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FACTORS AFFECTING REPRODUCTIVE SUCCESS IN THREE ENTOMOPHILOUS ORCHID SPECIES IN HUNGARY

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Abstract: The reproductive success of orchids is traditionally estimated by determining the fruit-set of individuals. Here, we investigated both the fruit and the seed production of three orchid species and the factors that may affect individual fruit-set, like pollination strategy, individual traits or the annual amount of precipitation. The species (*Dactylorhiza sambucina* (L.) Soó, *Dactylorhiza majalis* (Rchb.) P. F. Hunt & Summerhayes and *Platanthera bifolia* (L.) L. C. M. Richard) were studied in three consecutive years (2010–2012) in the Bükk Mountains, Hungary. All three species were proved to be non-autogamous by a bagging experiment. Data analyses showed significant differences between seed numbers but not between fruit-sets of species. There was no statistical difference in individual reproductive success between wet and dry years, however, the effect of the annual amount of precipitation is significant on the population level. Comparison of published fruit-set data revealed accordance with our results in *P. bifolia*, but not in *D. sambucina* and *D. majalis*. We assume that the surprisingly high fruit-set values of the two *Dactylorhiza* species may be due to the fact that the pollination crisis reported from Western European countries may not be an actual problem in the Bükk Mountains, Hungary.

Key words: *Dactylorhiza majalis* – *Dactylorhiza sambucina* – fruit-set – *Platanthera bifolia* – pollination crisis

Running title: Reproductive success of three orchid species

Introduction

Orchids are commonly known as a threatened group of plants due to several species being rare and endangered and recently showing strong decrease in their distribution areas [15]. The success of orchids in a habitat depends on several factors, but it is mostly affected by their reproductive success. Due to their conservation value the investigation of their reproductive success is quite a relevant topic today. It is proved that reproductive success is predicted by individual traits like pollination mode (rewarding, non-rewarding, autogamous), plant height, etc. and also predicted by community and environmental features like population size and annual precipitation.

The generally used method of quantifying the reproductive success of orchids is determining fruit-set (the proportion of flowers which developed into fruits) [28].

Although, many studies have discussed reproductive success of orchids by giving fruit-set data (e. g. [20]), quite a few attempted to deal with seed numbers [27]. Providing seed numbers per fruit or even per specimen may give further information on reproductive success, as the large number of seeds produced in every fruit compensates for low effectiveness of deceptive pollination and may ensure the persistence of the populations [10]. Extremely high seed number of higher quality seeds together with the energy-saving strategy of non-rewarding orchids may explain the success of this strategy.

In previous studies (e.g. [28, 4, 5]) pollination mode was proved to be the main predictor of reproductive success. Most orchid species have low reproductive success, as far as fruit-set is concerned [2]; especially non-rewarding species are not frequently visited [11]. The mean fruit-set of rewarding orchid species in Europe is 63.1%, while that of their deceptive relatives were found to be only 27.7% [28].

There is little and contradictory evidence available on the effects of individual traits and population characteristics on the reproductive success of orchids (for a review see [36]). Inflorescence size (number of flowers produced in the flowering season) may affect fruit-set, as plants with many flowers appear to be more attractive to pollinators [16]. Floral display (number of flowers open at the same time) is found to be an important factor especially concerning non-rewarding species [8]. Brys et al [6] proved that in *Listera ovata* (*Neottia ovata*) a larger inflorescence is more likely to be visited by pollinators and have its pollinaria removed than a smaller one. Larger floral displays were found to attract more pollinators in a given unit of time, and receive longer visitations as well [26, 30]. The bigger the inflorescence, the longer the flowering time is [10], which results in higher number of visitations and higher fruit-set [25].

Plant height appears to be less widely studied than other traits, however, pollinators are known to forage at a particular height [29]. Therefore, taller specimens may achieve greater reproductive success than shorter ones, independently of the size of floral display [19].

A larger population, with a high number of flowering plants, usually affects individual reproductive success positively [15], as fruit-set was proved to be significantly higher in populations with more flowering specimens [36]. In contrast to this, other populations with a high number of flowering specimens were found to have reduced reproductive success (i.e. *Lapeirousia oreogena* – [18], *Drakaea* spp. – [32]). Pollinators spend longer time periods and visit a higher number of flowers in larger populations [21, 22, 33], however, it may be disadvantageous where pollinators are scarce [6]. Fruit-set significantly positively correlates with local population density in the rewarding *Listera ovata* according to Brys et al. [6] Deceptive orchids, however, would not be successful in populations where plants are in either very dense or sparse patches [34]. Specimens in smaller, sparse populations often have lower

reproductive success than in larger ones [23, 3], and small populations are often exposed to increased geitonogamous self-pollination [17].

Reproductive success of orchids has an undoubted conservational importance, as several species are rare and endangered and recently showing strong decrease in their distribution areas [15]. Therefore the investigation of the reproductive success of orchid species is quite a relevant topic today.

According to the above mentioned we can see that quantifying reproductive success is complicated because these factors can conversely affect each other.

In this study our main research question aimed at identifying which factors actually determine the reproductive success of orchid species. For this reason we investigated three orchid species and those factors that may affect their individual fruit-set, like pollination strategy, individual traits or the annual amount of precipitation. This only makes sense if we exclude the possibility of facultative autogamy among the studied species. For the main question we set up four hypotheses:

- i) All three studied species are entomophilous (non-autogamous) taxa; their fruits are products of pollination events by insects.
- ii) Pollination mode may predict fruit-set of orchid species.
- iii) Fruit-set is dependent on individual traits like plant height, inflorescence length and size and population density.
- iv) Reproductive success is different in wet and dry years.

Materials and Methods

Field study

Field studies were carried out in populations of *Dactylorhiza majalis* (Rchb.) P. F. Hunt & Summerhayes 1965, *Dactylorhiza sambucina* (L.) Soó 1962 and *Platanthera bifolia* (L.) L. C. M. Richard in the Bükk Mountains (Northern Hungary) between 2010 and 2012. The biological characteristics of these species and locations are found in Table 1. All three species were sampled in a ‘wet’ (2010) and a ‘dry’ (2011 or 2012) year (Table 2).

Table 1

Characteristics of the studied taxa. Pollinators are provided according to Claessens and Kleynen [9].

Species	Pollination mode	Pollinators	Location	Study years
<i>Dactylorhiza sambucina</i>	Food deceptive	Hymenoptera: <i>Bombus</i> (9 species), <i>Apis mellifera</i> , <i>Andraena nigroaenea</i>	Felsőtárkány: Hereg-rét (N 48.05°; E 20.48°)	2010, 2012
<i>Dactylorhiza majalis</i>	Food deceptive	Hymenoptera: <i>Bombus</i> (7 species); Coleoptera (1 species)	Miskolc: Létrástető (N 48.10°; E 20.58°)	2010, 2011
<i>Platanthera bifolia</i>	Nectar rewarding	Lepidoptera (13 genera, 20 species)	Miskolc: Létrástető (N 48.10°; E 20.58°) &Miskolc: Miskolctapolca (N 48.07°; E 20.73°)	2010, 2011

Table 2

Meteorological characteristics of study years observed in Miskolc (N 48.10°, E 20.77°) according to the Hungarian Central Statistical Office [14].

Year	Average annual temperature (°C)	Number of annual sunny hours	Number of annual precipitation days	Annual precipitation (mm)
010	9.7	1893	185	1166
011	10.4	2289	109	484
012	10.9	2297	113	521

All populations were surveyed twice each year. (1) During anthesis the number of flowering specimens was estimated in the population (hereafter: ‘population size’). Individual characteristics: height of the flowering stem (hereafter: ‘height’) and inflorescence length were measured and the number of flowers (hereafter: ‘inflorescence size’) was recorded on randomly selected, individually marked specimens. Population density was measured by providing the distances of the 5 closest flowering specimens from the marked specimen. (2) During fruit ripening (4–6 weeks after flowering) the number of fruits was recorded individually and 1 half-mature (intact) capsule was collected from three regions (upper, middle and lower) of the infrutescence.

To exclude the possibility of facultative autogamy we tested the ability for autogamy on the studied species. Before anthesis 5–5 individuals of all three species were bagged with a fine net, in order to prevent pollination by insects.

Quantifying reproductive success

Individual fruit-set data were generated as quotient of the number of fruits and the number of flowers. Seed numbers per fruit (‘capsular seed number’) were determined under light microscope following the methodology of Sonkoly et al. [35]. Seed numbers per specimens were given by the mean of the three determined capsular seed numbers of a certain individual multiplied by the number of matured fruits of the same individual. Seed numbers per populations were calculated by the mean seed number per specimen multiplied by the estimated number of flowering individuals of the population.

Data analyses

R statistical environment (R Development Core Team 2010) was used for all statistical analyses. Normality was tested using Shapiro-Wilk Normality Test. Paired difference tests (Welch Two Sample t-test and Wilcoxon rank sum test) were used to test whether there is a significant difference between the fruit-sets, capsular seed numbers and seed numbers per specimens of species pairs, and between the fruit-set, capsular seed number and seed numbers per specimens of each species in different years. Correlation tests (Spearman's rank correlation) were used to search for correlations between fruit-set and individual traits (height, inflorescence length, inflorescence size and population density) and between pairs of certain individual traits (height and inflorescence length; inflorescence length and inflorescence size; height and inflorescence size; inflorescence size and number of fruits). In order to get the proper direction of the correlation between fruit set and population density, the distances of the five nearest individuals were summed and multiplied by -1.

Results

None of the bagged individuals produced any fruits in any of the species.

Altogether 258 capsules of 106 specimens were examined, and fruit-set data of 161 individuals were recorded during the studied years (Table 3). We found that capsular seed numbers and seed numbers per specimens significantly differed between *D. majalis* and *D. sambucina* and between *P. bifolia* and *D. sambucina*. *P. bifolia* and *D. majalis* significantly differed in their capsular seed numbers. Fruit-sets of the three species did not differ significantly (Table 4).

Table 3
Characteristics of the studied populations

Species	Year	FS \pm SE	CapSN \pm SE	SN/Sp \pm SE	Pop	SN/Pop
<i>Dactylorhiza sambucina</i>	2010	0.527 \pm 0.052 (n = 31)	2735 \pm 188 (n = 32)	14621 \pm 3093 (n = 16)	\approx 1000	14 621 000
	2012	0.535 \pm 0.038 (n = 45)	3426 \pm 304 (n = 34)	21890 \pm 4674 (n = 11)	\approx 500	10 945 000
<i>Dactylorhiza majalis</i>	2010	0.618 \pm 0.052 (n = 18)	10029 \pm 391 (n = 51)	121929 \pm 16897 (n = 18)	\approx 100	12 192 900
	2011	0.538 \pm 0.049 (n = 24)	7332 \pm 315 (n = 32)	82596 \pm 12838 (n = 19)	\approx 50	4 129 800
<i>Platanthera bifolia</i>	2010	0.624 \pm 0.064 (n = 24)	6440 \pm 387 (n = 74)	111492 \pm 16109 (n = 23)	\approx 150	16 723 800
	2011	0.534 \pm 0.047 (n = 34)	6262 \pm 524 (n = 35)	82505 \pm 13616 (n = 19)	\approx 70	5 775 350

FS: mean fruit-set. CapSN: mean capsular seed number, SN/Sp: mean seed number per specimen, Pop: estimated population size, SN/Pop: estimated seed number per population

Certain individual traits were found to correlate with fruit-set in two of the studied species. In *D. sambucina* no correlation was found between fruit-set and the particular traits. The fruit-set of *D. majalis* correlated with height, inflorescence length and population density, while the fruit-set of *P. bifolia* only correlated with inflorescence size (Table 5).

Table 4
Pairwise comparison of three studied reproductive characters
(Welch Two Sample t-test and Wilcoxon rank sum test).

<i>D. majalis</i> – <i>D. sambucina</i>	fruit-set	p = 0.448
	capsular seed number	p < 0.0001
	seed numbers per specimens	p < 0.0001
<i>D. majalis</i> – <i>P. bifolia</i>	fruit-set	p = 0.9522
	capsular seed number	p = 0.03892
	seed numbers per specimens	p = 0.8284
<i>P. bifolia</i> – <i>D. sambucina</i>	fruit-set	p = 0.1551
	capsular seed number	p < 0.0001
	seed numbers per specimens	p < 0.0001

Table 5

Correlations of studied traits with fruit-set (Spearman's rank correlation).

Species	Plant height (cm)	Inflorescence length (cm)	Nr. of flowers/ inflorescence	Population density (- cm)
<i>D. sambucina</i>	p = 0.2213 –	p = 0.2006 –	p = 0.06556 –	p = 0.391 –
<i>D. majalis</i>	p = 0.02698 r = 0.3412	p = 0.0045 r = 0.4297	p = 0.3299 –	p = 0.04144 r = 0.3160
<i>P. bifolia</i>	p = 0.9431 –	p = 0.06105 –	p = 0.01007 r=0.3928	p = 0.7431 –

We found significant correlation between plant height and inflorescence length in all species (*D. sambucina*: $r = 0.6511188$ $p < 0.001$, *D. majalis*: $r = 0.6451277$, $p < 0.001$ and *P. bifolia*: $r = 0.6960524$, $p < 0.001$). Significant positive correlation was revealed between height and inflorescence size ($R = 0.549755$, $p < 0.001$) and inflorescence length and inflorescence size ($R = 0.7774081$, $p < 0.001$) of *D. sambucina*. Positive correlation was found between the number of flowers and the number of fruits in all the three species (*D. sambucina*: $r = 0.2544691$, $p = 0.02$; *D. majalis*: $r = 0.59829$, $p < 0.001$; *P. bifolia*: $r = 0.7610293$, $p < 0.001$).

We found inconsistent patterns in the correlation of precipitation and fruit-sets, capsular seed numbers and seed numbers per specimens. These traits did not always show decline in dry years (2011, 2012) compared to a wet year (2010). We found insignificant correlations between these traits and precipitation in case of *D. sambucina* and *P. bifolia*. Merely the capsular seed numbers of *D. majalis* differed significantly between 2010 and 2011 ($p < 0.001$). When considering the number of flowering individuals of the populations as well, which appears to have dropped to approximately half of the previous number at every location, a substantial decline can be observed in seed numbers per populations (see Table 3).

Table 6

Comparison of the fruit-set data of the studied Hungarian populations with published international data.

	<i>D. sambucina</i>	<i>D. majalis</i>	<i>P. bifolia</i>
Present study	0.539 (2 years, 1 population)	0.572 (2 years, 1 population)	0.602 (2 years, 2 populations)
Claessens and Kleynen [9]	0.209 (16 years, 26 populations)	0.353 (11 years, 16 populations)	0.637 (13 years, 12 populations)
	p < 0.001 Wilcoxon test	p = 0.001457 Wilcoxon test	p = 0.5686 Wilcoxon test

Fruit-set values were surprisingly high in the two deceptive species, but our fruit-set data followed the European trend according to Claessens and Kleynen (2011). Among the examined species *P. bifolia* had the highest, *D. sambucina* the lowest and *D. majalis* the middle values. In case of the two deceptive *Dactylorhiza* species fruit-set data extracted from international literature sources were found to be significantly lower than our detected values. Fruit-set of the nectar rewarding *P. bifolia* did not differ significantly (Table 6).

Discussion

We revealed that all the studied species are entomophilous. As none of the bagged individuals produced any fruits, we can exclude the possibility of facultative autogamy in all the studied species, which is necessary for elucidating our study question. This result is in accordance with previous studies of Pellegrino et al. [31] on *D. sambucina* but in contrast with that of Brzosko [7], where four out of five bagged *Platanthera bifolia* individuals produced fruits. We assume that these results may be inconsistent due to the different bags used. According to our observations, a smaller mesh size would be more appropriate in orchids pollinated by Lepidoptera species, as our preliminary studies on the nectar rewarding *Gymnadenia odoratissima* (L.) Rich. showed that butterflies are able to reach flowers through the net with their long and thin proboscis.

We found that pollination mode did not affect the fruit-set of the studied species as it was hypothesized. We revealed that the reproductive success of the studied deceptive species neared to that of the nectar rewarding species.

According to Sabat and Ackerman [34] the effect of floral characteristics on reproductive success can be different regarding pollination modes. Insects pollinating nectar rewarding species often visit many flowers on the same individual causing higher fruit-set for those having larger inflorescences. In non-rewarding species pollinators learn to avoid deceptive flowers after a few visits, hence, large inflorescences are not beneficial there. Population density has a similar impact on individual fruit-set [34]. In a dense rewarding population fruit-set tends to be higher, but in deceptive populations due to negative reinforcement [1] a dense population is not necessarily more successful [6].

We found partly contradictory results to our third hypotheses. No correlation was found between the fruit-set of *D. sambucina* and the investigated traits. This corroborates with the results of Pellegrino et al. [31], who found that reproductive success was independent from plant density and inflorescence size as well.

Furthermore, the positive correlation found between the fruit-set of *D. majalis* and traits taking part in pollinator attraction (height, inflorescence length, population density) was in contrast with the findings of Hansen and Olesen [13], as they discussed fruit-set and population size to be uncorrelated. The positive correlation may be the consequence of the assumption that the number of naïve pollinators visiting deceptive flowers (flowers of *D. majalis*) is more than sufficient in the Bükk Mountains in Hungary.

Several studies proved inflorescence size to be influential regarding reproductive success, as plants with larger inflorescences seems to produce (absolutely) more fruits e. g. [32]. Correlation between the number of flowers and the number of fruits was found to be positively significant in all species. In contrast, correlation between the number of flowers and reproductive success (fruit-set) is not evident. Only the fruit-set of *P. bifolia* showed relatedness to inflorescence size as Maad [24] observed previously as well. Pollinators of the nectar rewarding *P. bifolia* may rather visit more flowers of a larger inflorescence at the same time and save energy, as it may be less time-consuming.

The correlation between plant height and inflorescence length may exist due to the fitness of the certain individual as larger ones are able to produce larger inflorescences. Those are more frequently visited by pollinators, being more attractive.

Differences between reproductive success of the species in wet and dry years were not significant. We found significant decrease of capsular seed number only in *D. majalis* between the dry and wet years. A possible explanation of this result is that this species has the highest moisture indicator value [12] among the three (*D. majalis* W-value = 8; *D. sambucina*

W-value = 4; *P. bifolia* W-value \approx 5). Therefore, it may have been affected more severely by the smaller amount of precipitation in 2011.

No reduction was observed in the reproductive success of *D. sambucina* and *P. bifolia* between the years, only if we weighted the results with the estimated number of flowering individuals of the populations. Specimens flowering in dry years did not have a reduced reproductive success, but only half of the populations flowered in 2011 and 2012 compared to 2010. In *D. sambucina* the seed number of the population decreased by one third, while in *D. majalis* and *P. bifolia* it decreased by two thirds of the 2010 value.

Due to the surprisingly high fruit-set values of the two *Dactylorhiza* species, we compared our results to other literature data [9] to see general trends in Europe. Pair tests showed substantial differences in the fruit-set of the two deceptive species, as our values were higher in both cases. We would like to note that these results must be handled cautiously, as Claessens and Kleynen [9] published data of a high number of populations from a broad period of time, while our results concerned only a few populations from two consecutive years. Nevertheless, we assume that the pollination crisis reported from Britain and The Netherlands by Biesmeijer et al. [3] may not be an actual problem in the Bükk Mountains in Hungary. They describe connected local extinctions of interacting plant and insect species. These coincident declines may be due to the preliminary decline of insects followed by that of plants or the plants preceding the loss of the linked pollinators. It also seems possible that this tandem decline is a response to some other environmental factor [3]. As the crisis may affect mainly species interacting with relatively few pollinators – like highly specialized deceptive orchids – the most substantial differences were found in the two *Dactylorhiza* species in the present study.

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