and the fruit set between populations. Then we used Spearman's rank correlation to test the association between some of the parameters of floral display (height of shoots, number of flowers per inflorescence, number of flowering individuals in the population, D_F , d_F), population size, pollinator abundance, and male and female reproductive success. All statistical analyses were performed in STATISTICA PL. ver. 10 (Stat-Soft Inc., 2011) software packages.

3. Results

3.1. Floral display

Populations differed in terms of floral display, described by different parameters. The average height of flowering individuals varied significantly among populations ($F=2.92$, $p<0.05$) (Fig. 2a), independent of the type of habitat. However, the tallest flowering plants were recorded in the anthropogenic A2 population (26.0 ± 3.6 cm), and the lowest in the natural N1 population (16.6 ± 3.3 cm). We noticed significant differences between anthropogenic and natural populations in terms of the average number of flowers per spike ($F=4.85$, $p<0.05$). We recorded the highest number of flowers in the anthropogenic A1 and A2 populations. One of the anthropogenic populations (A3) represented similar flowers number to natural populations (Fig. 2b).

Populations of *M. monophyllos* differed considerably in the number of flowering individuals (from 7 to 173). Moreover, despite the fact that all *M. monophyllos* populations were characterized by the aggregative spatial structure of flowering shoots, the sizes of groups and the mean distance between them differed among populations (N3 population stood out because of its extremely small size) (Table 1). In the A1 anthropogenic population, groups of flowering individuals were most numerous (from 3 to 25 flowering shoots) and the most dispersed ($D_F=12.9$). The two other anthropo-

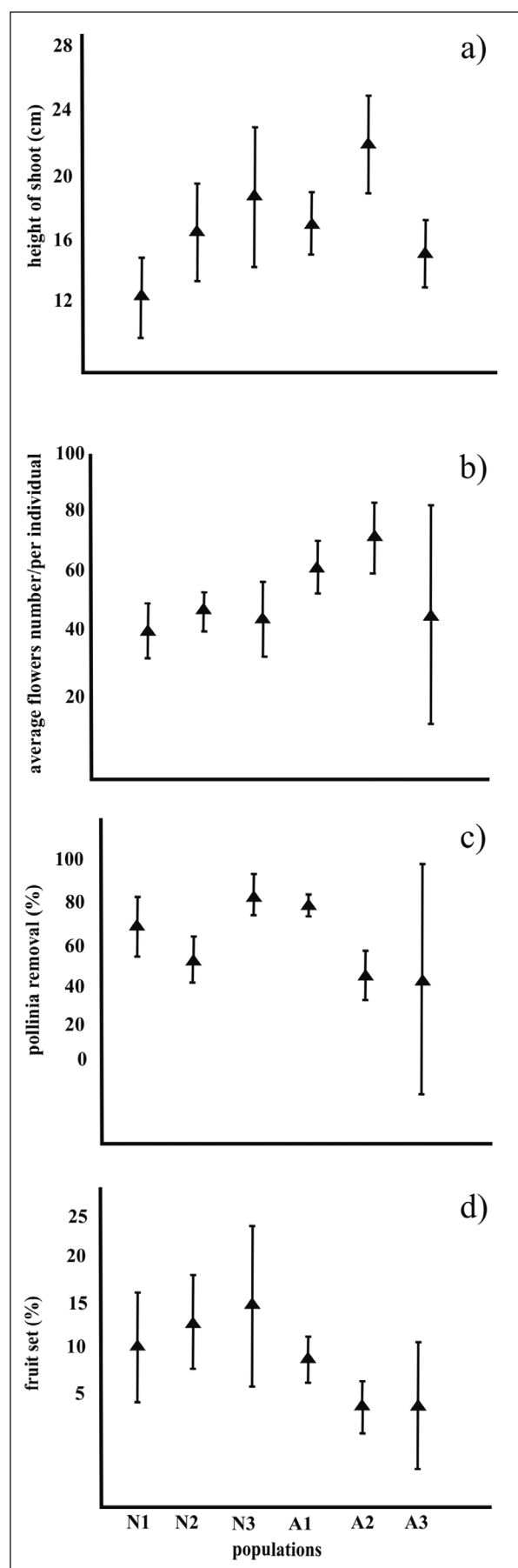


Fig. 2. Height of shoots (a), average flower number per inflorescence (b), proportion of flowers from which pollinia were removed (c) and proportion of flowers that developed into mature fruits (d) in six investigated *Malaxis monophyllos* populations

genic populations, A2 and A3, had a more cumulative structure ($D_F=6.2$ and 9.2) and much lower densities of flowering individuals in groups (2-3 flowering shoots). All the natural populations had a similar average density of flowering shoots in groups (around 4 shoots) (Table 1). However, the spatial structure of natural population, measured by average distance between single flowering shoots and group of shoots, differed considerably, from cumulative organization in the N1 and N2 populations ($D_F=4.8$ and 3.6 , respectively) to a dispersed structure in the N3 population ($D_F=10.2$).

3.2. Male and female reproductive success

The average pollinia removal differed considerably between populations (from 39.8% to 83.6% of pollinia removed from flowers per plant; $\chi^2=28.4$, $p<0.001$), independent of the type of habitat (Fig. 2c). Conversely, fruiting level was almost three times higher in natural populations than in anthropogenic ones (average for groups 12.3% and 4.4%, respectively; $\chi^2=15.47$, $p<0.001$; Fig. 2d). However, in the A1 anthropogenic population, the average fruit set was significantly higher (8.4%) than in the two other anthropogenic populations A2 and A3 (2.3%, 2.5% respectively).

If we consider the flowering individuals as a whole, we found a significant relationship between their heights and the proportion of the fruit set ($r=0.42$, $N=80$, $p<0.01$). When we consider flowering individuals from natural and anthropogenic populations separately, the relationship was clear and significant only for natural ones ($r=0.69$, $p<0.001$). The correlation between fruit

set and the number of flowers per inflorescence was also significant only for natural populations separately ($r=0.5$, $p<0.01$). Additionally, during the progressive development of the inflorescence, the frequency of fruit sets dropped in natural populations, and completely disappeared in anthropogenic ones (Fig. 3a-b).

However, neither flower number, nor the height of flowering individuals was related to pollinia removal. The rates of pollinia removal and level of fruit production were positively and significantly related with each other ($r=0.34$, $N=80$, $p<0.01$) which means that, in most cases, individuals with a high rate of pollinia removed, had also a higher proportion of fruit production in comparison to plants with low level of pollinia removed.

3.3. Pollinators' potential

We noticed some differences in the number of arthropods per sample (N_{Ins}) in the natural and anthropogenic populations, and in each of the investigated populations (Table 2). The lowest value of this parameter was recorded in the N1 population ($N_{Ins}=0.5$), while the highest was observed in the N3 population ($N_{Ins}=3.3$). However, the average diversity of arthropods

Table 2. Potential pollinators of *M. monophyllos* captured by sweep net in six populations

Population code	N_{Ins}	Frequency of given arthropods group
N1	0.5	- flies (<i>Diptera</i>) – 80% - hymenopterans (<i>Hymenoptera</i>) – 10% - beetles (<i>Coleoptera</i>) – 10%
N2	2.1	- flies (<i>Diptera</i>) – 87.8% - orthopterans (<i>Orthoptera</i>) – 7.4% - beetles (<i>Coleoptera</i>) – 1 (2.4%) - mites (<i>Acari</i>) – 1 (2.4%)
N3	3.3	- flies (<i>Diptera</i>) – 94% - hymenopterans (<i>Hymenoptera</i>) – 1.5% - spiders (<i>Aranea</i>) – 4.5%
A1*	2.4	- flies (<i>Diptera</i>) – 42.3% - hemipterans (<i>Hemiptera</i>) – 25.5% - hymenopterans (<i>Hymenoptera</i>) – 22.7% - spiders (<i>Aranea</i>) – 6.2% - moths (<i>Lepidoptera</i>) – 3.1% - beetles (<i>Coleoptera</i>) – 1%
A2	2.6	- flies (<i>Diptera</i>) – 57.7% - hemipterans (<i>Hemiptera</i>) – 19.2% - hymenopterans (<i>Hymenoptera</i>) – 17.3% - orthopterans (<i>Orthoptera</i>) – 3.9% - beetles (<i>Coleoptera</i>) – 2%
A3	1.5	- flies (<i>Diptera</i>) – 63.3% - hymenopterans (<i>Hymenoptera</i>) – 16.7% - spiders (<i>Aranea</i>) – 13.3% - beetles (<i>Coleoptera</i>) – 3.3% - hemipterans (<i>Hemiptera</i>) – 3.3%

Explanations: N_{Ins} – number of potential pollinators per sweep (about 3 m²) in each population, * – doubled sweep net sample (see Methods)

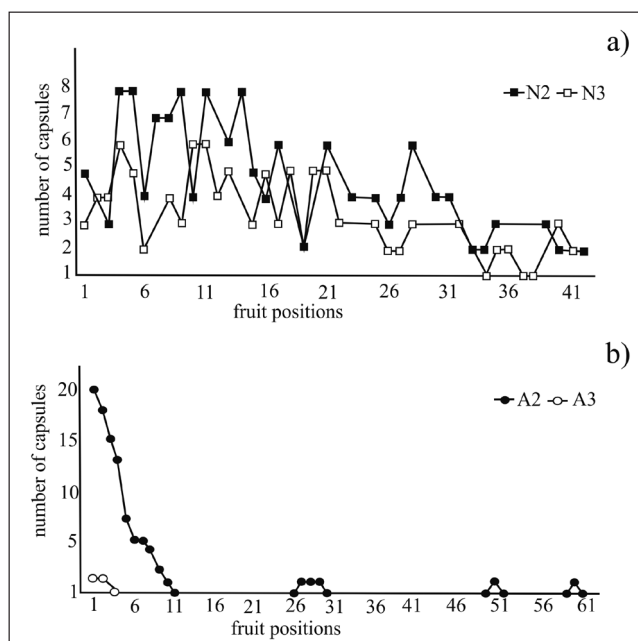


Fig. 3. Number of fruits recorded in different parts of the inflorescence in two natural (a) and two anthropogenic (b) populations. Numbers of fruits are the sum of fruits in a given position (from the base of the inflorescence)

(numbers of arthropod orders) was nearly twice higher in anthropogenic habitats than in natural ones (Table 2), and in all populations flies (the most probable group of *M. monophyllos* pollinators) were the most numerous insects representation. Additionally, in natural populations, the contribution of flies was very high (N1 – 80%; N2 – 87.7%; N3 – 94%), while in anthropogenic sites, it was significantly lower (A1 – 42.3%; A2 – 57.7%; A3 – 63.3%).

4. Discussion

Since the natural and anthropogenic habitats of *M. monophyllos* differed considerably in terms of humidity and vegetation, we suspected huge differences in the population reproductive parameters connected with these two types of habitats. Although anthropogenic habitats were often dryer and poorer in terms of vegetation than natural ones, we found there the most numerous populations and the biggest flowering shoots. However, floral display measured by individual size was significantly correlated with fruit set, but only in natural populations, clearly suggesting pollinator-dependent causes. Additionally, despite the fact that female success largely depended on population properties, like the number of compatible mates and its spatial structure (Ashman *et al.* 2004, Aguilar *et al.* 2006), we found no such correlations neither in natural nor in anthropogenic habitats. This may have been conditioned by different pollinator efficiency in different conditions, and the fact that in the case of non-rewarding orchids, floral display played a minor role. In these cases, flowering time and surrounding nectareous plants might be more important (Sabat & Ackerman 1996).

M. monophyllos, like other species of the *Malaxis* genus, is presumably an obligatorily cross-pollinated species which indicates that fruit set and seed production is fully dependent on pollinators (Vakhrameeva *et al.* 2008; Claessens & Kleynen 2011; Argue 2012). Additionally, the low to moderate level of fruit production observed in *M. monophyllos* populations (1.5 – 55%) (Vakhrameeva *et al.* 2008; Claessens & Kleynen 2011; Jermakowicz & Brzosko 2011) confirms this statement, and makes it comparable to other non-autogamous species (Neiland & Wilcock 1998; Brys *et al.* 2008; Argue 2012). Although there is a lack of information about nectar production in the case of *M. monophyllos*, available information suggests fungus-gnats from the Mycetophylidae family as the main group of pollinators (Claessens & Kleynen 2011). Fungus-gnats are usually found in humid and moist habitats, mainly in wooded areas, and the larvae of the majority of species are mycetophagous, feeding on the sporophores of fungus or on mycelium in dead wood (Rindal *et al.* 2009). However, our observations

showed presence of only simple representatives of this family in the Bukowno population, probably due to the crepuscular activity of Mycetophylidae (Claessens & Kleynen 2011). Flowers of *M. monophyllos* probably produce an unpleasant, mushroom-like scent (Vöth 1999) which can somehow attract female Mycetophylidae flies. Additionally, taking into account flower construction, we consider *M. monophyllos* to be a specialist that can attract different species of flies by both scents and colours. Thus, this information would suggest brood-site imitation as the deception mechanism in this species (Jersáková *et al.* 2006), although this requires further studies on the breeding system and pollination in *M. monophyllos* in general, as well in the context of different habitats.

Moreover, differences in fruit set caused by the habitat properties are also highlighted in the case of habitat modifications caused by natural and man-made factors. Comparisons of fruit production in natural and anthropogenic populations of orchids pointed out significant differences between these types of habitats. In anthropogenic populations of *Serapias cordigera* in Italy, for example, the fruit set was almost seven times lower than in populations from natural habitats (Pellegrino & Belusci 2014). Similarly, in a well-preserved population of *Oncidium ascendens* in Mexico, fruit production was almost two times higher than in populations from synanthropic habitats (Parra-Tabla *et al.* 2000). Our investigations of anthropogenic and natural *M. monophyllos* populations in Poland confirmed these results, because fruiting was significantly higher in natural populations (2-6 times). One of the possible explanations might be differences in pollinators' efficiency in completely different habitats. However, not clear connections between pollinia removal (male success) and pollinia deposition (female success) suggested that the fruit set was more limited in pollinia deposition in all the investigated populations. Such inconsistency, called pollen discounting, might be caused by different circumstances: (1) pollinators' behaviour, which can be modified by different environmental conditions, (2) population structure, when flowering individuals are spread over a huge area that cannot be effectively penetrated by pollinators, or (3) there may be a high frequency of visitors that are able to remove pollinia, but there is little chance that they will visit other flowering individuals of *M. monophyllos* (Ashman *et al.* 2004; Johnson *et al.* 2005; Aguilar *et al.* 2006; Brys *et al.* 2008). This was especially visible in the A1 anthropogenic population, which was the most extensive and abundant, and had only a moderate level of fruiting (in comparison to other populations). In such conditions, effective pollinators might be only a very small part of the total floral visitors, or because of the dispersed character of populations, pollinia were lost. Additionally, pollinia of *M. monophyllos* are especially

easy to remove from flowers, even by accidental visitors (authors observations) which can also promote pollen discounting. Moreover, pollinators' behaviour may change in response to new, abiotic conditions, and also as a response to the modification of plant phenotypes caused by changes in living conditions (O'Connell & Johnston 1998).

Additionally, analyses of the synchronization of flowering and pollinator abundance, indirectly through analyses of the fruit set in respect to position on the inflorescence, indicated spatial and temporal limits in pollinator occurrences. The highest proportion of the fruit set was in both sites at the bottom of the inflorescence, so they set at the beginning of flowering, in June. These results agree with the phenology of potential pollinators from the Mycetophyllide family which have the first peak of appearance in June, when they become active after the winter-spring lull, and before the autumn flush associated with the intensified appearance of fungus fruiting bodies (Rindal *et al.* 2009). Such data confirm that flowering time might be the most important trait affecting the fruit set, especially for orchids which do

not offer any reward to pollinators (Sabat & Ackerman 1996; Tuomi *et al.* 2015).

Our results imply that the availability of effective pollinators, and pollen discounting, were more important in shaping the level of female success than floral display, especially in anthropogenic populations of *M. monophyllus*. However, all population significantly differed in terms of floral display and, in natural populations, this factor seemed to be more important for increased fruit set. Simultaneously, our data highlighted the necessity to develop new conservation methods, including the preservation of potential pollinators in planning protection activities for *M. monophyllus* populations in both types of habitat. However, it does not change the fact that synanthropic habitats provide conditions for raising abundant populations of *M. monophyllus*, and they have great value in its effective conservation.

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