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Effect of Simulated Autogamy and Allogamy on the Success of *Cephalanthera longifolia* and *Cephalanthera rubra* (Orchidaceae) Fruit Set

Laurynas Taura * and Zigmantas Gudžinskas *

Laboratory of Flora and Geobotany, Nature Research Centre, Žaliųjų Ežerų Str. 47, 12200 Vilnius, Lithuania
* Correspondence: laurynas.taura@gamtc.lt (L.T.); zigmantas.gudzinskas@gamtc.lt (Z.G.)

Abstract: Detailed knowledge of reproductive traits and success is essential for the effective conservation of rare and endangered species. It has been reported that *Cephalanthera longifolia* and *C. rubra* (Orchidaceae) were abundantly fruiting in some regions several decades ago, but recent studies have shown a low natural fruit set rate for both species. The aim of this study was to experimentally test the fruit set of *C. longifolia* and *C. rubra* after simulated self-pollination and cross-pollination, and to investigate the mating system of both species. Simulated self-pollination and cross-pollination experiments were conducted on two populations of *C. longifolia* and one population of *C. rubra* in Lithuania in 2022. The results of the experiment showed that the fruit set rate of *C. longifolia* after simulated cross-pollination was 68.9%, and after simulated self-pollination, it was 70.4%, while in the control group (free pollination), the fruit set rate was 5.1%. The fruit set rate of *C. rubra* was 88.2% after simulated cross-pollination, 96.5% after simulated self-pollination and 17.8% in the control group (free pollination). No significant differences in fruit set rate were found between simulated cross-pollination and self-pollination, but significant differences were found compared to the control group (free pollination) in both study species. We conclude that both study species are self-compatible, can set fruit after self-pollination, and that natural low fruit set is not related to limited energy resources of the individual, but is caused by the inefficient pollination of flowers due to a lack of pollinators during the flowering period. The artificial pollination of flowers can be used to increase fruit set in rare species of the Orchidaceae family and improve their natural recruitment by seed.

Keywords: artificial pollination; conservation; endangered species; flower position; free pollination; Lithuania; mating system

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1. Introduction

Anthropogenic pressure on the environment, together with ongoing global climate change and their synergistic interaction, are among the major drivers of biodiversity change at both global and local scales [1–5]. Changes in ecosystems are most evident through changes in the composition of plant diversity caused by invasions of alien species and population decline of native species [6–8]. All these intricate processes affect the evolutionary traits of plants that once ensured the stability of their populations [9–11]. The key factor for the stability of plant populations is their constant recruitment, which depends on the reproductive success of the species [12,13]. For the effective conservation of rare and endangered species, a detailed understanding of their population demography, dynamics and reproductive success is essential [14–19].

The family Orchidaceae Juss. is one of the most species-rich plant families, and its species are among the most threatened worldwide, posing major conservation challenges [20–22]. Although studies on the reproduction of Orchidaceae species have been ongoing for many decades, there is still a lack of knowledge about their reproduction, and the available information is often fragmentary or even controversial [20]. Inadequate knowledge about plant reproduction makes it difficult to understand why species are declining and to develop conservation strategies [14,20]. The artificial pollination of flowers is increasingly used in the conservation of endangered Orchidaceae species to increase low natural fruit production, so that the seeds can be used to grow plants under artificial conditions and transplant them to their natural habitats. The use of this approach has shown good results, but the conservation of Orchidaceae has not yet achieved the expected effectiveness and there is still a lack of knowledge about the reproduction of many species in the wild and the natural recruitment of their populations [23–26].

The genus *Cephalanthera* consists of 19 species distributed in Europe, Asia, North America and North Africa [27,28]. Five species of the genus occur in Europe, *C. cucullata* Boiss. & Heldr., *C. damasonium* (Mill.) Druce, *C. epipactoides* Fisch. & C.A.Mey., *C. longifolia* (L.) Fritsch, and *C. rubra* (L.) Rich., which are endangered in all or at least part of their range [29–36]. Besides habitat loss, one of the reasons for their rarity and decline is poor fruit set and consequently reduced seed production [17,18,21,37].

The genus *Cephalanthera* includes species that are mainly autogamous and only occasionally allogamous (*C. erecta*, *C. longibracteata*, *C. damasonium*), as well as species that are mainly allogamous (*C. falcata*, *C. longifolia*, *C. rubra*), or have a mixed mating system [38–43]. Information on the mating system of *C. longifolia* and *C. rubra* is contradictory. It is generally accepted that both species are allogamous, and that the probability of autogamy is very low [38,39]. However, studies on *C. longifolia* in central Italy have shown that the level of heterozygosity in the populations is like that of other allogamous Orchidaceae species, and an increase in homozygosity has been explained by vegetative reproduction [39], but the increase in homozygosity could also be caused by the occurrence of autogamy [44]. *Cephalanthera rubra* is usually considered a species with a mixed mating system [39–41], although some researchers argue that it is a strictly allogamous species [42,45].

It has been reported that *C. longifolia* and *C. rubra* produce many fruits in some regions [41,46]. Nevertheless, the results of many studies in recent decades indicate a low fruit set rate in both species [37,47–50]. For example, in the UK, *C. rubra* produced only one fruit per decade, whereas in *C. longifolia*, only six cases of fruit set by solitary individuals have been recorded over two decades [48,51]. Researchers have suggested that low fruit set is caused by the peculiar mating system of the species and inefficient flower pollination. The results of our previous study on natural fruit set in Lithuania showed that from 5.2% to 19.5% of *C. longifolia* flowers and from 4.1% to 18.8% of *C. rubra* flowers produced fruits [17]. Fruit set appeared to be significantly lower in small or low-density populations than in large or high-density populations. In addition, we found that the percentage of fruit set in the lower flowers of the inflorescence was significantly higher than in the flowers at the top of the inflorescence. The results suggest that the relatively low fruit set rate was influenced by low pollinator activity, which was also dependent on habitat conditions [17].

There have been a few experiments on artificial pollination of *Cephalanthera* flowers to determine whether it is possible to increase fruit production in small and declining populations and thus stimulate their recovery [43,48,50,52]. Previous artificial pollination experiments have been conducted on a small scale, sometimes using only a few individuals [48,50], making it difficult to draw reliable conclusions from their results. Furthermore, the effect of self-pollination and cross-pollination on fruit set has not been investigated. Therefore, we decided to experimentally test the fruit set success of *C. longifolia* and *C.*

rubra after simulated self-pollination and cross-pollination, and to investigate the mating system of both species. The questions we wanted to answer were as follows: (a) What is the effect of simulated self-pollination and cross-pollination on fruit set compared to free pollination?; (b) What is the effect of flower position on fruit set after artificial and free pollination?; and (c) What is the effect of plant traits on fruit set in simulated self-pollination, cross-pollination and free pollination?

2. Materials and Methods

2.1. Study Species

Cephalanthera longifolia and *C. rubra* are nectarless, mycorrhizal perennials with thick rhizomes. The stem is erect, slender, with alternate narrowly lanceolate leaves [53]. *Cephalanthera longifolia* is 20–70 cm tall and has a lax inflorescence usually consisting of 8 (ranging from 5 to 23) flowers, whereas *C. rubra* is 30–80 cm tall and its inflorescence usually consists of 10 (ranging from 4 to 27) flowers [18]. The flowers of *C. longifolia* are creamy white, partially open, and have a yellow or yellowish lip, whereas the flowers of *C. rubra* are bright pink, with a curved labellum that has white markings to attract pollinators [54].

The flower of *Cephalanthera* species is characterised by a long column with a large stigma and a hinged anther containing two bipartite pollinia. Notably, the upper edge of the stigma of *C. longifolia* and *C. rubra* has a thin membranous rim that prevents contact between the pollinia and the stigmatic fluid, thus preventing spontaneous self-pollination [45].

2.2. Study Sites

The largest and most abundant populations of *C. longifolia* and *C. rubra* were selected for the study to ensure the necessary sample size for the experiment and to obtain sufficient pollen donor plants. The experiment with *C. longifolia* was conducted in two coenopopulations (hereafter referred to as 'populations') in the vicinity of Aukštieji Paneriai (Vilnius city, south-eastern Lithuania). The first population, Raisteliai, was selected in a young stand of *Betula pendula* that had developed naturally in an area of abandoned arable land (Table 1) [18]. The dominant *Betula pendula* trees in the stand were about 30 years old. The tree layer was moderately sparse and dominated by *Betula pendula*. The shrub layer was rather sparse. The herb layer consisted of a mixture of species characteristic of woodlands and grasslands. The second population, Paneriai, was selected in a mature forest consisting of approximately equal proportions of *Pinus sylvestris* and *Betula pendula* [18]. The dominant *Pinus sylvestris* trees were about 115 years old. The shrub layer was rather sparse. The herb layer was dense. The two sites were separated by a 500 m wide strip consisting of a *Pinus sylvestris* plantation and a stand of mature mixed forest.

Table 1. Location and vegetation characteristics of the experimental sites where artificial pollination of *Cephalanthera longifolia* and *Cephalanthera rubra* flowers was carried out.

Species and Site Name	**District**	**Latitude (°N)**	**Longitude (°E)**	**Cover (%)**		
				Tree	**Shrub**	**Herb**
Cephalanthera longifolia						
Raisteliai	Vilnius	54.6180	25.2101	40	30	30
Paneriai	Vilnius	54.6218	25.2065	40	40	60
Cephalanthera rubra						
Kapiniškiai	Varėna	54.0340	24.2915	0	0	70

The experiment with *C. rubra* was conducted in the village of Kapiniškiai in Dzūkija National Park (Varėna district, southern Lithuania). The population was selected in a dry grassland with a high diversity of herb species, located on a south-west-facing slope adjacent to a mixed pine-birch forest [18,55]. The habitat was free of trees and shrubs (Table 1), with only a little shade from the trees of the adjacent woodland affecting the habitat in the early morning and late evening hours. The herb layer was lush and covered 70% of the surface (Table 1).

2.3. Experiment Design

At each study site, an area of approximately 300 m^2 of uniform habitat containing at least 100 generative shoots of the study species was selected. The selected area was divided into three sub-plots of equal size for different experimental treatments and the corners of the sub-plots were marked with tags attached to the tallest plants. Normally developed shoots with four or more flowers and no signs of damage were selected for the experiment, while damaged shoots or shoots with fewer flowers were excluded. Before starting the artificial pollination, the opened flowers were inspected to assess the presence of both pollinia. Flowers were also inspected if they had not yet been pollinated (if there were any pollinia on the stigma brought in from other flowers). If at least one pollinia was removed from the flower or if the flower was already pollinated, the plant was not used in the experiment. In each subplot, 20 individuals fulfilling all the criteria were selected for each variant of the experiment. Since the safety of legally protected plant individuals must be paramount, we have not covered the flowers of the plants used in the pollination experiments, as this can cause damage to the plants both directly (covered plants are susceptible to wind damage) and indirectly (covered plants attract the attention of wild animals, which damage or destroy most or even all the plants tested).

Each shoot of both species selected for the study was marked at the base of the stem near the soil surface with a numbered silicone ring tag. The height of the shoot and the length of the inflorescence were measured, and the number of leaves and flowers was counted. The height of the plant was measured from the soil surface to the top of the plant and the length of the inflorescence was measured from the lower flower to the top of the upper flower. Leaf-like bracts and the lower sheaths without a developed leaf blade were excluded from the leaf count. Flowers in the inflorescence were numbered on the field record sheet consecutively from the base of the inflorescence to the apex, according to their attachment to the inflorescence axis. The number of flowers in the inflorescence included all normally developed flowers, regardless of their anthesis stage at the time of artificial pollination.

Flowers in the first subplot were pollinated with pollinia collected from flowers of plants growing at least 50 m away from the pollinia recipients to simulate allogamy. In the second subplot, the flowers were pollinated with pollinia from the same flower to simulate autogamy. The third subplot was left as a control and the plants were not subjected to any manipulation.

The pollinia for the simulated cross-pollination experiment were collected from fully opened flowers (one pollinia per flower) using curved tweezers and placed in a box made of electrostatic paper. The collected pollinia were immediately used to pollinate the flowers. One pollinia was placed on the stigma of each fully opened flower of the experimental individuals using curved tweezers. In the case of the induced self-pollination experiment, the flowers were pollinated by placing a pollinia from the same flower on the stigma with tweezers. The flowers of the control group were not manipulated. After each manipulation, the tweezers were thoroughly wiped with a damp cotton cloth to avoid cross-contamination.

The consecutive number of each artificially pollinated flower, starting from the bottom of the inflorescence, was recorded on the field record sheet.

Artificial pollination of *C. longifolia* flowers was undertaken on 8 and 9 June 2022 and the artificial pollination of *C. rubra* flowers was undertaken on 17 June 2022. One month after artificial pollination, each tagged individual was re-examined and the number of fruits produced by the individual was counted, indicating the consecutive number of the flower in the inflorescence that produced the fruit. By this time, the flowers that had not set fruit had already fallen off, leaving only bract (Figure 1).

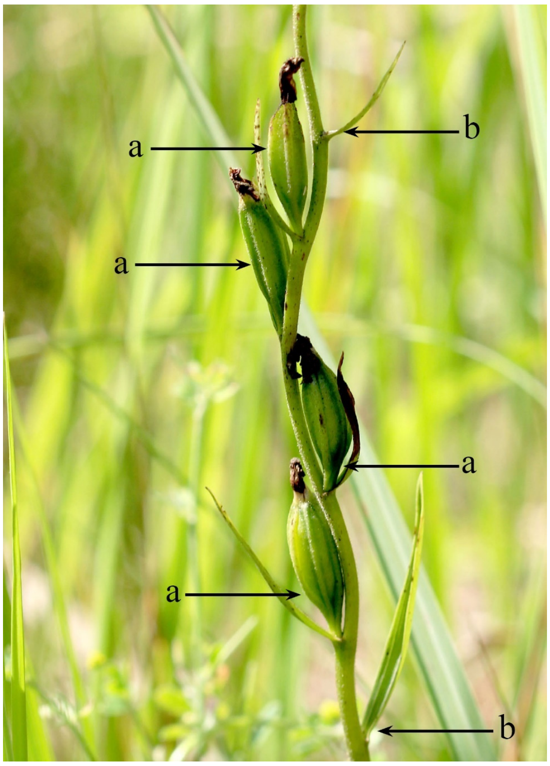

Figure 1. Fragment of *Cephalanthera rubra* inflorescence with developed fruits (a) and remaining bracts after abscission of unsuccessfully pollinated flower with ovary (b).

In the two experimental populations of *C. longifolia*, a total of 309 flowers were used for cross-pollination, 307 flowers for self-pollination and 336 flowers for the control. In the experimental population of *C. rubra*, 85 flowers were used for cross-pollination, 85 flowers for self-pollination and 214 flowers for the control.

2.4. Meteorological Conditions

According to the data provided by the Vilnius Meteorological Station, in June 2022, during the pollination experiment on *C. longifolia* flowers, the mean daily air temperature at the Paneriai site was 17.8 °C and the accumulated monthly precipitation was 129.4 mm. On the days of artificial pollination, 8 and 9 June, the mean daily temperature at the Paneriai site was 19.1 °C and 19.9 °C, respectively, and the daily precipitation was 0 mm and 5.4 mm, respectively. During the flowering period of *C. rubra*, in June 2022, the mean daily temperature at the Varėna Meteorological Station was 17.6 °C and the cumulative monthly precipitation was 132.1 mm. On the day of artificial pollination, on 17 June, the mean daily temperature at the Kapiniškiai site was at 16.2 °C and the daily precipitation was 0.2 mm.

2.5. Statistical Analysis

The data sets were tested for distribution using the Shapiro–Wilk test. As a part of the datasets in each category were not normally distributed, non-parametric methods of analysis were used. The Kruskal–Wallis H-test was used to compare multiple data samples, and the Mann–Whitney U-test was used for pairwise comparisons of experimental variants. The effect of fixed factors (population and experimental variant) on fruit set was assessed by two-way PERMANOVA (9999 permutations). The generalised linear model was used to test the relationships between plant traits and the number and percentage of developed fruits. The significance level of the results was set at $p < 0.05$. The results of the descriptive statistics were presented as mean and standard deviation (mean ± SD). All calculations and graphs were performed using PAST 4.17 software [56].

3. Results

3.1. Effect of Simulated Cross-Pollination and Self-Pollination on Fruit Set

Analysis of the results showed that the fruit set rate after artificial pollination of *C. lonfigolia* and *C. rubra* flowers by simulated cross-pollination and self-pollination was significantly higher than that after free pollination.

At the Raisteliai site, a total of 145 flowers of *C. longifolia* were artificially pollinated to simulate cross-pollination (Table 2) and 68.3% of them produced a fruit (99 fruits). The mean number of flowers per individual in this treatment was 7.3 ± 1.9 and 5.0 ± 1.5 flowers per individual set fruit (Figure 2). A total of 159 flowers were pollinated to simulate self-pollination and 115 of these (or 72.3%) set fruit. The mean number of flowers per individual in this treatment was 8.0 ± 2.3 and the mean number of fruits developed per individual was 5.8 ± 2.6. In the control, 179 flowers were examined of which only 9 (5.0%) developed fruit. The mean number of flowers and fruits developed per individual was 9.0 ± 2.1 and 0.5 ± 0.8, respectively (Figure 2). We found that at the Raisteliai site, there was no significant difference between the mean number of fruits developed by an individual of *C. longifolia* after simulated cross-pollination and self-pollination (U = 174.0, $p = 0.484$), while in the control, the mean number of fruits developed was significantly lower than in the cross-pollination (U = 1.5, $p < 0.001$) and self-pollination (U = 1.5, $p < 0.001$) treatments.

Table 2. The number of *Cephalanthera longifolia* and *Cephalanthera rubra* flowers used in the experiment, the number of fruits produced and the rate of fruit set.

Species and Site	Experimental Group	Number of Used Flowers	Number of Produced Fruits	Fruit Set Rate (%)
Cephalanthera longifolia				
Raisteliai	Cross-pollination	145	99	68.3
	Self-pollination	159	115	72.3
	Control	179	9	5.0
Paneriai	Cross-pollination	164	114	69.5
	Self-pollination	148	101	68.2
	Control	157	8	5.1
Pooled	Cross-pollination	309	213	68.9
	Self-pollination	307	216	70.4
	Control	336	17	5.1
Cephalanthera rubra				
Kapiniškiai	Cross-pollination	85	75	88.2
	Self-pollination	85	82	96.5
	Control	214	38	17.8

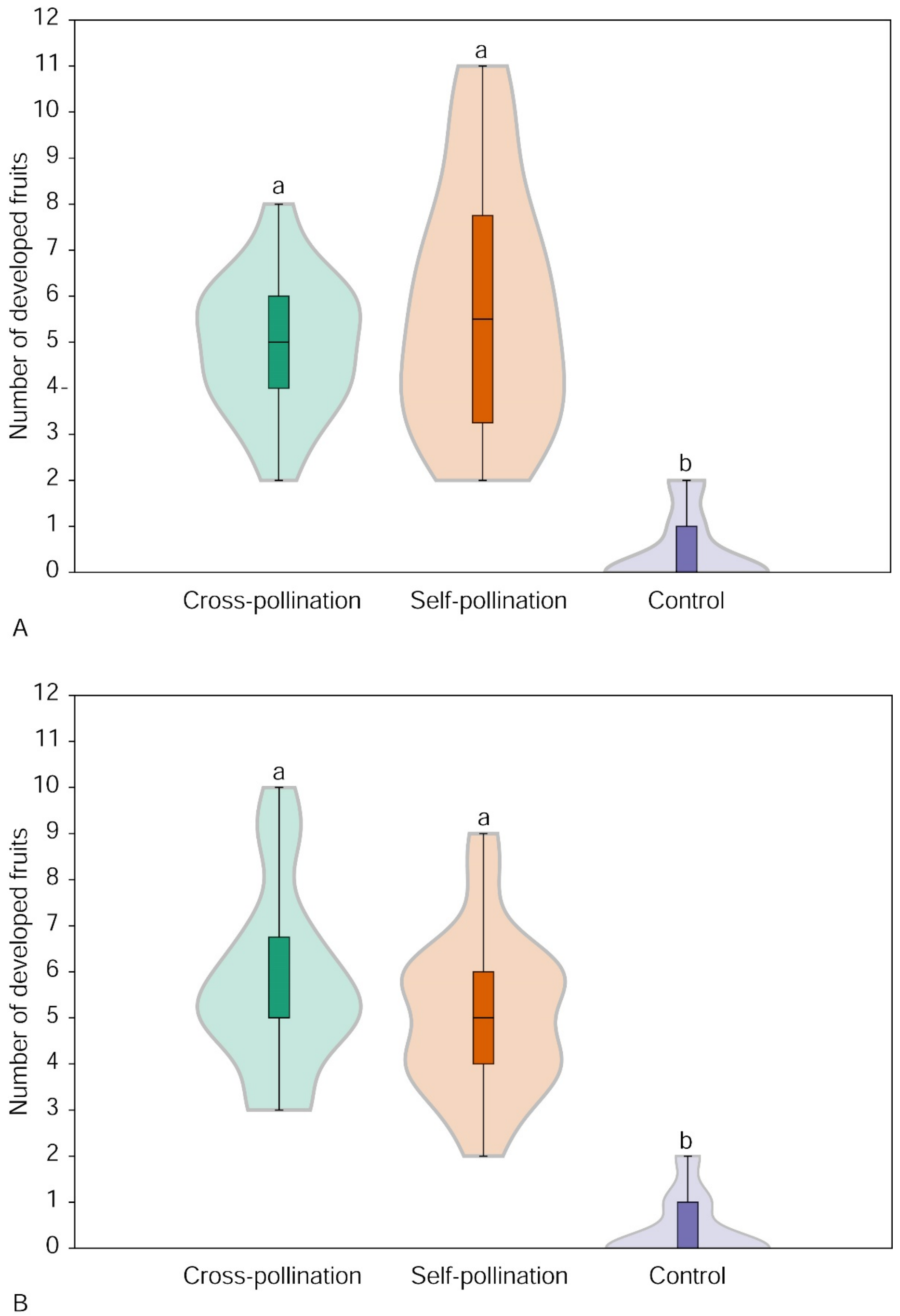

Figure 2. Mean number of developed fruits per *Cephalanthera longifolia* individual after simulated self-pollination and cross-pollination and in the control group at the Raisteliai (**A**) and Paneriai (**B**) sites. Different letters above the box plots and violins indicate significant differences according to the Mann–Whitney test.

A total of 164 flowers of *C. longifolia* were used to simulate cross-pollination at the Paneriai site, and 114 flowers (69.5%) set fruit (Table 2). The mean number of flowers per individual in this treatment was 8.2 ± 2.5 and 5.7 ± 1.9 flowers per individual set fruit (Figure 2). In the simulated self-pollination treatment, a total of 148 flowers were artificially pollinated, of which 101 (68.2%) set fruit. The mean number of flowers and fruits developed per individual was 7.4 ± 2.0 and 5.1 ± 1.7, respectively. In the control, 157 flowers were examined, of which only 8 (5.1%) developed fruit. The mean number of flowers per individual in the control was 7.9 ± 2.5 and the mean number of fruits developed per individual was 0.4 ± 0.7. At the Paneriai site, there was no significant difference between the self-pollination and cross-pollination treatments in the number of fruits developed (U = 165.0, $p = 0.341$), while significantly fewer (U = 1.0, $p < 0.001$) fruits were produced in the control than in both treatments.

When comparing the Raisteliai and Paneriai site treatments, there were no significant differences in the mean number of fruits produced after simulated cross-pollination (U = 161.5, $p = 0.294$) and self-pollination (U = 177.0, $p = 0.536$). There was also no significant difference (U = 197, $p = 0.933$) in the mean number of fruits produced between the control of the two study sites.

In the experiment with *C. rubra* at the Kapiniškiai site (Table 2), a total of 85 flowers were artificially pollinated to simulate cross-pollination, of which 75 (or 88.2%) produced a fruit. The mean number of flowers per individual in this treatment was 4.3 ± 1.1 and 3.8 ± 1.2 flowers per individual set fruit (Figure 3). A total of 85 flowers were pollinated to simulate self-pollination, and 82 of these (or 96.5%) set fruit. The mean number of flowers per individual in this treatment was 4.3 ± 1.3, and the mean number of fruits developed per individual was 4.1 ± 1.3. In the control, 214 flowers were examined, of which 38 (17.8%) developed fruit. The mean number of flowers and fruits developed per individual was 10.7 ± 3.3 and 1.9 ± 1.9, respectively. No significant difference (U = 166.0, $p = 0.349$) was found between the mean number of fruits developed after simulated cross-pollination and self-pollination of *C. rubra* flowers. However, the mean number of fruits developed by an individual in the control was significantly lower than after the simulated cross-pollination (U = 85.5, $p = 0.002$) and self-pollination (U = 76.5, $p < 0.001$) treatments.

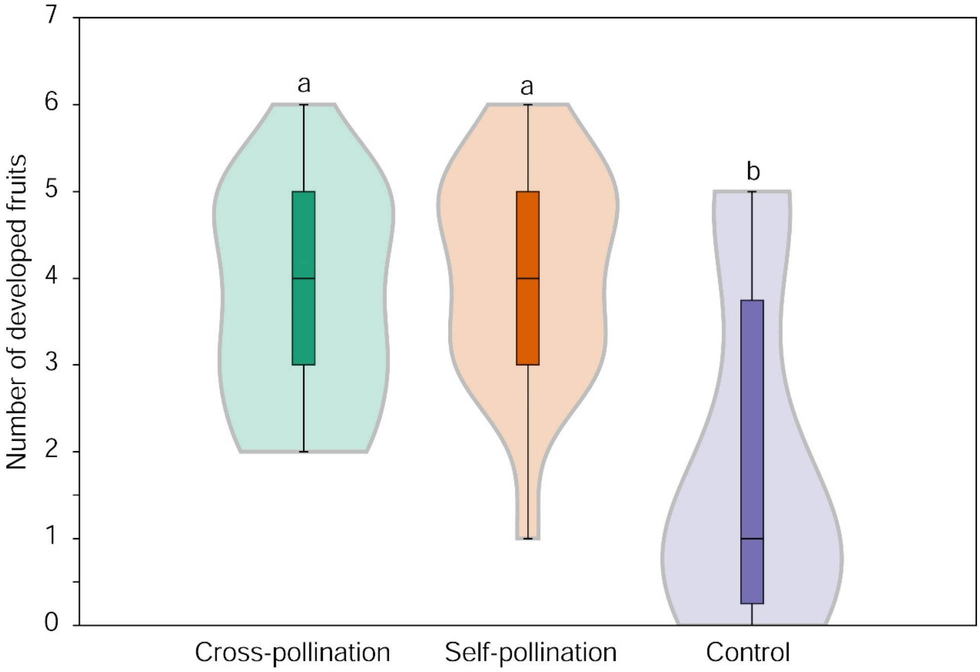

Figure 3. Mean number of developed fruits per *Cephalanthera rubra* individual after simulated self-pollination and cross-pollination and in the control group at the Kapiniškiai site. Different letters above the box plots and violins indicate significant differences according to the Mann–Whitney test.

According to the results of the two-way permutation analysis, the fruit set of *C. longifolia* was significantly affected by the experimental condition (F = 115.96; df = 2; $p = 0.0001$), while the study site (F = 0.52; df = 1; $p = 0.974$) and the interaction of the two factors (F = 1.88; df = 2; $p = 0.159$) had no significant effect. When the pooled fruit set data for *C. longifolia* and *C. rubra* were analysed, it was found that species had no significant effect on fruit set (F = 3.31; df = 1; $p = 0.071$), while experimental condition (F = 119.84; df = 2;

$p = 0.0001$) and the interaction of the two factors (F = 14.43; df = 2; $p = 0.0001$) significantly affected fruit set.

Part of the flowers, mainly upper and apical flowers, of the *C. longifolia* individuals involved in the experiment were not subjected to artificial pollination, as they were not open at the start of the experiment. At the Raisteliai site, 41 flowers of all plants used for simulated cross-pollination and 18 flowers of plants used for simulated self-pollination were not manipulated. At the Paneriai site, plants used for these treatments had 7 and 17 flowers, respectively. None of these flowers developed fruit. However, significantly different results were found for *C. rubra* at the Kapiniškiai site. The plants used for the simulated cross-pollination and self-pollination treatments had 117 and 120 flowers that were not manipulated, but 24 (or 20.5%) and 38 (or 31.7%) of them produced fruits, respectively.

The results of the experiment showed that 68.9% of *C. longifolia* flowers developed fruits after simulated cross-pollination and the fruit set rate was similar at both study sites (Table 2). A slightly higher, but insignificant, fruit set rate of 70.4% was found after simulated self-pollination. In the control groups, the fruit set rate was 5.1% at both study sites and was significantly lower than in both treatments (Table 2). A quite different pattern of fruit set success was found after the experiment on *C. rubra*. After simulated cross-pollination the fruit set rate was 88.2%, after simulated self-pollination 96.5% and in the control group the fruit set rate was 17.8%. The fruit set rate of *C. rubra* was significantly higher after simulated cross-pollination ($p < 0.05$) and in the control group ($p < 0.01$) than in the corresponding groups of *C. longifolia* at both sites.

3.2. Effect of Flower Position on Fruit Set

Analysis of the relationship between fruit set and position of the flower in the inflorescence of *C. longifolia* revealed significant differences between treatments and control (H = 44.21; $p = 0.005$). However, pairwise comparisons between self-pollination and cross-pollination treatments regarding the position of the flower in the inflorescence and the number of fruits developed did not reveal significant differences, except for the significant difference ($p = 0.047$) between the eighth and the third flower in the self-pollination treatment. In the control, flowers developed significantly fewer fruits ($p < 0.05$) than in the self-pollination and cross-pollination treatments, regardless of their position in the inflorescence. The percentage of fruit set (Figures 4 and 5) ranged from 50% (first flower) to 90% (fifth flower) in the cross-pollination treatment, from 50% (eighth flower) to 90% (third flower) in the self-pollination treatment, and from 0% (first, second, seventh, and eighth flowers) to 25% (third flower) in the control.

Analysis of the relationship between flower position in the inflorescence and fruit set in *C. rubra*, in contrast to *C. longifolia*, showed no significant differences between the two treatments and the control (H = 16.54; $p = 0.454$). Pairwise comparisons between simulated self-pollination and cross-pollination treatments for flower position and number of fruits developed also showed no significant differences. However, the fifth flower of the control developed significantly fewer fruits ($p < 0.05$) than the flowers of the cross-pollinated and self-pollinated treatments. The percentage of fruit set (Figure 6) ranged from 52.6% (first flower) to 100% (third, fourth, fifth and sixth flowers) in the cross-pollination treatment, from 90.9% (fifth flower) to 100% (third, fourth and sixth flowers) in the self-pollination treatment and from 15% (fifth flower) to 30% (sixth flower) in the control group.

The analysis of the relationship between the position of the flower in the inflorescence and the fruit set rate of *C. longifolia* and *C. rubra* showed that pollination success, rather than the position of the flower in the inflorescence, is the determining factor.

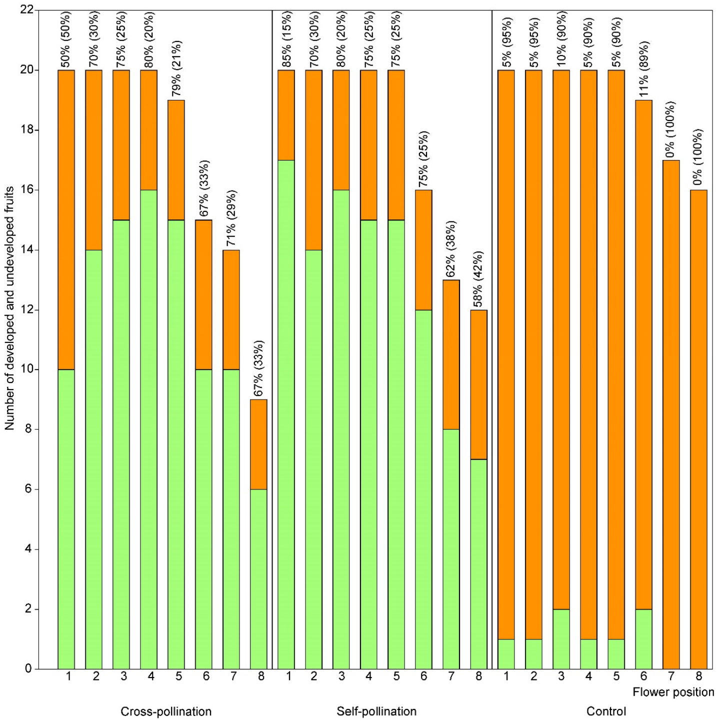

Figure 4. Effect of flower position in *Cephalanthera longifolia* inflorescence on fruit set in induced cross-pollination and self-pollination treatments and free pollination (control) at the Raisteliai site. The number of developed fruits is indicated by light green bars and the number of undeveloped fruits by dark orange bars. The numbers above the bars indicate the percentage of developed and undeveloped (in brackets) fruits at that position.

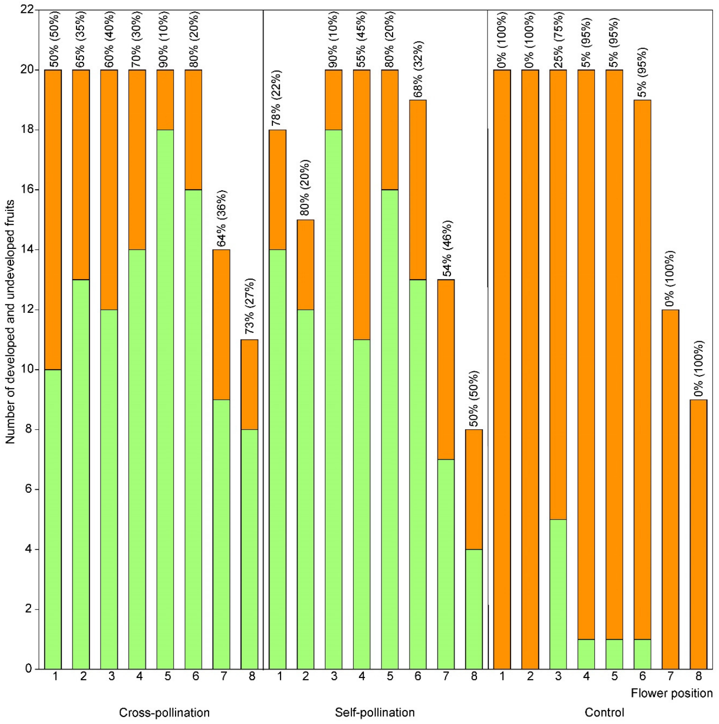

Figure 5. Effect of flower position in *Cephalanthera longifolia* inflorescence on fruit set in induced cross-pollination and self-pollination treatments and free pollination (control) at the Paneriai site. The number of developed fruits is indicated by light green bars and the number of undeveloped fruits by dark orange bars. The numbers above the bars indicate the percentage of developed and undeveloped (in brackets) fruits at that position.

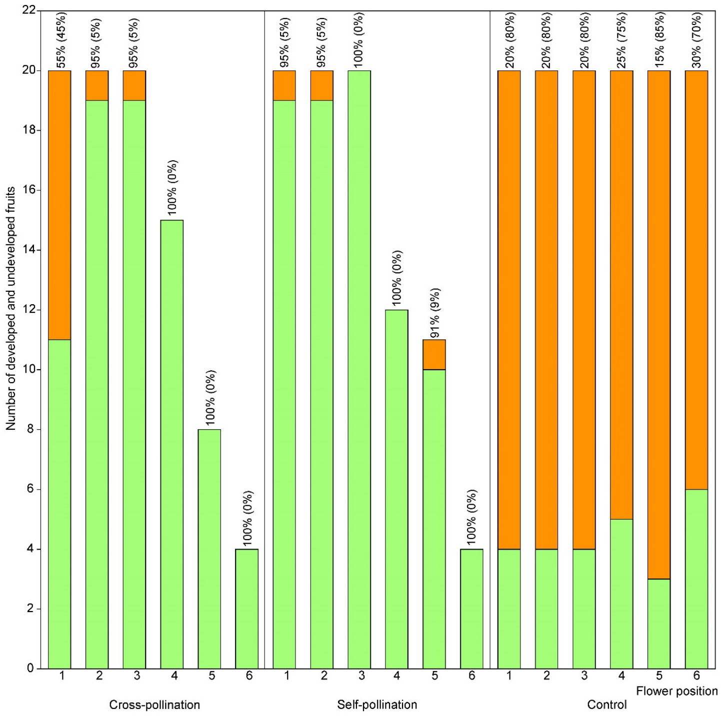

Figure 6. Effect of flower position in *Cephalanthera rubra* inflorescence on fruit set in induced cross-pollination and self-pollination treatments and free pollination (control) at the Kapiniškiai site. The number of developed fruits is indicated by light green bars and the number of undeveloped fruits by dark orange bars. The numbers above the bars indicate the percentage of developed and undeveloped (in brackets) fruits at that position.

3.3. Relationships Between Plant Traits and Fruit Set

Although the individuals in the study populations were selected for the experiment to be as similar as possible to minimise the effect of their differences on the results, some differences in their traits were unavoidable. *Cephalanthera longifolia* individuals selected for the experimental groups at the Raisteliai site did not differ in height, inflorescence length and number of flowers. However, individuals selected for the simulated self-pollination treatment differed significantly in leaf number from the cross-pollination treatment (p = 0.023) and from the control (p = 0.004), whereas there were no significant differences between the cross-pollination treatment and the control (Table 3). At the Paneriai site, *C. longifolia* individuals selected for the experimental groups did not differ in number of leaves, inflorescence length and number of flowers, but did differ in plant height. Plants selected for the simulated cross-pollination treatment were significantly taller (p = 0.049) than plants selected for the self-pollination treatment.

The results of the generalised linear model showed no significant relationships between the number (p = 0.115) and percentage (p = 0.636) of fruits developed of *C. longifolia* and plant height. No significant relationships were found between the number of leaves and the number of fruits developed (p = 0.204), but the relationship with the percentage of fruits developed was significant (p = 0.041). A significant relationship was found between inflorescence length and the number of developed fruits (p < 0.001), but the relationship with the percentage of developed fruits was not significant (p = 0.722). Similar results were obtained when analysing the relationship between the number of flowers in the inflorescence and fruit set. The relationship between the number of fruits developed and the number of flowers was significant (p < 0.001), but not with the percentage of fruits developed (p = 0.790).

Table 3. Comparison of characteristics of *Cephalanthera longifolia* and *Cephalanthera rubra* individuals used in treatments and control (mean ± standard deviation). Different superscript letters indicate significant differences between experimental groups as determined by Kruskal–Wallis post hoc test.

Species, Site and Experimental Group	Plant Height (cm)	Number of Leaves	Inflorescence Length (cm)	Number of Flowers
Cephalanthera longifolia				
Raisteliai				
Cross-pollination	40.2 ± 7.1^{a}	7.5 ± 0.6^{a}	8.9 ± 2.6^{a}	9.1 ± 2.4^{a}
Self-pollination	38.0 ± 8.9^{a}	7.1 ± 0.6^{b}	9.3 ± 2.9^{a}	8.8 ± 3.3^{a}
Control	40.6 ± 6.0^{a}	7.8 ± 0.8^{a}	9.3 ± 2.8^{a}	9.0 ± 2.1^{a}
Paneriai				
Cross-pollination	41.8 ± 6.7^{a}	7.8 ± 0.7^{a}	10.3 ± 3.1^{a}	8.5 ± 2.6^{a}
Self-pollination	36.2 ± 7.7^{b}	8.0 ± 1.4^{a}	9.2 ± 2.7^{a}	8.1 ± 2.1^{a}
Control	39.6 ± 7.2^{ab}	8.1 ± 0.9^{a}	9.1 ± 3.3^{a}	7.9 ± 2.5^{a}
Cephalanthera rubra				
Kapiniškiai				
Cross-pollination	41.8 ± 5.6^{a}	5.2 ± 0.7^{a}	9.4 ± 2.7^{a}	10.1 ± 2.8^{a}
Self-pollination	41.4 ± 10.8^{a}	4.8 ± 0.9^{a}	11.1 ± 4.4^{a}	10.0 ± 4.0^{a}
Control	40.1 ± 5.1^{a}	5.0 ± 0.8^{a}	8.3 ± 4.1^{b}	10.7 ± 3.3^{a}

Individuals of *C. rubra* at the Kapiniškiai site did not differ in height, number of leaves and number of flowers in all experimental groups but differed significantly in inflorescence length (Table 3). The inflorescences of the control plants were significantly shorter than those of the simulated cross-pollination ($p = 0.045$) and self-pollination ($p = 0.006$) treatments.

Slightly different results were obtained from the generalised linear model analysing the relationship between *C. rubra* traits and fruit set. Plant height had a significant ($p < 0.001$) relationship with the number of fruits developed, but the relationship was not significant with the percentage of fruits developed ($p = 0.234$). Number of leaves showed no significant relationships with number of fruits developed ($p = 0.672$) and percentage of fruits developed ($p = 0.827$). Inflorescence length had a significant relationship with the number ($p < 0.001$) and percentage ($p = 0.021$) of developed fruits. The same regularity was found when analysing the relationship between the number of flowers in the inflorescence and the number ($p < 0.001$) of fruits developed, but the relationship with the percentage of fruits developed was insignificant ($p = 0.993$). These results suggest that effective flower pollination, rather than plant traits, has a significant effect on fruit set in *C. rubra*.

4. Discussion

Several conflicting opinions have been published on the mating system of *C. longifolia* and *C. rubra*. Some authors have suggested that both species are mainly allogamous, and that autogamy is unlikely or very rare [38,39], while others have claimed that they have a mixed mating system [40,42,43]. The results of the experiment of artificial pollination of *C. longifolia* and *C. rubra* flowers in their natural habitats, simulating self-pollination and cross-pollination, confirmed that both species are self-compatible. The study confirmed that *C. longifolia* and *C. rubra* are equally successful at setting and ripening fruits after simulated cross-pollination and self-pollination of flowers. It was not possible to assess the quality of the ripened seeds in this study, but judging from the external characteristics of the fruits, they appeared to be fully developed, and therefore, it is likely that the ripened seeds were viable. Tałałaj et al. [50] reported that *C. rubra* produced fully developed seeds after induced autogamy during an experiment in the northeastern part of Poland. Nevertheless, further research should focus on evaluating the quality and viability of seeds produced after artificial cross-pollination and self-pollination.

Research on artificial pollination of *C. rubra* flowers in natural habitats has been very limited, and experiments have involved small numbers of individuals. In Poland, studies of induced autogamy have been conducted in different years with only two or three individuals (with a total of 11 to 29 flowers), resulting in fruit set of 80.0% to 90.0% [50]. An even smaller study of artificial pollination of flowers was carried out in England. Only six flowers were hand-pollinated, three of which set and ripened fruit [48]. The largest study of artificial pollination of *C. longifolia* published to date involved 40 individuals and 223 flowers [52]. The results of the study showed that 48.4% to 53.5% of the artificially pollinated flowers set fruit [52]. Our experimental treatments for artificial pollination of *C. longifolia* flowers in Lithuania included 80 individuals and 616 flowers, while the treatments for *C. rubra* included 40 individuals and 170 flowers. However, we were not able to perform the same experiment on *C. rubra* in the forest habitat due to the low number of individuals in such populations in Lithuania [17,18]. Nevertheless, we assume that the results of artificial pollination of *C. rubra* flowers in forest habitats would have been like the results of the experiment with plants occurring in the grassland habitat.

We believe that the results of this experiment, based on a substantially large sample, are a reliable and important addition to the knowledge of the pollination biology of both species studied. This assumption is indirectly confirmed by comparing the control group with the results of a three-year study on natural fruit set of *C. longifolia* and *C. rubra* [17]. The natural fruit set rate of *C. rubra* at the Kapiniškiai site during the three years (2021–2023) was 18.8%, while the results of the present study showed an insignificant difference in fruit set rate. At the same site in the control group (free pollination), 17.8% of the flowers produced fruit in 2022. At the Paneriai site, the natural fruit set of *C. longifolia* over the three years was 7.9% [17], while in the present study the fruit set rate in the control group was 5.1%. At the Raisteliai site, almost the same fruit set rate was found in the control group (5.0%). Therefore, we concluded that the significant increase in fruit set could only be explained by the effect of simulated cross-pollination and self-pollination of the flowers.

In pollination experiments, flowers are usually covered before flowering to completely protect them from accidental pollination [43]. In the pollination experiments with *C. longifolia* and *C. rubra*, we did not cover the flowers for several reasons. The first reason for not covering the inflorescences was the strict requirement for non-destructive studies on legally protected plants. Covering the inflorescences of the plants used in the experiment would, in the event of failure, have a significant impact on seed production in the year of the study, which could theoretically have a significant negative impact on a small population such as *C. rubra*. The second motivation for not covering inflorescences was previous negative experience in experiments with other plants. We found that any extraneous material, in this case the fabric used to cover the flowers, attracted the attention of animals (particularly wild boars, badgers, foxes and possibly raccoon dogs), which damaged or destroyed most or even all the tested plants. In natural habitats, such experiments could only be carried out in enclosures that protect the experimental plants from wild animals. However, legal regulations prevent the installation of fences, as such interventions are considered a significant disturbance to the habitat [36,57]. We believe that the most important principle to follow when conducting research on protected plants is to avoid harming the individuals, population and habitat of the species being studied.

Although the inflorescences of *C. longifolia* and *C. rubra* were not protected by covers against double pollination during the experiment, we believe that the results obtained are reliable and clearly demonstrate the effectiveness of artificial pollination. We found highly significant differences between fruit set in both experimental treatments and the control.

Fruit set after artificial pollination was several times higher than in the control. As a result, even if the same proportion of flowers in the experimental plants could have been free pollinated as in the control, the results would be almost unchanged. The fruit set of *C. longifolia* after artificial pollination, subtracting the possible rate of pollination by insects, would range from 63.8% to 65.3% and would be significantly higher than in the control (5.1%). In the case of *C. rubra*, fruit set after artificial pollination would range from 70.4% to 78.7% and would be significantly higher than in the control (17.8%).

Previous studies have shown that the position of the flower in the inflorescence of *C. longifolia* has a significant effect on natural fruit set, while the position of the flower in *C. rubra* has no significant effect. In *C. longifolia*, flowers lower in the inflorescence are more likely to set fruit, whereas in *C. rubra* there was no clear relationship between fruit set and flower position [17]. The same relationship was found in the control groups of this study. In the experimental groups, fruit set was significantly higher because of artificial pollination and did not depend on the position of the flower in the inflorescence. This suggests that the naturally low fruit set of *C. longifolia* and *C. rubra* is not caused by a lack of resources to support fruit development [43], but by inefficient flower pollination by insects. Artificial pollination compensates for the lack of insect pollinators and significantly increases fruit set.

Analysis of the results of the *C. longifolia* and *C. rubra* fruit set experiment showed that even after artificial pollination, the first (lower) flower in the inflorescence was less likely to set than the other pollinated flowers above it. One reason for this phenomenon may be that at the time of artificial pollination, the first flower in the inflorescence was already at the end of flowering and the stigma was no longer receptive. The exact timing of stigma receptivity in *C. longifolia* and *C. rubra* is unknown, and the timing of stigma receptivity in different Orchidaceae flowers varies greatly depending on the species and environmental conditions [58–60]. Although it has been reported that the stigma of some temperate Orchidaceae species, such as *Platanthera chlorantha* (Custer) Rchb. [61], remains receptive for up to 15 days, the prevailing dry and very warm weather during our experiment may have influenced its receptivity. During the artificial pollination of *Cephalanthera rubra* flowers, the average daily temperature was 16.2 °C, and the total rainfall was only 0.2 mm. Although the pollinated flowers showed no external signs of end of flowering, the stigma of some of the flowers may have been dry and non-receptive. It is almost impossible to assess the condition of the stigma in the field without damaging the plant and flower.

Results from several studies have shown that in Orchidaceae species with a highly specialised pollination system, pollen transfer between populations is limited, increasing differentiation between populations but decreasing genetic diversity within populations [62,63]. In nectarless species that attract pollinators by deception, gene flow may facilitate the increase of genetic diversity within populations [64,65]. Orchidaceae species with a mixed mating system, such as *C. longifolia* and *C. rubra*, maintain population equilibrium through autogamy, which ensures reproductive security under unfavourable conditions for cross-pollination, whereas allogamy facilitates the increase in and stabilisation of genetic diversity under favourable conditions for cross-pollination [66,67]. However, the genetic diversity of *C. longifolia* and *C. rubra* has been relatively little studied. Studies on the enzymes of *C. damasonium*, *C. longifolia* and *C. rubra* in Italy suggested that the genetic diversity of these species is determined by the mating system [39], but the results of subsequent studies only partially confirmed this conclusion [67,68]. The authors [39] considered *C. longifolia* to be an allogamous species, whereas our study confirmed that it is a self-compatible species, setting and ripening fruits after pollination by its own pollen. However, the frequency of autogamy is unknown and may be quite rare.

Based on plastid microsatellite studies, four haplotypes of *C. longifolia* were found in Europe, of which only one is widespread and the other three are rare and restricted to certain parts of Europe, while nine haplotypes were found in populations of *C. rubra* from southern and western Europe, and no variation was found in the European population of *C. damasonium* [69]. The genetic diversity of *C. rubra* has been studied more extensively, but research has focused on populations from northeastern Poland. In three populations, polymorphic loci were found to account for 25% to 31.3% [67], while in the other nine populations, only eight of the 16 loci studied were polymorphic, accounting for 6.3% to 37.5% [68]. A strong correlation was found between the level of genetic polymorphism in *C. rubra* and population size [68], suggesting that the probability of clonality and homozygosity is higher in small populations than in larger populations.

The experience and results of this study can be used to develop a conservation strategy for *C. longifolia*, *C. rubra* and other nectarless Orchidaceae species. Artificial pollination of flowers could be very useful for maintaining small populations where natural fruit set is extremely low, irregular or absent [70]. For example, it has been reported that a very small population of *C. rubra* in the UK produced only one fruit per decade [48], and such erratic fruiting cannot ensure recruitment and survival of the population. Artificial pollination of flowers in small or extremely sparse populations can significantly increase annual fruit production and thus the probability of population recovery, especially if favourable habitat conditions are created through the implementation of management measures [18]. The results of the experiment show that with a small investment of time and money, it is possible to significantly increase the fruit set, and therefore seed production, of rare and endangered Orchidaceae species.

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