

DOI: 10.5586/aa.1706

Publication historyReceived: 2016-11-24
Accepted: 2017-02-02
Published: 2017-03-31**Handling editor**Marcin Zych, Faculty of Biology,
University of Warsaw, Poland**Authors' contributions**EB: research designing, field work, writing the manuscript;
BO: field work, data analyses;
PM: field work, data analyses, writing the manuscript; EJ: field work, manuscript preparation;
IT: field work, advising, AW: field work, advising**Funding**

This study was carried out in the course of preparing a conservation management plan for the Biebrza Valley Nature 2000 area (PLH200008) and supported from the budget of this task.

Competing interests

No competing interests have been declared.

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INVITED ORIGINAL RESEARCH PAPER

Pollinator limitation affects low reproductive success in populations of nectarless orchid in the Biebrza National Park

Emilia Brzosko*, Beata Ostrowiecka, Paweł Mirski, Edyta Jermakowicz, Izabela Tałałaj, Ada Wróblewska

Department of Plant Ecology, Institute of Biology, University of Białystok, K. Ciołkowskiego 1J, 15-245 Białystok, Poland

* Corresponding author. Email: emilka@uwb.edu.pl**Abstract**

The deficiency of pollinators is indicated as the main factor limiting fruit set in orchids. Nectariferous species are more successful in setting fruits than nectarless species. In the present study, we tested whether pollinator limitation lowers reproductive success in populations of *Cypripedium calceolus* on environmental islands in the Biebrza National Park, NE Poland. Moreover, we analyzed how population size and structure affect pollination success. Our observations and results of experiments document the low level of fruiting in populations of nectarless *C. calceolus* (9.2% on average) and strong pollen limitation. Generally, we noted a positive relationship between pollination success and all parameters of population size (measured by both occupied area and number of clumps or shoots) and parameters measuring floral display (number of flowers in population, number of flowers in clumps, spatial structure of flowering shoots). We suggest that information about factors influencing the reproductive success of this endangered species may be useful for planning appropriate conservation actions.

Keywords*Cypripedium calceolus*; female success; male success; mineral islands; self-compatibility**Introduction**

For the maintenance of plant populations and their viability and stability, successful recruitment from seeds is essential. On the other hand, this success depends on the production of fruits/seeds, which is often recognized as a factor limiting the recruitment of new individuals [1–3]. A low level of fruiting (even below 10% in non-autogamous species) is indicated as a common characteristic of one of the largest families, Orchidaceae [4,5]. The deficiency of pollinators is indicated as the main factor limiting fruit set in orchids [2,6,7]. Despite pollinator restriction, fruiting can be increased in many orchid species if they are self-compatible, which enables reproductive assurance due to spontaneous or facilitated selfing [2]. Available studies suggest a dichotomous pattern of pollination effectiveness in orchids. Neiland and Wilcock [6], analyzing worldwide data on fruit set in representatives of the Orchidaceae family (117 species growing in different parts of the globe), stated that nectariferous species are more successful in setting fruits than nectarless species. Other authors confirmed

this finding [2,8]. Differences in reproductive success between nectarless orchids and species offering nectar are explained through pollinators' behavior. In the case of rewarding plants, they both visit flowers more frequently and spend more time on the single flower and the whole inflorescence (if present) than in non-rewarding species (insects soon learn to avoid plants without a reward).

Many studies have documented a great differentiation in fruiting levels in orchid populations in space and time [9–14]. Variation of environmental parameters and changes of weather conditions are pointed out as the main causes of this differentiation. In animal-pollinated plants, pollination success is also influenced by population size (especially the participation of flowering individuals) and their distribution in space (density) ([8] and papers cited therein). Some studies have shown that in nectarless orchids fruiting is less affected by reduced population size, while nectariferous orchids are more vulnerable to the Allee effect [15,16]. Recently, changes in environmental conditions due to anthropogenic alteration of habitats and climate change often lead to disturbances of insect communities, which can affect pollination processes [17,18]

Although pollen limitation as a factor determining seed production has been studied over the last few decades, knowledge on this topic is still not satisfactory. The lack or deficit of pollinators is also suggested as a factor limiting fruit set in the rare nectarless orchid, *Cypripedium calceolus* [11,12,19: p. 121]. Despite this suggestion, evidence of pollinator-limited fruiting in this species is lacking. Therefore, we tested whether, and if so how greatly, pollinator deficiency concerns populations of *C. calceolus* on environmental islands in the Biebrza National Park, and whether flowers of this species can be self-pollinated. Moreover, we analyzed how population structure (in terms of area cover, number of clumps or shoots, including the proportion of flowering shoots and their density) affects pollination success. The presence of relatively large numbers of populations of this very rare species under the same climatic and very similar environmental conditions is a unique case, which gives the possibility to test the relationship between population properties and the effectiveness of pollination. Such information not only enhances the knowledge about factors influencing the reproductive success of this endangered species, but is also useful for planning appropriate conservation actions.

Material and methods

Study species and area

Cypripedium calceolus L. (Orchidaceae) is a very rare orchid distributed in the Northern Hemisphere [20]. It is a boreal species and occurs in deciduous and mixed forests (rarely in full sunlight), mainly on calcareous soils [20–22]. The species can grow under the forest canopy, but under strong shading it does not flower [19: p. 118]. *Cypripedium calceolus* is a herbaceous, perennial, rhizomatous plant that reproduces sexually and asexually. Its individuals can grow as single shoots or form clumps of many shoots. One clump can build one or more genetic individuals [23]. It is pollinated mainly by small bees from the genera *Andrena*, *Lasioglossum*, and *Halictus* [24,25]. In Poland, this species has the highest conservation status – listed in the Polish red list (Category V) and in the Polish red book of plants (VU) [26,27]. It is protected by the Washington Convention, Bern Convention, and Habitats Directives [28]. Of the 240 localities of this species known in Poland, more than half are historical [27,29].

Our research was conducted in one of the country's largest populations of this species existing on mineral islands in the Biebrza National Park (NE Poland). There are several hundred mineral islands among the widespread peat bogs of the Biebrza River valley, which differ in size, shape, and vegetation cover [12,30]. Among them, we found 13 mineral islands holding 30 populations of *C. calceolus*. On some islands, individuals formed one patch, in which clumps or single shoots were close to one another (max. a few meters away), and on other islands we observed more patches, spatially distanced from a few tens to thousands of meters, and which differed in character of occupied habitat. If there was a distinct habitat barrier or a long distance

(a few hundred meters) between individuals on the same mineral island, we treated it as a separate population (potentially subpopulation). Flowering and fruiting monitoring was carried out in 2012, while experiments with artificial pollination were done in 2014.

Data collection

To test the self-compatibility of *C. calceolus*, we used a hand-pollination experiment in the biggest population (Tab. 1; No. 6). We covered nine flowers with cotton nets before flowering, and during anthesis we deposited one pollinarium on the stigma surface within the same flower. To test the presence of spontaneous autogamy, we isolated five flowers with cotton nets until the fruiting phase. To test the deficiency of pollinators, we artificially supplemented pollination in 50 flowers, each from three large populations (Tab. 1; Nos. 6, 22, 23). To avoid crossing between relatives, flowers were pollinated by pollinaria collected from the most distanced plants. Additionally, male pollination success (pollinaria removing) was observed in one population (pop. No. 6; Tab. 1).

To test the influence of population structure and environmental conditions on reproductive success, during the flowering period in each population we estimated population size measured by occupied area (m²), total number of clumps and shoots, and number of flowering shoots. The number of flowers on each shoot was also noted. A few weeks after flowering, fruits were counted and fruiting level was expressed as the number of fruits divided by the number of flowers in a given population. Moreover, in each locality we documented the presence and proportion of other plants using the Braun-Blanquet method, and we noted the flowering species.

Apart from data gathered in the field, *C. calceolus* populations were characterized by minimum and average distance to other populations in the Biebrza National Park, as well as by the distance to the closest mineral island and the area of the occupied mineral island. Measurements were done in ArcGIS 10.3 software [31] under the PUWG 1992 projection.

Data analyses

We examined the relationship between population properties, characteristics of mineral islands, and fruiting ratio (non-normal distribution; Kolmogorov–Smirnov test, $p < 0.01$) using Spearman's rank correlation. The same traits were also tested with one-way ANOVA between groups of populations in which fruiting was present and absent, despite the second flowering being observed. Linear regression was done for number of flowering shoots, flowers per m² and per clump to visualize its relation with the percentage of fruiting individuals. Data from phytosociological relevés were used to correlate the fruiting percentage of populations with understory cover level, number of species in understory, and number of species flowering at the same time as *C. calceolus*. The impact of shading was measured by the estimation (rounded to 5%) of summer cover of trees and shrub layer. Statistical analyses were done in VassarStats [32].

Results

Effectiveness of artificial pollination

The fruit set noted after induced autogamy was very high and reached 88.9%, but no fruit was set in the experiment of spontaneous autogamy (unmanipulated flowers). The effectiveness of fruiting obtained in the supplemental pollination experiment reached 100% in each of three populations.

Tab. 1 Characteristics of *Cypripedium calceolus* populations studied in 2012 on mineral islands in Biebrza National Park (NE Poland).

| Pop. No. | Area occupied (m ²) | No. of clumps | No. of shoots | No. of flowering shoots (% ratio) | No. of flowers | Fruiting (%) |
|----------|---------------------------------|---------------|---------------|-----------------------------------|----------------|--------------|
| 1 | 20 | 5 | 14 | 12 (85.7) | 14 | 0 |
| 2 | 100 | 17 | 40 | 19 (47.5) | 22 | 9.1 |
| 3 | 500 | 42 | 185 | 50 (27) | 47 | 10.6 |
| 4 | 100 | 34 | 301 | 75 (25) | 76 | 19.7 |
| 5 | 50 | 8 | 38 | 5 (13.2) | 6 | 0 |
| 6 | 250 | 79 | 666 | 411 (61.7) | 505 | 0.8 |
| 7 | 300 | 72 | 483 | 247 (51.1) | 298 | 5.7 |
| 8 | 15 | 3 | 10 | 2 (20) | 3 | 0 |
| 9 | 10 | 3 | 4 | 2 (50) | 3 | 0 |
| 10 | 300 | 55 | 240 | 40 (16.7) | 45 | 0 |
| 11 | 100 | 38 | 83 | 11 (13.3) | 13 | 0 |
| 12 | 20 | 12 | 14 | 0 | 0 | 0 |
| 13 | 1 | 1 | 15 | 0 | 0 | 0 |
| 14 | 1 | 1 | 1 | 0 | 0 | 0 |
| 15 | 150 | 5 | 45 | 19 (42.2) | 24 | 0 |
| 16 | 100 | 25 | 90 | 20 (22.2) | 24 | 12.5 |
| 17 | 100 | 10 | 31 | 5 (16.1) | 5 | 0 |
| 18 | 0.8 | 7 | 38 | 4 (10.5) | 4 | 0 |
| 19 | 1 | 1 | 2 | 1 (50) | 1 | 0 |
| 20 | 0.8 | 14 | 24 | 1 (4.2) | 1 | 0 |
| 21 | 2 | 1 | 2 | 0 | 0 | 0 |
| 22 | 125 | 32 | 53 | 22 (41.5) | 26 | 0 |
| 23 | 500 | 67 | 213 | 37 (17.4) | 44 | 6.8 |
| 24 | 200 | 33 | 56 | 1 (1.8) | 1 | 0 |
| 25 | 500 | 131 | 321 | 20 (6.2) | 23 | 8.7 |
| 26 | 500 | 30 | 83 | 23 (27.7) | 21 | 0 |
| 27 | 200 | 18 | 61 | 32 (52.5) | 37 | 0 |
| 28 | 200 | 11 | 60 | 2 (3.3) | 2 | 0 |
| 29 | 50 | 13 | 29 | 7 (24.1) | 7 | 0 |
| 30 | 5 | 1 | 3 | 0 | 0 | 0 |

Population properties

In total, in 30 localities on 16 mineral islands in the Lower Basin of the Biebrza valley we observed 769 clumps and 3205 shoots of *C. calceolus*. Population size ranged from 1 to 134 clumps and from 1 to 666 shoots (Tab. 1). The average number of shoots per clump in populations fluctuated between 1 and 15. The populations found occupied areas from <1 to about 500 m².

Flowering was observed in 25 populations among the 30 populations studied (Tab. 1). The ratio of flowering shoots in populations ranged from 1.8 to 85.7%. In one half of the populations, flowering was at a level below 20%. The average number of flowering shoots per clump fluctuated between 0.03 and 5.2. The total number of flowers in populations ranged between 0 and 505 (average values per clump: 0.01–6.4). On the majority of shoots, one flower was noted, while two flowers emerged in only 18% of shoots. Flowering was negatively affected by shading of trees and shrub canopy ($r = -0.31$; $p = 0.05$).

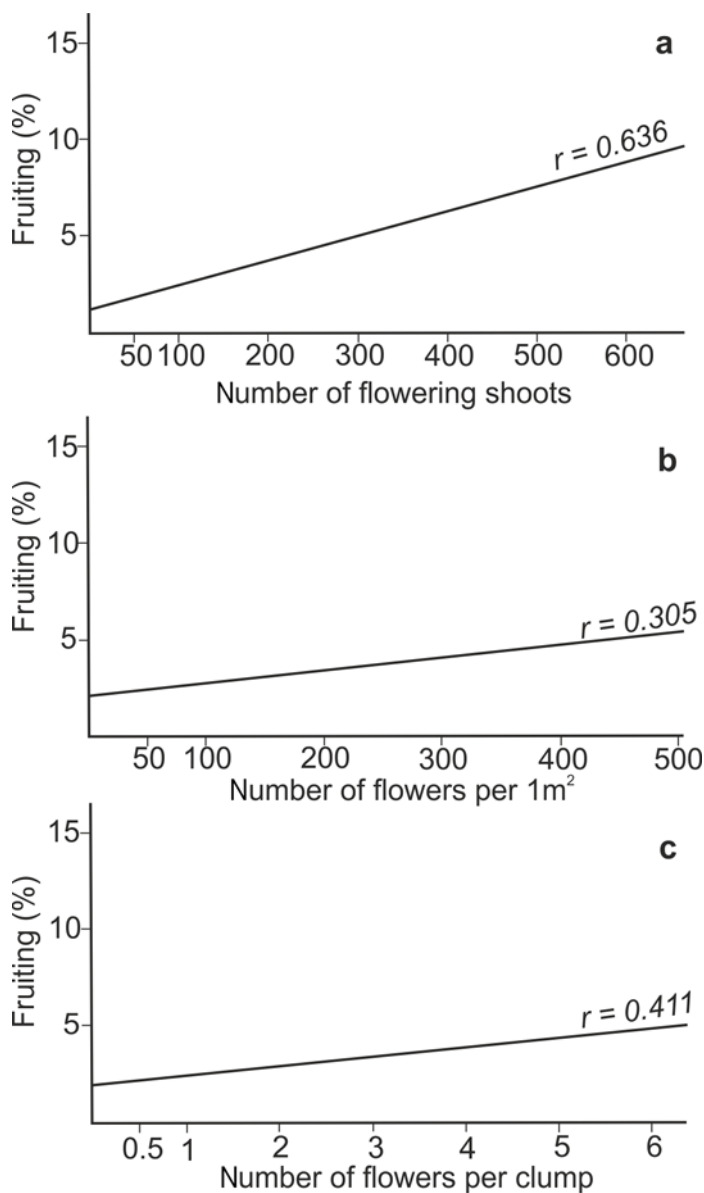


Fig. 1 Relation between fruiting ratio and number of flowering shoots (a), flowers per 1 m² (b), and per clump (c) in *Cypripedium calceolus* populations in the Biebrza National Park (NE Poland). Curves were obtained with linear regression. r – Spearman rank correlation coefficients.

Effectiveness of natural pollination and factors affecting it

The effectiveness of natural pollination was very low in *C. calceolus* populations from the Biebrza National Park. Fruiting was noted in only eight populations, and its level ranged between 0.8 and 19.7% (9.2% on average; Tab. 1). The lowest fruit set was observed in the population with the highest number of flowers. Several population characteristics associated with population size showed significant positive correlations with fruiting ratio: number of clumps ($r = 0.45$; $p < 0.00$), total number of shoots ($r = 0.64$; $p < 0.000$), and number of flowering shoots in populations ($r = 0.63$; $p < 0.000$), as well as total number of flowers ($r = 0.64$; $p < 0.000$) and area occupied by a given population ($r = 0.36$; $p = 0.03$) (Fig. 1). Also, density-weighted traits, like number of flowers per clump, showed a positive relation with fruiting ($r = 0.41$; $p = 0.01$), while the number of flowers per 1 m² was marginally positively correlated ($r = 0.30$; $p = 0.05$). Fruiting was also correlated with the number of other species in the understory level ($r = 0.46$; $p < 0.00$) and the percentage of cover in this layer ($r = 0.34$; $p = 0.032$), but most markedly with the number of species which were observed to flower at the same time as *C. calceolus* ($r = 0.54$; $p = 0.00$). Shading did not affect fruiting ($r = -0.09$; $p = 0.31$).

Male pollination success was very low. Only 19 (3.5%) pollinaria were removed from 375 observed flowers. In total, 15 flowers were effectively visited.

Populations in which fruiting was observed showed greater values than fruitless (but flowering) ones in the following traits: number of clumps ($F = 5.7$; $p = 0.03$), total number of shoots ($F = 20.1$; $p < 0.000$), flowering shoots ($F = 8.3$; $p = 0.00$) and flowers ($F = 7.7$; $p = 0.01$), but not with a density-weighted trend.

Lack of effect of mineral islands' isolation

There were no significant correlations between fruiting ratio and distance to the closest *C. calceolus* population ($r = 0.09$; $p = 0.31$), nor with the mean distance to all populations ($r = 0.14$; $p = 0.22$). Correlation coefficients of distance to the closest mineral island ($r = -0.19$; $p = 0.15$) or area of mineral island ($r = -0.23$; $p = 0.11$) were negative, but not significant.

Discussion

Our results document the low level of fruiting in populations of nectarless *C. calceolus* existing on mineral islands in the Biebrza National Park. The low fruit set observed in 2012 in all known populations in this area is similar to that given by Brzosko [12] for three populations from this area monitored over 11 years. The results are also in accordance with the effectiveness of pollination in populations of *C. calceolus* observed in other parts of its geographical range. For example, in Sweden populations 0–25% fruiting was noted [24], while in Estonian populations of *C. calceolus* the average fruit set was 11% [11]. A low level of fruiting was also found in Denmark (6–7.5% [33]) and in Russia (in Moscow Province 4–14%, and in Murmansk Province 3.6% [19: p. 121]). Low, strongly pollen-limited fruit set is also documented for other species from the *Cypripedium* genus [8,34–36].

We found that supplemental pollination enriched fruiting success dramatically. The same result was obtained for *C. calceolus* in England [37] and for other species of this genus. For example, Gill [38] found 100% fruit set after hand pollination in *C. acaule*, while natural fruiting during a 10-year study was below 5%. Similar results for this species were noted by O'Connell and Johnston [8] – 100% vs. 5.4–11.9%. Although we confirmed that *C. calceolus* is self-compatible, we excluded the potential for selfing in this species. Firstly, because we recorded the lack of spontaneous autogamy, and secondly, the very low level of natural fruiting indicates allogamy and lack of mixed-mating (crossing plus geitonogamy between ramets of the same genet) [2]. The low visitation rate (3.5% pollinaria removal) in the biggest *C. calceolus* population confirms pollinator deficiency. Therefore, we can conclude that pollinator limitation is a crucial factor causing low reproductive success in *C. calceolus* populations in the Biebrza valley. During almost 30 years of observation of *C. calceolus* populations on mineral islands, single *Andrenas* were observed, and in experiments with traps no specimens of these insects were noted (Brzosko, personal observation). Also, Ramsay and Stewart [37] stated that despite *Andrena* being a common genus, only a few of these bees were seen in the British locality of *C. calceolus*. The simplest explanation for pollinator restriction in this area is the paucity of suitable habitats for them. Species from the *Andrena* genus require open areas to build their nests, while mineral islands are mostly covered by dense vegetation. Moreover, species composition on mineral islands (during *C. calceolus* flowering, single individuals of single species are in flowers) and neighboring marsh communities, dominated by sedges, seems to be unsuitable for pollinators. Despite this, our results show that even a low proportion of flowering species positively affects fruit set in *C. calceolus* populations. Other authors have also found that pollination success in orchids is often higher in populations co-occurring with species attractive for pollinators [39,40].

We noted a positive relationship between pollination success and all parameters of population size (measured by both occupied area and number of clumps or shoots) and parameters measuring floral display (number of flowers in population, number of flowers in clumps, spatial structure of flowering shoots in population). This indicates that a reduction in pollinator attraction to small populations decreases pollination success. This is in accordance with findings concerning the influence of population size, its density and floral display on reproductive success [10,14,41–45], but does not confirm other findings that fruiting is less or not affected by reduced population size in nectarless orchids [11,15,16]. Although an appropriate, minimum population size is required to attract pollinators, exceeding the threshold of population size may have the opposite effect when the pollinators are limited. For example, Internicola

et al. [46] found that increasing the aggregation of *Dactylorhiza sambucina* negatively influenced reproductive orchid success. This occurred in the biggest of the studied *C. calceolus* populations, where fruit set was the lowest. Moreover, this population had the lowest level of fruiting among three populations observed over 11 years by Brzosko [12], despite the percentage of flowering shoots and total number of flowers being permanently the highest. This suggests that under conditions of floral overabundance the population of pollinators is insufficient to serve so big an orchid population. In such a situation, plants probably compete for pollinators, as stated by Steven et al. [47]. Goulson [48] gave an interesting explanation of lower visitation rate in larger patches. According to him, it is connected with insects' memory. Searching for unvisited flowers is easier in smaller patches than in larger ones. Moreover, in terms of the economy of pollinators foraging, visitation of neighboring flowers is less costly than those more distanced in larger patches.

Although in different parts of its range *C. calceolus* exists mainly in different types of forests, which by definition are shaded, it prefers more light for intensified flowering [19: p. 118]. Our results confirm that under better light conditions the rate of flowering increases without affecting fruit set. Moreover, the character of vegetation on mineral islands in the Biebrza National Park is changing due to natural processes (succession) and human activity (illegal cutting of trees). These changes strongly influence the maintenance of *C. calceolus* populations. Natural succession provides the expansion of trees and shrubs and increases shading. Human activities produce open areas, quickly dominated by expansive grasses and sedges that eliminate other flowering plants, including orchids, which are vulnerable to competitors. Changes in the character of vegetation on mineral islands restrict both sexual reproduction and the vegetative growth of individuals, and may further threaten population maintenance.

Our results clearly suggest that the majority of *C. calceolus* populations in the Biebrza National Park are not in good condition. A few of them are too small to survive in the occupied area. On the other hand, in many populations reproductive processes are restricted due to unsuitable environmental conditions, including pollinator limitation. In effect, the maintenance of *C. calceolus* populations in this area seems to be threatened. We suggest that appropriate conservation measures should be applied to preserve localities of this rare orchid. Knowledge about the population structure and environmental conditions in which populations exist could be used in planning effective conservation strategies.

Acknowledgments

Experiments on *C. calceolus* were done by permission of the Ministry of the Environment, Republic of Poland (decision No. DLPpn-4102-316/36993/12/DP).

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Deficyt zapylaczy przyczyną niskiego sukcesu reprodukcyjnego w populacjach nienektarycznego storczyka w Biebrzańskim Parku Narodowym

Streszczenie

Jedną z głównych przyczyn niskiego poziomu owocowania u storczyków jest deficyt zapylaczy. Generalnie, storczyki nektaryczne charakteryzują się niższym owocowaniem niż nienektaryczne. Celem badań było sprawdzenie czy niski sukces reprodukcyjny w populacjach *Cypripedium calceolus* na wyspach mineralnych w Biebrzańskim Parku Narodowym spowodowany jest niedostatkami zapylaczy. Ponadto, analizowano wpływ wielkości i struktury populacji tego gatunku na sukces zapyleniowy. Obserwacje i wyniki eksperymentów dokumentują niski poziom owocowania w populacjach *C. calceolus* (średnio 9.2%) oraz limitację pyłkiem. Odnotowano pozytywne zależności między sukcesem zapyleniowym a badanymi wskaźnikami wielkości populacji (mierzonymi zarówno wielkością zajmowanego arealu, jak też liczbą kęp i pędów) oraz wielkością wystawy kwiatowej (liczba kwiatów w populacji, liczba kwiatów/kępę, rozmieszczenie pędów kwitnących). Znajomość czynników wpływających na sukces reprodukcyjny zagrożonego gatunku jest bardzo istotna w planowaniu odpowiednich działań konserwatorskich.