

Research Article

Differences in reproductive performance traits of two *Platanthera* species

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The species *Platanthera bifolia* and *P. chlorantha* (Orchidaceae) are both declining in Europe, but *P. bifolia* has been diminishing relatively more. Loss of habitats and landscape heterogeneity due to agricultural intensification or abandonment of less fertile areas are primary drivers of decreasing orchid populations. We measured reproductive traits (fruit and flower count per plant, fruit weight, seed number and weight, the proportion of seeds with embryos and germination success) to compare the reproductive success of the two species in different habitats across Estonia. *Platanthera chlorantha* tends to reach its full fruiting capacity in smaller size classes, despite a relatively low base level. Germination success was low for both species but twice as high for *P. chlorantha* than its congener. We propose that higher proportion of embryos in fruits of *P. chlorantha* in shade and woodland growing conditions is related to higher availability of diverse pollinators. *Platanthera chlorantha*, albeit growing on its northern distribution limit in Estonia, seems to be tolerating the abandonment of its habitats more effectively than *P. bifolia*, at least with respect to reproductive performance traits.

Keywords: fitness, *Platanthera*, pollination, reproduction rates, seed quality

Introduction

The fitness of plants is commonly evaluated by their abundance, size, germinability of seeds, number of flowers, photosynthetic efficiency or plant biomass. Changing landscapes and increasing human impact influence orchid populations and their fitness worldwide (Kull et al. 2016). Orchid pollination is often one-sided and specialised, with the orchid relying more on its pollinator than vice versa (Tremblay 1992, Roberts and Dixon 2008, Joffard et al. 2019). In general, the reproduction of orchid species tends to be more pollinator-limited than resource-limited. However, the high cost of increased fruit set has been noted in many orchid species (Calvo 1993, Jersáková et al. 2006). Orchid populations are also affected by several environmental factors. For example, Jacquemyn, Brys and Jongejans (2010) showed that light conditions were essential for fruit and seed production of *Orchis purpurea*, which were higher in open populations than shaded



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ones. On the other hand, Janečková et al. (2006) found that in *Dactylorhiza majalis*, shaded plants grew longer stalks and bigger leaves, however, seed weight was not affected by reduced light levels. Seed production of *Spiranthes spiralis* was higher for plants that were artificially shaded (Willems et al. 2001).

Although the recruitment of seeds and the formation of offspring rely on the availability of safe sites for germination and growth, this is given less importance when the production of fruits and viable seeds become limiting factors (Faast et al. 2011). Several methods are available to assess orchid fitness, most of which involve various seed quantity and quality parameters. Fruiting proportion is easy to measure, and if insufficient, it indicates either a shortage of pollination or a lack of different resources. Seed quality can be assessed by counting seeds (Cunningham 2000, Kildisheva et al. 2020), measuring fruit mass (Vallius 2001, Wallace 2003) and estimating seeds mass or number of seeds per fruit (Wallace 2003, Brys et al. 2004, Janečková et al. 2006, Case and Bierbaum 2013, Metsare et al. 2015). More sophisticated lab-based methods involve the estimation of the proportion of seeds with embryos (Vallius et al. 2008, Johnson et al. 2009, Case and Bierbaum 2013, Metsare et al. 2015, Sun et al. 2015) and evaluation of germination success (Kauth et al. 2011, Metsare et al. 2015).

Platanthera bifolia Rich. and *P. chlorantha* (Custer) Rchb. are terrestrial orchids with Eurasian distributions (Hultén and Fries 1986). They are closely related and widely distributed. *Platanthera chlorantha* reaches its northern distribution limit in Estonia (Kuusk 1984) and *P. bifolia* reaches it farther north. Both species are nectar rewarding and have very similar ecological requirements (Bateman and Rudall 2012). Esposito et al. (2018) have demonstrated that these two closely related species have distinct ecological preferences: *P. chlorantha* favours dry, calcareous grasslands, while *P. bifolia* is typically found in more humid meadows with more acidic soil. Both species are sensitive to shading by surrounding vegetation; as with many other orchid species, they cannot compete with taller and dominating plant species (Janečková et al. 2006). Bateman et al. (2012) found that both species can grow in several habitats, with *P. chlorantha* being the most shade-tolerant. Plants of *P. chlorantha* in shaded locations have been shown to be less detected by the pollinators responsible for producing fruits (Nilsson 1978); hence, reduced visitation rates may lower the reproductive success of this and other *Platanthera* species. Patt and others (1989) have shown that for *Platanthera stricta* capsule production is pollinator-limited and seed set may be pollen-limited. So, plants growing in large but sparse populations may receive less pollen of the same species than plants in small dense populations (Wilcock and Neiland 2002), thus having a negative impact on their seed quality. Finally, it has been shown in *P. bifolia* that a shortage of resources (caused by defoliation) may critically affect reproductive success of this species (Vallius and Salonen 2006).

Mõtsep and others (2018) reported that in Estonian habitats, high pollinator abundance had a positive effect on the fruit set of *P. bifolia* but not on that of *P. chlorantha*. It

has been demonstrated that the fruit set of *P. bifolia* varies between 41.7 and 93.0 depending on year and population (Nilsson 1983, Maad 2000, Maad and Alexandersson 2004, Vojtkó et al. 2015). The same characteristic for *P. chlorantha* seems to vary to a smaller extent (51.8–83.0%). Metsare et al. (2015) showed that self-pollination in *P. bifolia* resulted in a lower number of seeds per fruit than in fruits resulting from cross-pollination. In Finland, Mattila and Kuitunen (2000) considered *P. bifolia* to be both resource- and pollination-limited within a given year. In the Estonian case, both species are assumed to be pollinator-limited (Mõtsep et al. 2018).

Loss of habitats and landscape heterogeneity due to agriculture intensification are some of the main determinants of decreasing biodiversity (Tilman et al. 2001). Both *P. bifolia* and *P. chlorantha* grow in seminatural communities characterised by high species richness, which is beneficial for surrounding agricultural areas and offers diverse ecosystem services (Gonthier et al. 2014). The gradual overgrowing of former seminatural pastures and grasslands is a reason for the decline of many meadow species in Estonia (Kull and Zobel 1991, Kana et al. 2008, Peciña et al. 2019). Kull and Hutchings (2006) noted that the population of *P. bifolia* and *P. chlorantha* decreased by 64 and 46% in England and by 12 and 7% in Estonia, respectively, during the second half of the 20th century. Efimov (2012) also reported a better state of *P. chlorantha* compared to *P. bifolia* in northwest Russia.

With this study, we aim to analyse whether the potential drivers of reproduction of these two closely related species are different and whether these differences could explain the dissimilarities in their rates of decrease in the Estonian area. We compare the flowering characteristics (number per spike), fruiting characteristics (size, proportion and number of flowers per spike) and seed quality characteristics (weight, germination and embryo proportion) in different populations to differentiate the species or their favourite habitats and understand how habitat openness, population size and density affect fertility in either species.

Materials and methods

Study species

The two *Platanthera* species are very similar in morphology; both produce inflorescences (spikes) with up to 20 hermaphroditic flowers with a nectariferous spur of somewhat varying lengths in different geographical regions (Nilsson 1983, Bateman and Rudall 2012). In Estonia, both species grow in wooded meadows, pastures and light forests. *Platanthera chlorantha* can also be found in alvar grasslands and among juniper bushes. Despite often growing in mixed populations, the small number of hybrids corroborates the significance of pre-mating barriers in maintaining species boundaries (Mõtsep et al. 2021). Estonian populations show no clear correspondence to the subspecies recognized by Scandinavian botanists (Nilsson 2008, Durka et al. 2017, Pedersen and Lange 2021).

Study sites

In 2017 and 2018, we studied seven pure *P. bifolia* populations, seven pure *P. chlorantha* populations and three mixed populations, comprising a total of 17 populations (Fig 1, Supporting information).

We determined the habitat (grassland and woodland) for each study site (Appendix 1) and divided the studied populations into two categories by size (bigger versus smaller than about 5 ha) and by density (denser versus sparser than about 0.007 plants per m²). These divisions are somewhat subjective and artificial, as the actual areas range up to several square kilometres in some cases, and the numbers of plants commonly fluctuate from year to year. In general, due to natural variability and rareness of studied species, the database remained unbalanced and some factor combinations are missing (Table 1, Supporting information). Beside site-dependent factors we also described the light availability for each studied plant (not shaded – the majority of stalks of neighbouring plants within 5 m radius were shorter than their distance from the studied plant; shade – the surrounding vegetation was higher). The study sites were heterogeneous with respect to this factor and therefore both shaded/non-shaded plants could be found in most sites.

Data collection

In all populations, we collected one fruit (capsule) from 15 plants per species for seed quality assessment. In some of the sites, only a few plants were found, and in such cases, we collected up to three fruits per plant. All flowers and fruits on each plant were counted to determine fruiting proportion (fruit%). We analysed 269 fruits: 124 from *P. bifolia* and 145 from *P. chlorantha*. For seed quality assessment, we weighed the collected dried fruits and seeds in a fruit using a digital scale with an accuracy of 0.001 g (Radwag AS 62.R2). The embryos of approximately 200 seeds per fruit were counted under a binocular microscope to determine the

proportion of seeds with an embryo (embryo%). The ratio of germination success (Germ%) was assessed in the lab after pre-treatment conducted in 5% sodium hypochlorite + 0.1% Tween 80 solution for 10 min. Seeds were sown in a commercial agar medium, F522 – Fast Terrestrial Orchid Medium (PhytoTechnology Laboratories), which had previously been sterilised by autoclaving. The seeds from each fruit were sown in two repetitions in petri dishes and sealed with parafilm; the dishes were kept in the dark at room temperature. Three months after sowing, we counted the sown seeds and the germinated seeds to calculate the proportion of germinated seeds.

Statistical analysis

Despite the limited amount of available populations in Estonia, the number of studied sites, however, was deemed large enough to diminish the impact of any dominating site in our analyses. Furthermore, no study site showed the highest or lowest average for all parameters studied (Supporting information). For statistical analysis, we used the SAS System ver. 9.4 software package. At first, the data were analysed using the non-parametric Spearman rank correlation test in the CORR procedure (Supporting information) to test for associations between measured plant and site variables. We used general linear model analysis of covariance (ANCOVA) to study the relationship between fruiting proportion (response variable) and fruit number per inflorescence (covariate) using species and habitat as categorical factors. Comparison of the reproductive characteristics of the two studied species was conducted with Student's t-test assuming unequal variance. In order to study germination success, we restricted the analysis to values greater than zero under the assumption that the absence of germination could have resulted from methodological factors. In this case Wilcoxon test was used to compare the two species. For detailed analysis, the significance of majority of factors was evaluated with the GLM analysis

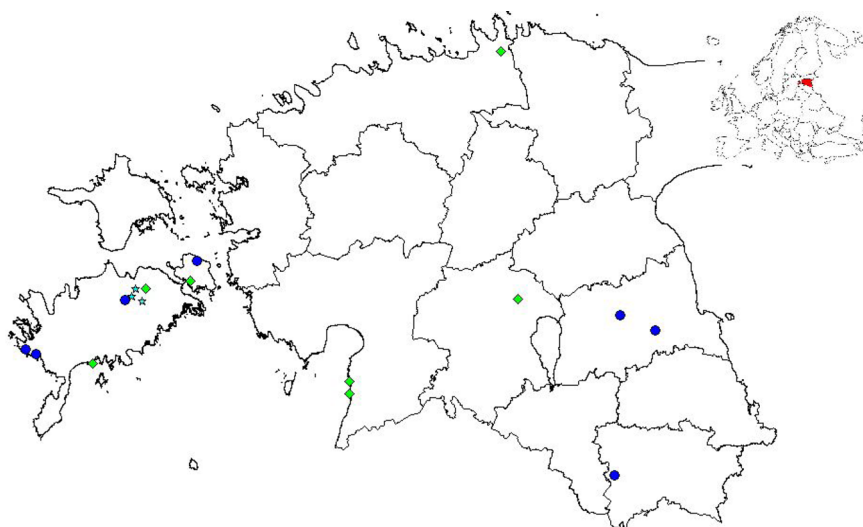


Figure 1. Map of the *Platanthera* population studies in Estonia and the location of Estonia in Europe. ◆ – *P. bifolia*; ● – *P. chlorantha*; ★ – mixed populations.

Table 1. Number of sites (per species) in the alternative categories: grassland/woodland, big/small, dense/sparse.

	Grassland	Woodland	Big	Small	Dense	Sparse
<i>P. bifolia</i>	3	4	4	4	2	6
<i>P. chlorantha</i>	6	1	2	5	1	6
Mixed	1	2	1	2	2	1

of variance (ANOVA) procedure. Here, the dependent factors (embryo%, fruit%, flower no., fruit no., seed and fruit weights) were considered as continuous variables and the independent factors describing their growing conditions as categories. The differences between the average values of various groups were detected with the Ryan-Einot-Gabriel-Welsh (REGW) Multiple Range test. In this case, each category of plants was labelled as a separate group (e.g. *P. bifolia*/Grassland, ... *P. chlorantha*/Woodland, Fig. 3a).

Results

Comparison of species

The average seed and fruit weight of the two species were found to be similar, but some differences in the reproductive characteristics were observed (Table 2). Overall, *P. chlorantha* had significantly fewer flowers, fruits per spike and fruiting proportions than *P. bifolia*. Although the germination success under artificial conditions was low for both species, *P. chlorantha* showed twice the level of germination compared to *P. bifolia*.

Fruiting proportion

An interesting difference between the species appeared when plotting the fruit number against the fruiting proportion (Fig. 2). *Platanthera chlorantha* tends to reach its full fruiting capacity more rapidly, starting from relatively low levels.

Regardless of the species and habitat, we found that the number of fruits on the spike significantly positively determined the fruiting proportion of a plant ($p < 0.001$) (Fig. 2). Individuals with the most fruits per spike tended to reach 100% fruiting proportions in both species. In woodlands, plants of both species reached the maximum fruiting probability, with a higher number of fruits than in grasslands ($p < 0.005$).

The overall fruiting proportion of *P. bifolia* tended to be higher than that of *P. chlorantha*, and both species showed higher fruiting proportions in woodland populations

(Fig. 3a). Fruiting% of *P. bifolia* was significantly higher in light growing conditions, but no such pattern was found for *P. chlorantha* (Fig. 3b).

A closer inspection of the data revealed that *P. bifolia* had the highest fruiting proportions in big and dense populations and the lowest in small and sparse populations, with big and sparse populations being intermediate (Fig. 3c). In contrast, *P. chlorantha* performed best in small, dense populations (big and dense populations were not available for this species) and equally well in big and small sparse populations.

Seed quality

Seed quality measured as the proportion of seeds with embryos was non-significantly higher for *P. bifolia*, whose population averages ranged from 0.56 to 0.75, a little less than the variation recorded for *P. chlorantha* (0.57–0.77). Results were relatively uniform in woodland versus grassland and light versus shaded populations, with the exception that *P. chlorantha* had better seed quality in woodland sites (Fig. 4a, b).

As expected, *P. bifolia* had the highest numbers of seeds with embryos in large and dense populations and the lowest in small and sparse populations (Fig. 4c). For *P. chlorantha*, the highest seed quality was found in small and sparse populations.

Wilcoxon test revealed clear difference ($p = 0.0011$) between species in germination success (Fig. 5). According to Spearman correlation test, no statistically significant patterns of germination success due to habitat characteristics were detected (Supporting information).

Discussion

We have studied two closely related orchid species that have been declining in numbers at different rates (Kull and Hutchings 2006, Efimov 2012). Our study was performed in multiple populations located all over the Estonian distribution range of these species. Despite our efforts we were not

Table 2. Comparison of the reproductive characteristics of the studied species. SE=standard error, p=statistical significance according to Student t-test assuming unequal variance (n=95–124 and n=105–145 for *P. bifolia* and *P. chlorantha*, respectively).

Parameter	<i>P. bifolia</i>		<i>P. chlorantha</i>		p
	Average	SE	Average	SE	
Flowers per spike	18.90	0.50	14.80	0.36	< 0.001
Fruits per spike	13.96	0.49	9.71	0.43	< 0.01
Fruit weight (mg)	18.35	0.64	19.97	0.64	n.s.
Seed weight (mg)	6.32	0.48	6.08	0.46	n.s.
Fruit%	75.10	2.09	65.20	2.37	< 0.001
Embryo%	68.30	1.71	67.40	1.69	n.s.

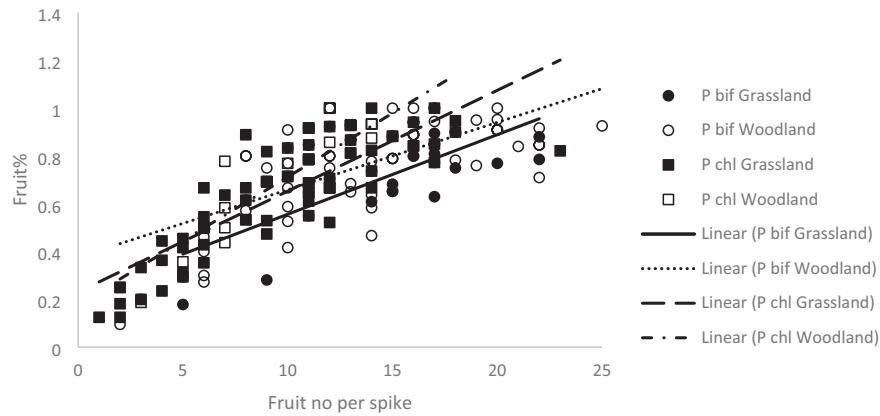


Figure 2. Relation between the fruit number per spike and the fruiting proportion of the individual plants separated by species and habitat.

able to find populations with certain combinations of environmental factors (which largely resulted from the rarity of dense *P. chlorantha* populations) but assume that the database is geographically and environmentally representative enough to draw some conclusions.

European Floras and botanical references claim that *P. chlorantha* plants may be slightly taller and have more flowers per spike than *P. bifolia*, depending on local habitat and growth-conditions. Our data clearly show the opposite - *P.*

bifolia plants had more flowers and more fruits per spike, on average, than *P. chlorantha*. There are studies available indicating that growing conditions may have an impact on flower traits. Boberg and Ågren (2009) found that woodland plants of *P. bifolia* exhibited larger flowers than their alvar counterparts. For *P. bifolia*, inflorescence size (flower number per spike) and fruit number were correlated with the environmental factors we monitored, which may indicate that the flowering rate of this species tends to be resource-limited. For

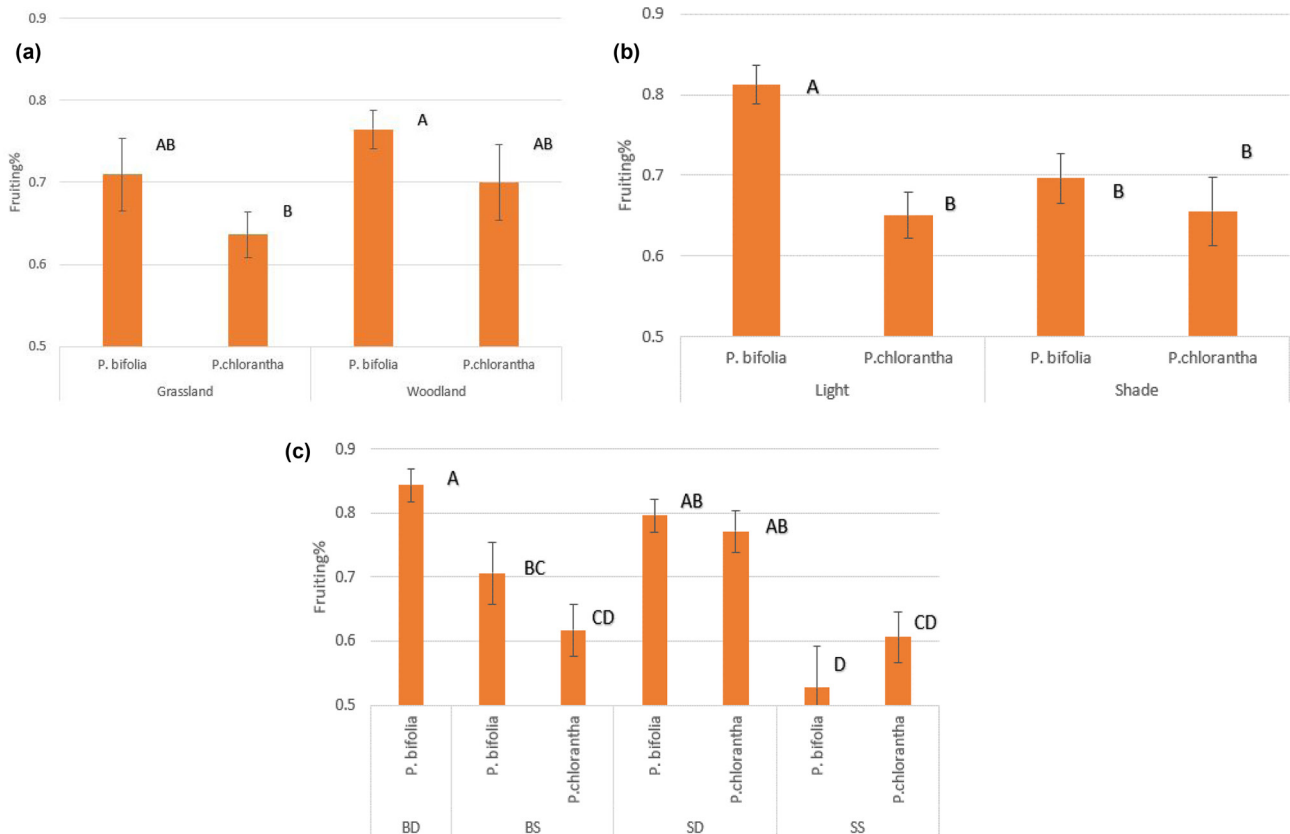


Figure 3. Fruiting proportions for both species in grassland and woodland habitat types (a), in light and shade (b) and in populations of different size/density categories (c). SS: small and sparse; SD: small and dense; BS: big and sparse; BD: big and dense.

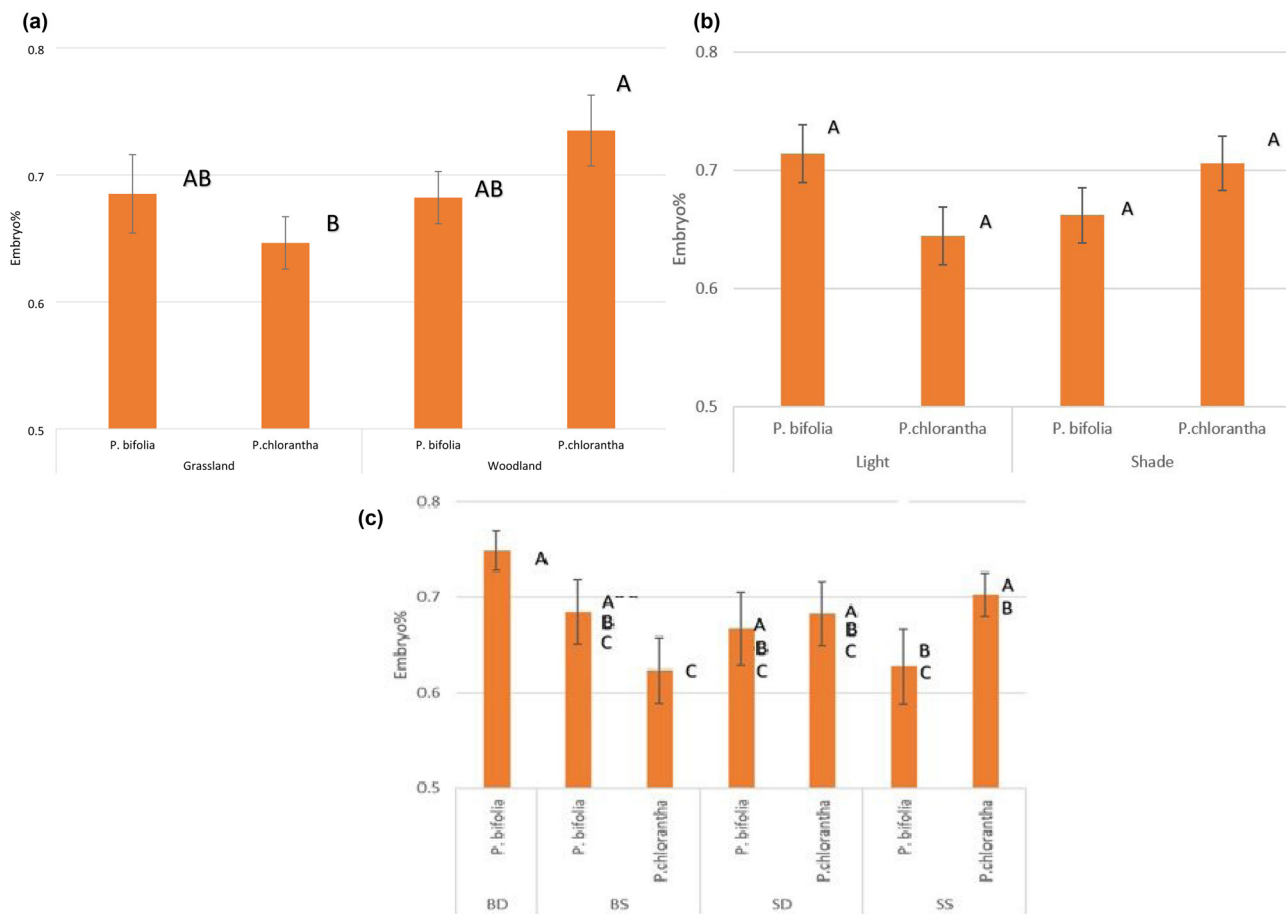


Figure 4. Proportion of seeds with embryos for both species in grassland and woodland habitat types (a), in light and shade (b) and in populations of different size/density categories: SS: small and sparse; SD: small and dense; BS: big and sparse; BD: big and dense populations (c).

P. chlorantha, no such pattern was detected, and therefore we assume this species to be more pollinator-limited as noted also by other scientists (Calvo 1993, Jersáková et al. 2006). In contrast, fruit and seed weights did not differ significantly between the species. This result may reflect methodological limitations or genuine similarities between the species. Consequently, we did not include these factors in subsequent analyses on the impact of growing conditions.

More detailed analysis revealed that *P. chlorantha* with more fruit also had higher fruiting proportion, i.e. a plant with many flowers had greater potential to grow them into fruits. The fruiting success of *P. bifolia* did not depend to the same extent on the number of its flowers. In many deceptive orchid species, the proportion of flowers that develop fruit is independent of the number of flowers in the inflorescence (Kindlmann and Jersáková 2006). However, in our case woodland plants with a particular number of fruits had higher fruiting ratios than those in the grasslands. We assume that this may be the result of better light or visibility for pollinators in woodland with poor understorey. The abundance of pollinators for both species, and especially more rare pollinator species, is reported to be higher in woodland areas. It has been reported earlier that visibility is important for both

Platanthera species, and a clear display often guarantees up to 100% pollination (Mötelp et al. 2018).

In *P. bifolia*, a larger plant size is shown to be beneficial for reproductive effort (Mattila and Kuitunen 2000). In our case *P. bifolia* had significantly higher fruiting proportion in better light conditions. No such pattern was found for *P. chlorantha*. This result was similar to Kirillova and Kirillov (2024), who noted that the more closed the canopy was, the bigger

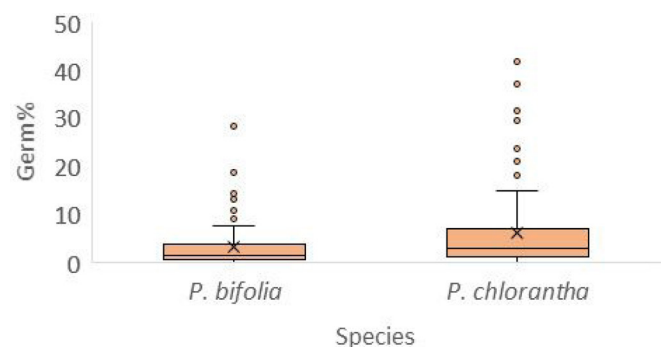


Figure 5. Variation in germination success within and between the species studied.

decrease in values of some indicators of reproductive success of *Platanthera* plants. So, we may confirm resource limitations in this species as shown also by Shefferson and others (2020, 2006).

Light preferences explain also the higher average fruiting success of *P. bifolia* in woodlands which usually have limited understorey. Higher light demand can become crucial in the future if the area of managed seminatural habitats is declining and forming bushlands resulting in losing suitable habitats for *P. bifolia*. However, considering climate change, areas with less management may provide better shelter from direct sunshine or drought, resulting in better growing conditions for both plants and pollinators. Population density is important for fruit production in both species. Fruiting proportion of both species tended to be higher in dense populations, indicating better availability of pollen in them. In small and sparse populations shortage of suitable pollinators can be expected.

To evaluate the reproductive success of a species, it is not so relevant how many fruits a plant has, but rather how good is the quality of the seeds, i.e. how many of these contain an embryo or how many of these are able to germinate. In many cases, the number of fruits does not guarantee high seed quality or, by extension, a high reproductive performance of a plant (Metsare et al. 2015). One factor that may enhance seed quantity as well as quality is the availability of the diverse and abundant pollen load (Proctor and Harder 1994, Neiland and Wilcock 1998, Vallius and Salonen 2006). Averages for embryo percentage did not statistically differ between the species. Interestingly according to our analysis *P. bifolia* fruiting proportion does not predict seed quality (in terms of embryos per seeds), at the same time for *P. chlorantha* these factors show a clear statistical correlation (data not shown, but see Fig. 3a, 4a). Therefore, we assume that there is another unknown factor at play that has an impact on *P. bifolia* embryo proportion. This assumption is supported by some reports speculating that *P. bifolia* may tolerate or experience more self-pollination, especially under pollinator-limited conditions. Selfing can lead to low embryo viability, even if fruits form normally (Mattila and Kuitunen 2000, Esposito et al 2018, Brzosko et al 2022). Furthermore, Mõtlep et al. (2018) revealed that only a few moth species can pollinate *P. bifolia*, while about 14 moth species may be suitable for pollinating *P. chlorantha*. This difference may play an important role in the reproductive success of these species. For example, in *P. chlorantha*, the embryo proportion in woodland fruits was significantly higher than that in grasslands, indicating the presence of more efficient pollinators in this habitat. *P. bifolia* is assumed to experience shortage of well-adapted pollinators, contrary to many deceptive orchids that have evolved to make the most out of every casual encounter (de Jong et al. 1993, Perez-Hernandez et al. 2011).

The proportion of seeds with embryos in sparse versus dense and large versus small populations showed opposing patterns in the two species. While the seed quality of *P. bifolia* decreased in the predicted order from large and dense to small and sparse populations, *P. chlorantha* had the highest embryo

proportion in small and sparse populations. The latter conditions are the most typical for *P. chlorantha* in Estonia, with single plants growing randomly over large areas of former pastures now abandoned and overgrown. Therefore, the ability to attract suitable pollinators, even in very sparse populations, may be one of the advantages that slows its decline compared to the other species.

Orchid seed germination in laboratory is strongly affected by several factors, including light, temperature, germination medium, different levels of seed dormancy, and several researchers have observed low levels of germination (Rasmussen 1995, Baskin and Baskin 1998, Whigham et al. 2006, Metsare et al 2015). In our research the germination success was also relatively low and the germination of seeds from many fruits failed totally. At a first glance the average germination success of *P. chlorantha* in our study was higher than that of *P. bifolia*. However, the number of fruits with germinated seeds was small and numerous outliers were found in the germination data for both species. Thus, we conclude that more detailed studies, based on improved germination protocols, are needed to understand the role of different habitat factors in determining the germination success of the two *Platanthera* species. To clarify the differences in greater detail, it would be particularly interesting to focus on mixed populations, considering both ecological and phenological aspects.

Conclusions

So, we can conclude with some confidence that *Platanthera chlorantha*, which decline in Estonia has been slower than that of *Platanthera bifolia*, exhibits smaller size, lower flower and fruit numbers and lower fruiting ratio but higher germination proportion than the other study species.

As a declining species, *P. bifolia* still performs larger plants with more flowers and fruits, but higher fruiting proportion of this species is not followed by better seed quality (both in terms of embryo percentage and in vitro germination) indicating unfavourable conditions for reproductive success. Hence, we propose that this species requires traditional management of its habitats – wooded meadows and light forests – than its congener *P. chlorantha*, which is indifferent to the shaded conditions in abandoned grasslands.

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Author contributions

Marilin Mõtlep: Conceptualization (lead); Data curation (equal); Methodology (equal); Writing – original draft (equal). **Aigi Ilves:** Data curation (equal); Writing – original draft (supporting). **Tiiu Kull:** Conceptualization (equal);

Methodology (equal); Resources (equal). **Katrin Heinsoo**: Formal analysis (equal); Software (lead); Writing – review and editing (supporting). **Kadri Tali**: Conceptualization (equal); Formal analysis (equal); Methodology (equal); Resources (equal); Supervision (equal); Writing – original draft (equal); Writing – review and editing (lead).

Data availability statement

Data are available from the EMU dspace, <https://doi.org/10.15159/eds.dt.25.03> (Mõtlep et al. 2025).

Supporting information

The Supporting information associated with this article is available with the online version.

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