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ORIGINAL RESEARCH PAPER

Genetic diversity of *Dactylorhiza incarnata* (Orchidaceae) in northern Poland

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Abstract

The genetic structure of *Dactylorhiza incarnata* var. *incarnata* populations is shaped not only by historical events such as recolonization after ice sheet retreat or limited seed and pollen dispersal, but also the bottleneck effect. During the last decade, *D. incarnata* var. *incarnata* has also experienced a strong decline in population numbers and sizes, due to habitat loss and fragmentation. In the present research genetic diversity was examined in eight populations located in northern Poland, using six nuclear microsatellites loci. At the species level our results showed a moderate mean level of genetic diversity (A = 4.67; A_e = 2.73; R_s = 4.48; H_o = 0.438; F_{IS} = 0.224), which varied among the studied populations (A: 2.17–3.67; A_e: 1.55–2.69; R_s: 1.31–1.61; H_o: 0.292–0.631; F_{IS}: −0.283–0.340). A considerable overabundance of homozygotes was detected in four populations (F_{IS} within the range of 0.067–0.340), and in the remaining populations an excess of heterozygotes was observed. The average apparent out-crossing rate was also calculated (t_a = 0.980), and primarily indicated a tendency to out-cross within the species. Moderate genetic differentiation was found among the studied populations (F_{ST} = 0.149; R_{ST} = 0.174; $p < 0.05$). The differentiation of the populations corresponded to relatively low gene flow value (N_m = 0.426) among populations, which amounted to only one migrant every second generation.

Keywords

Dactylorhiza incarnata; gene flow; genetic variation; habitat fragmentation; nuclear microsatellites

Introduction

In recent decades the processes of the extinction of vascular plant species has accelerated considerably as a result of human activity causing disturbances within their natural habitats [1,2]. Additionally, habitat fragmentation had also a great impact on the genetic variation of plant populations. Such fragmentation contributes to population subdivisions, reinforces immigration barriers, increases random genetic drift [3], decreases population sizes, increases distances among populations, and therefore, weakens gene flow.

Orchidaceae is a family of flowering plants which is particularly vulnerable in Europe [4]. This is due to, inter alia, their unique reproductive strategies, specific interactions with symbionts, and often specific habitat requirements which make them very sensitive to changes in their habitat. Orchids usually form small populations that are vulnerable to such evolutionary processes as the bottleneck effect or inbreeding. The combination of these processes can lead to genetic erosion and, consequently, to the loss of genetic diversity [5]. Moreover, the breeding system and the dispersal

characteristics of pollen and seeds are the most significant factors shaping the genetic structure of their natural populations [6]. In highly selfing species with limited seed and pollen dispersal, inbreeding coefficients are expected to be high, and most of the genetic variation is expected to be observed among populations. In contrast, outcrossing species with wide seed and pollen dispersal are presumed to be characterized by low inbreeding coefficients, and indicate higher levels of variability within populations and lower levels of differentiation among them [7].

Molecular studies have greatly increased our understanding of the *Dactylorhiza* genus. Based on allozyme variation [8], it is known that all the *D. majalis* (Rchb.) P.F. Hunt and Summerh. tetraploid marsh orchids recognized in Europe are allotetraploids that may have originated by hybridization between parental lineages related to contemporary *D. incarnata* (L.) Soó s. l. and *D. maculata* (L.) Soó s. l. Further analyses using AFLPs [9], PCR-RFLPs [10,11], VNTRs [12] and internal/external transcribed spacers (ITS/ETS) [13] have identified differentiation of genetic diversity within the *D. maculata* s. l. lineage and have revealed a division into *D. maculata* subsp. *fuchsii* and *D. maculata* subsp. *maculata*. These markers have also been used to elucidate the origin of the European allotetraploid taxa. Nonetheless, genetic variation in *D. incarnata* s. l. based on DNA polymorphism is not as well understood yet. Based on allozyme variation, Hedrén [14] distinguished three varieties within this group: (***i***) *D. incarnata* var. *incarnata* – with long, rising leaves without spots, with pink flowers, (***ii***) *D. incarnata* var. *cruenta* Hyl. – with short leaves, spotted on both sides, with dark red flowers, and (***iii***) *D. incarnata* var. *ochroleuca* Jagiello and Kuusk – with leaves without spots and with yellow flowers. Additionally, subsequent studies using allozyme markers [15,16] and the AFLP method [9] demonstrated that the aforementioned varieties of *D. incarnata* show very low genetic variation, high levels of inbreeding, and in terms of the analyzed loci they are almost identical. Furthermore, Hedrén and Nordström argued that these varieties represent distinct inbred lines and gene flow across them is rare [17].

Dactylorhiza incarnata var. *incarnata* is a herbaceous, perennial, food-deceptive orchid species and has Eurosiberian distribution. Populations of this species are particularly vulnerable to habitat destruction, subjecting them to risk of local extinction, because they have a relatively narrow ecological tolerance and prefer only habitats with neutral or slightly alkaline pH [18]. In northern Poland, *D. incarnata* var. *incarnata* seems to be especially endangered. The number of local populations has dramatically declined in Pomerania [19]. Recent field studies confirmed individuals of the early marsh orchid in only 8 out of 56 previously known localities. The decline in the number of populations is largely the result of changes in peat bogs and meadows management starting secondary succession later on. Therefore, the need to place *D. incarnata* var. *incarnata* in the VU – vulnerable to extinction category within the regional red lists of endangered flora – has been recognized [20]. Nonetheless, the last regulation of the Ministry of Environment, Republic of Poland has changed the status of the species from strict to partial protection. Additionally, it is highly probable that the decline of *D. incarnata* populations is masked by the rather frequent occurrence of *D. majalis*, because the improper identification of species within the *Dactylorhiza* genus is still problematic.

The main aims of this study were: (***i***) to assess the levels and patterns of genetic variation in the studied populations using microsatellite markers, and (***ii***) to determine the putative factors influencing genetic differentiation in the populations of *D. incarnata* var. *incarnata*. This work is one of the few examples of studies on variation in the *Dactylorhiza* genus, conducted with the use of nuclear microsatellite markers.

Material and methods

Molecular methods

Field studies were performed from 2009 to 2011 in Gdańsk Pomerania and Middle Pomerania (Fig. 1). The exact location of sites, approximate population size and sample sizes in the data sets are included in Tab. 1. In this study all populations were

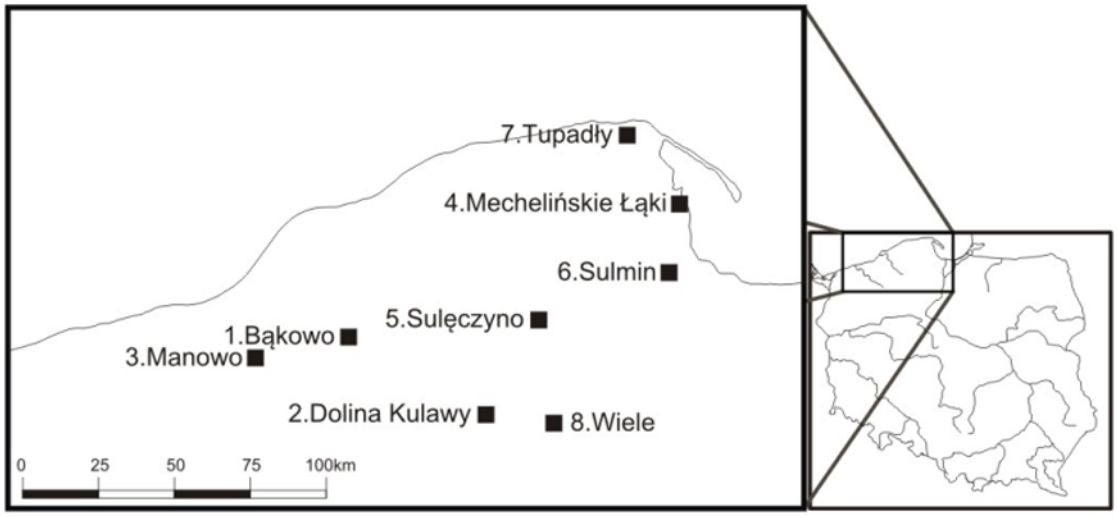

Fig. 1 Localities of *Dactylorhiza incarnata* var. *incarnata* populations in northern Poland.

identified as *D. incarnata* var. *incarnata*. For subsequent DNA extraction the collected leaf fragments from the specimens were dried in silica gel following the procedure described by Chase and Hills [21].

Total DNA was extracted from the collected leaf samples following the procedure described by Bekesiova et al. [22]. A total of 169 individuals in eight populations of *D. incarnata* var. *incarnata* were examined for six nuclear microsatellites loci. The six selected loci – *ms3*, *ms6*, *ms8*, *ms10*, *ms11*, and *ms13* – were developed for *Dactylorhiza* by Nordström and Hedrén [23] and consist of trinucleotide repeats. Available microsatellite loci show different levels of diversity. Only loci with the highest levels of variation were selected for further analysis, according to Nordström and Hedrén [23].

All fragments were amplified simultaneously in the same PCR by multiplexing. For the multiplex reaction PCRs were carried out in a total volume of 10 µL containing: 0.25 µM primer for each *ms3* and *ms8* locus, 0.075 µM primer for *ms6* locus, 0.05 µM primer for *ms10* locus, 0.025 µM primer for *ms11* locus, 0.1 µM primer for *ms13* locus, and template DNA (2 ng/µL) in 2× Multiplex PCR Master Mix (Qiagen). One of the primers used for amplification was dye-labelled at the 5′-end (Applied Biosystems Inc.), which made it possible to visualize the amplified fragments. A touchdown protocol was used for PCR amplification: the initial denaturation round (95°C for 15 min) was followed by 9 cycles of 94°C for 30 s, 60°C (reducing 0.5°C per cycle) for 1 min 30 s, and 72°C for 1 min. The next 24 cycles used 94°C for 30 s, an annealing of 55°C. The final round used 72°C for 10 min. The PCR product from each reaction was mixed with appropriate size standards to enable exact size determination. All samples for the length-variable fragment analyses were run on a 3130xl genetic analyzer (Applied Biosystems Inc.). The size of each fragment was determined using Genescan and Genotyper software v. 3.7 (Applied Biosystems Inc.).

Genetic analysis

The following parameters of genetic diversity were calculated for *D. incarnata* var. *incarnata*, at the species and population levels: the allele frequency, the mean number of alleles per locus (A), the effective number of alleles per locus (A_e), the expected (H_e) and observed (H_o) heterozygosity. Because sample sizes differed among populations, an added measure of allelic richness (R_s) was computed using the rarefaction method [24]. Furthermore, measurements of allelic richness are generally considered most effective in detecting loss of genetic variation in fragmented populations regardless of historic or recent anthropogenic disturbances [25]. Fixation indices, F_{IS} (an inbreeding coefficient) were estimated based on Weir and Cockerham [26] estimators. The statistical significance of F_{IS} was calculated by a permutation test for each locus and

Tab. 1 Survey of *Dactylorhiza incarnata* var. *incarnata* populations in northern Poland.

No.	Population	Coordinate	*N*	*A*	A_e	R_s	H_o	H_e	F_{IS}	[*p*]	t_a	Collector/Voucher No.
1	Bąkowo	54°10′N, 17°00′E	16	3.50	2.47	1.55	0.552	0.549	−0.005	0.574	1.011	Kazimierski UGDA2010/375-394
2	Dolina Kulawy	53°58′N, 17°32′E	17	2.83	2.16	1.51	0.631	0.510	−0.283	0.971	1.622	Naczk UGDA2011/555-574
3	Manowo	54°06′N, 16°18′E	15	3.33	2.35	1.53	0.374	0.527	0.299	0.000	0.550	Naczk UGDA2011/609-623
4	Mechelińskie Łąki	54°37′N, 18°30′E	40	3.50	2.44	1.53	0.353	0.531	0.340	0.000	0.498	Naczk UGDA2009/1-40
5	Sulęczyno	54°13′N, 17°47′E	20	2.67	1.73	1.31	0.325	0.303	−0.075	0.837	1.157	Naczk UGDA2009/218-238
6	Sulmin	54°19′N, 18°28′E	39	3.00	1.78	1.38	0.455	0.384	−0.189	1.000	1.454	Naczk UGDA2011/783-824
7	Tupadły	54°48′N, 18°17′E	14	3.67	2.69	1.61	0.488	0.610	0.206	0.006	0.667	Naczk UGDA2009/20-37
8	Wiele	53°55′N, 17°50′E	8	2.17	1.55	1.31	0.292	0.311	0.067	0.411	0.885	Naczk UGDA2010/256-263
	Means		-	3.08	2.15	1.47	0.434	0.466	0.045	-	0.980	

N – number of samples analyzed in genetic studies; *A* – mean number of alleles per locus; A_e – effective number of alleles per locus; R_s – allelic richness; H_o – observed heterozygosity; H_e – expected heterozygosity; F_{IS} – inbreeding coefficient; [*p*] – probability of the hypothesis $F_{IS} = 0$; t_a – apparent out-crossing rate. Voucher specimens and the names of collectors were included.

across loci for each population ($p = 0.05$). The apparent out-crossing rate was determined considering the formula $t_a = (1 - f)/(1 + f)$, where f was estimated as $f = (H_e - H_o)/H_e$ [27]. To study concordance with the Hardy–Weinberg equilibrium (HWE) the MCMC approximation of the exact test was tested at each polymorphic locus in every population, at the significance level $p < 0.05$ [28]. The mentioned statistics were performed by means of the computer programs: GENEPOP v. 4.0 [29] and FSTAT v. 2.9.3.2 [30].

In order to compare the differentiation of genetic variation between populations, two measures of genetic diversity were used: F_{ST} (takes into account the effect of unequal sample size and the number of analyzed populations [26]) and R_{ST} [an analogue of F_{ST} developed specifically for microsatellite data that takes into account the stepwise mutational model (SMM) [31]]. The statistical significance for the individual parameters was assessed with the use of the permutation test, at a significance level of $p = 0.05$. The calculations were performed for each pair of populations and for the entire sample. Furthermore, the theoretical number of migrants (N_m) entering every population per generation was estimated based on the method proposed by Barton and Slatkin [32]. Data analyses were performed with the use of the computer programs: FSTAT v. 2.9.3.2 [30] and GENEPOP v. 4.0 [29]. The differentiation between all studied populations was illustrated by means of a principal coordinates analysis (PCO [33]), which was performed on the resulting matrix of genetic distances, by means of the computer program PAST v. 2.14 [34]. To investigate the population structure and to assign individuals to populations, the Bayesian clustering method was used, as implemented in STRUCTURE v. 2.3.4 [35]. Data were analyzed with an admixture model, assuming correlated allele frequencies compiled by Falush et al. [36]. Twenty replicates were run for all possible values of the number of clusters (K) up to $K = 1–8$. The ad hoc statistic ΔK was calculated following the references by Evanno et al. [37]. All runs were based on 500 000 iterations after a burn-in of 100 000 steps. The results were summarized on the online platform Harvester [39], which implements the Evanno method to assess the most likely K value given the data [37].

The program BOTTLENECK v. 1.2.2 [39] was used to test a recent reduction of effective population size because allelic diversity is generally lost faster than heterozygosity during a bottleneck. As a consequence, recently bottlenecked populations will display an excess of heterozygosity in comparison to the level expected on the basis of the number of alleles found in the sample if the population was at mutation drift equilibrium. The test was performed with the use of the stepwise mutational model for microsatellites as genetic markers [40]. The significance of differences between the excess and deficiency of genetic diversity was examined with the Wilcoxon test (1000 permutations). The population of Sulmin was excluded from the analysis due to a significant lack of data in the input file.

Tab. 2 Parameters of genetic diversity estimated for *Dactylorhiza incarnata* var. *incarnata*.

Locus name	*A*	A_e	R_s	H_o	H_e	F_{IS}	[*p*]	*p*-value
ms3	5	3.10	4.71	0.307	0.680	0.554	0.000	0.000
ms6	2	2.00	2.00	0.976	0.501	−0.953	1.000	0.000
ms8	8	3.99	7.36	0.325	0.752	0.568	0.000	0.000
ms10	2	1.10	2.00	0.071	0.091	0.216	0.043	0.042
ms11	4	3.17	4.00	0.390	0.690	0.437	0.000	0.000
ms13	7	2.99	6.80	0.560	0.668	0.163	0.001	0.001
Means	4.67	2.73	4.48	0.438	0.563	0.224	-	-

A – mean number of alleles per locus; A_e – effective number of alleles per locus; R_s – allelic richness; H_o – observed heterozygosity; H_e – expected heterozygosity; F_{IS} – inbreeding coefficient; [*p*] – probability of the hypothesis $F_{IS} = 0$; *p*-value – significance level for the hypothesis of HWE equilibrium.

Results

Genetic diversity

At the species level the average number of alleles *A* was equal to 4.67 (Tab. 2). Furthermore, the effective number of alleles reached values in the range from 1.10 (*ms10*) to 3.99 (*ms8*). The observed heterozygosity (H_o) in a given locus was usually lower than the expected value (H_e) due to the significant deficiency of heterozygotes in relation to the presumed genetic equilibrium. The observed deficiency of heterozygotes is reflected in the positive average values of the inbreeding coefficient. The F_{IS} statistics reached the average value of 0.224. In the analyses carried out for a single locus the permutation test confirmed the lack of HWE in all tested loci (Appendix S1).

In the eight populations of *D. incarnata* var. *incarnata* the mean number of alleles per locus in a population was equal to 3.08 (Tab. 1). The population of Tupadły was characterized by the highest degree of variation, the population of Wiele by the lowest one. The level of expected heterozygosity (H_e) ranged between 0.303 and 0.610. The observed heterozygosity (H_o) took a value between 0.292 and 0.631, with an average of 0.434. The average value of the inbreeding coefficient (0.045) was relatively low at the population level. However, an excess of heterozygotes in relation to genetic equilibrium was observed, which is reflected in the negative values of the inbreeding coefficient for individual populations. The apparent out-crossing rate (t_a) ranged from 0.498 to 1.622, with an average of 0.980.

The allele frequency distribution analysis showed that all the populations of *D. incarnata* var. *incarnata* experienced a reduction in the number of individuals (i.e., bottleneck effect). A significant excess of expected heterozygosity was found in all studied populations (Appendix S2).

Tab. 3 Genetic differentiation F_{ST} and R_{ST} for *Dactylorhiza incarnata* var. *incarnata* populations.

Locus name	F_{ST}	[*p*]	R_{ST}	[*p*]
ms3	0.266	0.000	0.253	0.000
ms6	0.005	0.001	0.005	0.001
ms8	0.246	0.000	0.230	0.000
ms10	0.049	0.034	0.049	0.038
ms11	0.120	0.005	0.161	0.007
ms13	0.065	0.000	0.072	0.004
Means	0.149	-	0.174	-

[*p*] – probability of the hypothesis $F_{ST} = 0$ or $R_{ST} = 0$.

Genetic differentiation and gene flow

The genetic differentiation for *D. incarnata* var. *incarnata* amounted to F_{ST} = 0.149 and R_{ST} = 0.174 (Tab. 3). The obtained average values indicate a moderate level of genetic differentiation for the studied species. Genetic differentiation (F_{ST}) between populations of the early marsh

Tab. 4 Genetic differentiation F_{ST}-values (below diagonal) and R_{ST}-values (above diagonal) between studied populations of *Dactylorhiza incarnata* var. *incarnata* in northern Poland.

Population	1	2	3	4	5	6	7	8
1	-	0.085	0.148	0.218	0.007	0.166	0.226	0.153
2	0.109	-	0.121	0.079	0.123	0.021	0.155	0.027
3	0.046	0.176	-	0.341	0.165	0.186	0.010	0.109
4	0.060	0.036	0.130	-	0.312	0.119	0.361	0.117
5	0.138	0.242	0.155	0.191	-	0.208	0.266	0.231
6	0.124	0.161	0.154	0.143	0.322	-	0.225	0.111
7	0.092	0.177	0.007	0.140	0.236	0.198	-	0.122
8	0.164	0.225	0.191	0.117	0.221	0.371	0.204	-

Statistically significant values are in bold ($p < 0.05$). Studied populations: 1 – Bąkowo; 2 – Dolina Kulawy; 3 – Manowo; 4 – Mechelińskie Łąki; 5 – Sulęczyno; 6 – Sulmin; 7 – Tupadły; 8 – Wiele.

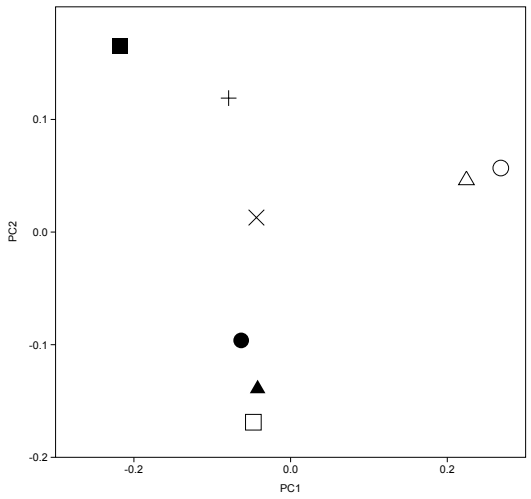

Fig. 2 Principal coordinates analysis (PCO) showing differentiation between the *Dactylorhiza incarnata* var. *incarnata* populations. The two first axes account for 42.74 and 23.87% of the total variation, respectively. Symbols: black circle – Bąkowo; "+" – Dolina Kulawy; white square – Manowo; "×" – Mechelińskie Łąki; white triangle – Sulęczyno; black square – Sulmin; black triangle – Tupadły; white circle – Wiele.

orchid ranged between 0.007 (Manowo–Tupadły) and 0.371 (Sulmin–Wiele), and in case of the R_{ST} parameter – between 0.007 (Sulęczyno–Bąkowo) and 0.361 (Tupadły–Mechelińskie Łąki; Tab. 4). In the PCO analysis the first ordination axis allowed the distinguishing of Bąkowo, Tupadły, and Manowo from other populations, whereas the second axis mostly separated Wiele and Sulęczyno populations (Fig. 2). The most likely number of clusters as determined by STRUCTURE was $K = 4$ (Fig. 3). The studied populations were structured into four almost distinct gene pools (Bąkowo, Dolina Kulawy, Manowo, and Mechelińskie Łąki), and the rest of the populations had a high degree of admixture and represented mixed genetic backgrounds. On the basis of the private alleles frequency distribution, estimated gene flow N_m equaled only 0.426 for the studied species (around one migrant every second generation). However, the lowest estimate was observed between the Dolina Kulawy and Sulęczyno populations ($N_m = 0.075$) separated by approximately 49 km, and the highest was obtained between the Manowo and Tupadły populations ($N_m = 1.412$), separated by approximately 199 km (Tab. 5). For the other pairs of populations, the values of gene flow were lower than 1.

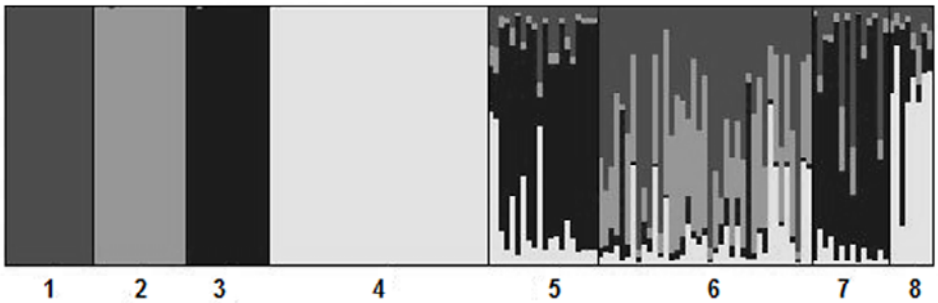

Fig. 3 Structure clustering results for all populations of *Dactylorhiza incarnata* var. *incarnata* at $K = 4$. Each population is represented by one chart, showing the relative proportion of membership to the different clusters, where: 1 – Bąkowo; 2 – Dolina Kulawy; 3 – Manowo; 4 – Mechelińskie Łąki; 5 – Sulęczyno; 6 – Sulmin; 7 – Tupadły; 8 – Wiele.

Tab. 5 Number of individual migrants per generation (N_m, below diagonal) and geographical distance (km, above diagonal) between pairs of *Dactylorhiza incarnata* var. *incarnata* populations in northern Poland.

Population	1	2	3	4	5	6	7	8
1	-	77.1	122.0	64.3	43.2	71.8	68.5	94.1
2	0.354	-	103.0	133.0	49.0	102.0	137.0	60.1
3	0.399	0.168	-	197.0	133.0	186.0	199.0	134.0
4	0.660	0.543	0.126	-	83.2	44.9	36.9	116.0
5	0.256	0.075	0.254	0.332	-	58.3	103.0	51.2
6	0.795	0.291	0.383	0.333	0.112	-	70.0	77.9
7	0.240	0.155	1.412	0.211	0.178	0.178	-	142.0
8	0.401	0.211	0.297	0.478	0.374	0.106	0.243	-

Studied populations: 1 – Bąkowo; 2 – Dolina Kulawy; 3 – Manowo; 4 – Mechelińskie Łąki; 5 – Sulęczyno; 6 – Sulmin; 7 – Tupadły; 8 – Wiele.

Discussion

Genetic diversity

A relatively low level of genetic diversity was observed in the northern part of the early marsh orchid range [14,15,41]. These results indicated that the level of allozyme variation for the populations of the early marsh orchid is the lowest among the studied species within the *Dactylorhiza* genus. In Pomerania the observed heterozygosity based on allozyme variation was only $H_o = 0.003$ [20], and the overall outcome in the whole country was $H_o = 0.008$ [42]. The extremely low levels of genetic variation of *D. incarnata* may indicate inbreeding, as well as the loss of individual alleles during the recolonization from southern refugia, along with the withdrawal of the ice sheet [43]. A slightly higher level of variability was observed in populations from Turkey [44], also based on allozyme data. Refugia for *D. incarnata* were presumably located in Southern and southwestern Europe, and previous studies of the genetic structure showed that these populations are characterized by the highest levels of genetic variation in Europe [12,17,45].

In the research of Hedrén and Nordstöm [17], which was conducted using microsatellite markers, regarding the genetic variation of the three varieties present on Gotland, the average expected heterozygosity ranged from 0.12 (*D. incarnata* var. *ochroleuca*) to 0.59 (*D. incarnata* var. *incarnata*). However, the same analysis provided somewhat lower values when the calculations were performed at the population level: he took values between 0.09 (*D. incarnata* var. *ochroleuca*) and 0.39 (*D. incarnata* var. *incarnata*). Also, in the present study lower average values for expected heterozygosity (0.466) were observed when the analyses were also performed at the population level (see Tab. 1).

In our study the inbreeding coefficient was higher than expected ($F_{IS} = 0.224$), based on species level. An equally high level of inbreeding was observed within populations on Gotland ($F_{IS} = 0.58$ [17]). Many authors have suggested that the levels of genetic variability may be a consequence of a breeding system [5,7,46]. Autogamy especially affects the value of the inbreeding coefficient, but in fact allogamy is postulated to be the dominant type of breeding system in the early marsh orchid [14]. Vallius et al. [47] argued that different varieties of *D. incarnata* (i.e., *incarnata*, *cruenta*, and *ochroleuca*) maintain a high level of inbreeding, which reduces gene flow and conserves the integrity of a given taxon. This claim was also supported by other researchers based on allozyme variation [41]. Thus, a population might consist of several inbred lines that are fixed for characters such as flower color, leaf shape, and leaf spotting.

The results of recent experimental research suggest that in natural conditions the reproductive success of *D. incarnata* may be the result of three modes of pollination – apomixis, autogamy, and allogamy, as proved by the development of the fruit set in all the respective experimental groups [48]. Likewise, other pollination experiments have demonstrated that autogamy is possible in *D. incarnata* but uncommon [49]. In turn, a predominantly cross-fertilization mating system with a small amount of inbreeding within the studied species was indicated by the resulting mean value of apparent out-crossing rate (t_a = 0.980). However, the mixed mating system was also recognized in four studied populations (Manowo, Mechelińskie Łąki, Tupadły, and Wiele; see Tab. 1).

The apparent out-crossing rate can be less than 1.0, when alike genotypes mate among themselves more often than expected; equal to 1.0, when genotypes mate with each other in proportion to their frequency within the populations; or higher than 1.0, when different genotypes mate more frequently than expected [50]. The latter situation was also observed for the studied populations. This may be due to the disassortative mating which occurs when individuals of different genotypes pair much more often than in panmictic populations. In disassortative mating there is negative phenotypic correlation between the mating plants with regard to the considered trait – the mating plants resemble each other less than plants belonging to pairs of random plants. The population genetic effect of disassortative mating with regard to some traits is an increased frequency of plants with heterozygous genotypes for the loci affecting the traits [51].

One should keep in mind that in animal-pollinated species crossing events are often unpredictable. More than half of the animal-pollinated species of plants produce a mixture of selfed and outcrossed progeny within a season [52]. The proportion of selfed vs. outcrossed progeny is determined by the timing and relative amount of self- and outcross-pollination and by post-pollination processes [53]. On the other hand, in animal-pollinated plants patterns of reward production may affect pollinator behavior, and may therefore be expected to influence the frequency of visits, the effectiveness of pollination, pollen dispersal distances and population structure [54].

Genetic differentiation

The available literature data show that populations of *D. incarnata* exhibit very large inter-population differentiation. Research conducted with the use of allozyme markers showed a high value of G_{ST} = 0.733 for the Polish populations of the early marsh orchid [42]. In Pomerania, a similar pattern of differentiation was also observed, with G_{ST} = 0.55 [19]. A slightly lower value of genetic differentiation, but still high, was reported by Vandepitte et al. [55] within populations along the Belgian-French coast (F_{ST} = 0.35), using the AFLP markers. In turn, in our study the genetic differentiation of the early marsh orchid populations was low (F_{ST} = 0.149 and R_{ST} = 0.174). A clearly higher level of differentiation was obtained in the analysis of the R_{ST} than the F_{ST} parameter, which may indicate that the genetic differences between populations are the result not only of restricted gene flow, but also of mutations in microsatellite loci [56]. F_{ST} is based on the infinite allele model, whereas R_{ST} is estimated under the stepwise mutation model (SMM). In SMM, alleles can be identical in state because of mutation, which affects to increase or decrease the size of the microsatellite repeats and, in consequence, the electrophoretic mobility of an allele by one repeat unit [57]. According to Slatkin [31], this mutation model is more appropriate for the analysis of microsatellites. In the estimation of R_{ST} the estimation procedure accounts for potential genotyping errors due to stutter bands or peaks that are typical of dinucleotide microsatellite loci [58]. In contrast to the estimate of F_{ST}, the impact of inaccuracies in allele size statement is minimized during the estimation of R_{ST}.

Gene flow is an important force for maintaining genetic diversity in small populations of plants because it may reduce genetic drift and inbreeding, and moderate directional selection, and can preserve their adaptability to changing environmental conditions. The obtained level of gene flow for *D. incarnata* var. *incarnata* was relatively low (N_m = 0.426; one migrant every second generation). It is a very low value for species such as orchids, with wind-dispersed seeds. They are expected to show higher

levels of gene flow (e.g., N_m = 5.33 [59]). In cases when the gene flow is restricted or does not occur, it leads to genetic divergence of populations, as a result of natural selection or genetic drift [60]. Gene flow and its consequences depend largely on the number of individuals and the distance between the populations. Additionally, it should be kept in mind that gene flow in plants occurs via two independent processes – the dispersion of pollen and seeds. Indirect estimates of gene flow among populations of the early marsh orchid reflect a historical capacity for gene flow through pollen dispersal by deceived bumblebee pollinators and/or long-range dispersal of dust-like orchid seeds. However, current levels of gene flow may be impacted by habitat destruction, fragmentation, and reduced population size.

In our case, pollen transfer by insects among the populations seems to be very difficult due to large geographical distances, exceeding 100 km (e.g., a distance of 197 km between Manowo–Mechelińskie Łąki, 137 km between Dolina Kulawy–Tupadły, 142 km between Tupadły–Wiele; see Tab. 5). Field studies have shown that bumblebee species forage mostly at distances of 50–600 m up to 2.2 km from the nest [61]. In turn, orchid seeds are generally believed to be capable of long-distance dispersal by the wind, due to their small size. Although wind-dispersal of seeds may be highly localized, generating a fine scale genetic structure, Trapnell and Hamrick [62] pointed out that some seeds can enter the air column and be dispersed over long distances, minimizing the effects of isolation and population differentiation (e.g., *Cephalanthera longibracteata* Blume [63], *Laelia rubescens* Lindl. [62]). However, some authors have noted that most orchid seeds fall within only a short distance of the maternal plants, which is consistent with significant local spatial genetic structure in orchid populations (e.g., [64]). The highest value of gene flow was observed between populations in Tupadły and Manowo (N_m = 1.412), separated by a distance of 199 km. Nevertheless, even if seeds are transported over long distances along the coast of the Baltic Sea by strong westerly blowing winds, it is required to encounter the mycorrhizal symbiont needed for their germination [65]. It is therefore possible to deduce that local pollen dispersal is largely responsible for the gene flow observed here. In a situation when pollen dispersal is also restricted, this leads to inbreeding, reinforcing the accumulation of more intense genetic structure and subdividing the population over time under a process of isolation by distance [66]. In addition, pollen dispersal distances can be limited when plants occur in clearly delimited subpopulations and pollinators move around within patches before flying to another subpopulation. Moreover, it is likely that geographic isolation, habitat fragmentation, short distance pollinator movements, and limited seed dispersal influence restricted gene flow among the *D. incarnata* var. *incarnata* populations, which was also confirmed by Bayesian clustering analysis (see Fig. 3). This revealed that a population-level admixture was present in half of the studied populations, suggesting some dispersal and gene flow into these sites, in contrast with populations from Bąkowo, Dolina Kulawy, Manowo, and Mechelińskie Łąki, in which a single gene pool predominated. We suggest that, as in other orchid species, in *D. incarnata* var. *incarnata* both pollen and seed dispersal are greatly restricted and occur on a local scale [67].

Many studies have implicated limited gene flow as the major factor affecting the spatial genetic structure within populations, as well as being the result of microhabitat selection. In this study the level of migration among the early marsh orchid populations was below one, so gene flow will not prevent continued divergence among populations [68]. Likewise, within several genetic studies on the spatial genetic structure of terrestrial orchid populations, including research on *D. incarnata* [49], a significant pattern has been found, which was explained in most cases by spatially restricted patterns of gene flow (e.g., [63,64]).

Bottleneck effect analysis revealed that all the tested populations of this taxon have experienced a reduction in the number of specimens. Two genetic consequences of a small population size have been indicated: genetic drift and inbreeding at both population and species level [69]. In small isolated populations genetic drift may reduce genetic diversity, because effective population size is usually much smaller than the number of reproductive individuals in a population. The small size of the population, as well as the unavailability of suitable habitat for *D. incarnata*, leads to the spatial isolation of the populations.

In recent years human activities have led to extensive modification and fragmentation of habitats, and these activities may also have an impact on the genetic structure of natural populations. Habitat fragmentation reduces the sizes of the populations and increases isolation between them [70]. As a consequence, a decrease of genetic variation and an increase of interpopulation genetic divergence due to the increase of random genetic drift, inbreeding and reduced gene flow is observed [1]. In conclusion, species with more continuously distributed populations should experience higher gene flow than species with discrete, isolated populations. They should also have relatively lower variation among the local populations. Therefore, fragmented populations of *D. incarnata* var. *incarnata* may be exposed to reduced current gene flow and subsequent loss of allelic variation as the consequence of long distances between neighboring localities [71], leading finally to a decrease of the species' genetic diversity.

Conclusions

Long-term reduction in the numbers of individuals (bottleneck effect) occurred in the recent history of the *D. incarnata* var. *incarnata* populations. It was caused by the colonization of areas available shortly after the withdrawal of the continental ice sheet or changes in the environment due to anthropogenic pressure. It seems that genetic drift plays a significant role in the populations of *D. incarnata* var. *incarnata*, where the probability of inbreeding is very high. Moreover, there is a little chance that new alleles will be transferred to other populations with such observed limited gene flow. All these circumstances contribute to the danger of extinction for populations of *D. incarnata* var. *incarnata* at the regional scale, and may also be expected in other parts of its range.

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Supplementary material

The following supplementary material for this article is available at http://pbsociety.org.pl/journals/index.php/asbp/rt/suppFiles/asbp.3496/0:

Appendix S1 Allele frequencies in the study populations of *Dactylorhiza incarnata* var. *incarnata*.

Appendix S2 Probability of bottleneck effect in the recent history of *Dactylorhiza incarnata* var. *incarnata* populations.

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