Concordant Patterns of Population Genetic Structure in Food-Deceptive *Dactylorhiza* Orchids

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Keywords: *Dactylorhiza fuchsii; Dactylorhiza incarnata* var. *incarnata; Dactylorhiza majalis;* FIS; spatial genetic structure

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Article

Concordant Patterns of Population Genetic Structure in Food-Deceptive *Dactylorhiza* Orchids

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Abstract: Background: The patterns of inbreeding coeficient (F_{IS}) and fine-spatial genetic structure have been evaluated regarding mating system and inbreeding depression of food-deceptive orchid populations, *Dactylorhiza majalis*, *Dactylorhiza incarnata* var. *incarnata*, and *Dactylorhiza fuchsii* from NE Poland. Methods: We used 455 individuals representing nine populations of three *Dactylorhiza* taxa and AFLP markers to estimate percent polymorphic loci (*P*%) and Nei's gene diversity (*H*), calculated using the Bayesian method, F_{IS}, F_{ST}, the spatial autocorrelation analysis with the pairwise kinship coefficient *Fij*, and AMOVA in populations. The genetic diversity parameters were discussed applying data from fruit set, in vitro seed germination and inbreeding depression of three *Dactylorhiza*. Results: We detected a relatively high proportion of polymorphic fragments (*P* = 40.4.5–68.4%) and Nei's gene diversity indices (*H* = 0.140–0.234). The overall F_{IS} was relatively low-to-moderate 0.071–0.312. The average *F*ij for the populations of three *Dactylorhiza* taxa showed significantly positive values, which were observed in the short-distance classes (1-10m (20 m)). F_{ST} values were significant in each *Dactylorhiza* taxon, ranging from the lowest values in *D. fuchsii* and *D. majalis* (0.080-0.086, $p < 0.05$), and the higher value (0.163, $p < 0.05$) in *D. incarnata* var. *incarnata*. The molecular variance was highest within populations (AMOVA: 76.5-86.6%; $p < 0.001$). Conclusions: We observed the concordant genetic diversity patterns in food-deceptive, allogamous and pollinator-dependent, although also self-compatible with the mix-mating system three *Dactylorhiza*. In our survey, F_{IS} is often substantially higher than *F*ij at the first class of SGS, suggesting that selfing (meaning of geitonogamy) is at least responsible for homozygosity. The strong SGS may have additional and unexplored evolutionary consequences in *Dactylorhiza*, and combined with low inbreeding depression, may strongly influence establishing the inbred lines in the case of *D. majalis* and *D. incarnata* var. *incarnata*.

Keywords: *Dactylorhiza fuchsii*, *Dactylorhiza incarnata* var. *incarnata*, *Dactylorhiza majalis*, F_{IS}, spatial genetic structure

1. Introduction

The mating system affects plant population genetic structure by modifying the drift/migration equilibrium characterized by the effective size of a population [1,2]. The mating system is also related to inbreeding in a complex way. Theoretical and experimental studies often supported that pollen transfer in outcrossers leads to lower genetic structure and higher genetic diversity compared to selfers resulting from a strong founder event, which increases the inbreeding [2,3,4]. However, pollen flow may act in concert with typical life-history traits related to dispersal mechanisms, such as seeds dispersed by wind or animals, and they can show together high power to predict the magnitude of FSGS within populations [5,6]. When pollen and seed dispersal are limited, resulting in strong intra-population structure, biparental inbreeding can also affect F_{IS}. Therefore, the strong fine spatial genetic structure (FSGS) is frequent even within allogamous or potentially allogamous plants following an isolation-by-distance model [6]. The higher levels of fine-spatial genetic structure (FSGS) have also been highlighted for selfing and clonal species and in low-density populations [6,7,8]. Variations in mating systems and different edaphic and climatic may additionally translate into substantial differences in FSGS conditions [9]. F_{IS} also reflects inbreeding in previous generations of perennials, resulting in the Wahlund effect on the population [10]. Mating system and seed dispersal also influence F_{ST} via its effect on pollen-mediated and short and/or leptokurtic gene flow and effective population size, especially in selfing and mating between relatives by increasing inbreeding,

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which enhances genetic drift. A summary report by Duminil et al. [11] based on data from 263 plant species revealed that the F_{IS} observed at the adult plant stage allows testing of the impact of both biparental inbreeding and inbreeding depression on population genetic structure. In the earliest stages of the plant life cycle, inbreeding depression mainly affects inbred progeny. Therefore, the F_{IS} of adult plants includes information on the selfing rate and inbreeding depression.

In Orchidaceae, the mating system and gene flow within and among populations can generate common genetic diversity patterns and FSGS [12]. First, pollinator-mediated gene flow among populations, e.g. is higher in deceptive than in rewarding orchids [13,12]. The deceived pollinators typically visit only a few flowers among plants within populations, thereby promoting cross-pollination and reducing the likelihood of inbreeding [14,15,16,17,19]. Therefore, it can be hypothesized that FSGS of these orchids is weak. In contrast, in rewarding ones, within-population genetic structure could be stronger due to geitonogamy and mating among close relatives. However, based on earlier orchid studies, the dispersal of dusty-like seeds seems to be limited [e.g. 20,21,22,23,24,25]. These results agree with studies that have investigated FSGS both of deceptive orchids e.g. *Caladenia tentaculata* [26], *Cephalanthera longibracteata* [22], *Orchis cyclochila* [23], *Orchis purpurea* [27], and *Orchis mascula* [28,29], *Cymbidium goeringii* [30] and rewarding ones e.g. *Gymnadenia conopsea* [31], *Pogonia ophioglossoides* [32], *Epipactis thunbergii* [33]. Moreover, orchid germination success has been reported to be higher in the vicinity of mother plants because a mycorrhizal could favour the establishment of seedlings [34,35].

In this study, we focused on *Dactylorhiza* taxa, which are food-deceptive orchids with no rewards for their pollinators [36]. This genus can be considered as a model one due to plant-pollinator interactions, natural selection, and consequent female reproductive success and their impact on genetic structure in food-deceptive plant groups [37,38,39]. In this context, the mating system and ID in food-deceptive *Dactylorhiza majalis*, *Dactylorhiza incarnata* var. *incarnata*, and *Dactylorhiza fuchsii* populations from NE Poland have been documented in detail comprehensively [40,41,4243]. A mixed mating system was observed in all three studied *Dactylorhiza* taxa similar to Hedrén and Nordström study [44]. Ostrowiecka et al. [40] found that pollinator behavior in *D. majalis* likely promotes geitonogamy, explaining the development of selfed seeds in fruits at different levels of the inflorescence with germination potential similar to that of outcrosses within populations. Vallius et al. [45] and Hedrén and Nordstöm [44] argued that different *D. incarnata* varieties maintain a high level of inbreeding, and populations might consist of several inbred lines that were fixed for characters such as flower colour, leaf shape, and leaf spot. Wróblewska et al. [42] results corroborate with previous studies on *Dactylorhiza*, concerning the low or medium level of fruit set ranged from 7.4% to 77.5% [36,46,45]. In vitro experiments revealed that seed germination of three *Dactylorhiza*taxa both from natural pollination and hand-treatments (selfing and outcrossing) shaped at a relatively low level, up to 35% (exception of *D. fuchsii*, outcrossing experiments) [42]. The *in vitro* asymbiotic seed germination was similar or slightly higher in selfing than crossing experiments in *D. incarnata* var. *incarnata* and *D. majalis*, while it was reversed in *D. fuchsii* [42]. Spontaneous autogamy in three *Dactylorhiza* taxa existed in < 1% of pollination in the studied populations and most likely did not affect reproductive success [47,48]. Taxa are assumed to be terrestrial, long-lived, self-compatible, and tuberous perennial orchids that reproduce either by seeds or (rarely) vegetatively [49,40]. Pollination occurs by different taxonomical groups of insects (Hymenoptera, Diptera, and/or Coleoptera, mostly bees and bumblebees) [40,41]. Molecular markers such as cpDNA (trnL, trnF and psbC–trnK), internal transcribed spacer (ITS) sequences, and flow cytometry data confirmed the taxonomic status of the studied three orchids [41].

Based on estimates of earlier ecological survey, e.g. natural fruit set, mix-mating system, inbreeding depression from controlled crosses treatment and of studied orchid taxa from NE Poland [40,42,43] and genetic report of Hedrén and Nordstöm [44], Naczk et al. [49] we tested the hypotheses that that inbreeding coefficient is shaped at a high level the food-deceptive orchids *D. majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii*. We also assumed that seed dispersal is mainly a short distance in orchids close to the mother plant, as was observed by many authors who researched experimentally seed dispersal; therefore, the fine-scale genetic structure is stronger due to the effect

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of inbreeding and short-distance dispersal. Finally, the purpose of this study is (1) to estimate the inbreeding coefficient and the intensity of FSGS using AFLP markers, (2) to discuss how similar mating systems and different ID shape genetic diversity patterns of three food-deceptive *Dactylorhiza* taxa.

2. Materials and Methods

2.1. Study sites

The present study was performed from May to July in 2014 to 2017 in three *D. majalis* populations (KA, SKI, and SKII), three *D. incarnata* var. *incarnata* populations (ZB, RO, and MR), and three *D. fuchsii* populations (BR, CM, and GR) located in northeastern Poland (Figure 1). *D. majalis* grows in wet meadows with abundant, entomophilous, and rewarding plants. The study sites differed in the abundance of *D. majalis* individuals, from *ca*. 120–200 flowering individuals in SKI and SKII, to *ca*. 1,000 flowering individuals in KA. All meadows were extensively used, mown every year in late July or early August, and not artificially fertilized. Three populations of *D. incarnata* var. *incarnata* had similar sizes (MA, *ca*. 68–100 flowering plants; ZB, *ca*. 30–100 flowering plants; and RO, *ca*. 35–200 flowering plants; Figure 1). The populations in the Biebrza Valley and Rospuda Valley occupied sedge communities with a low cower of the herb layer by rewarding plant species (*ca.* 10%). *D. fuchsii* was observed in open hornbeam forests, with a low number of rewarding plants, in the Białowieża Primeval Forest and its vicinity (CM and BR, population sizes *ca*. 84–133 flowering plants). One *D. fuchsii* population (GR, population size *ca*. 140–193 flowering plants) was located in the Biebrza Valley [42].

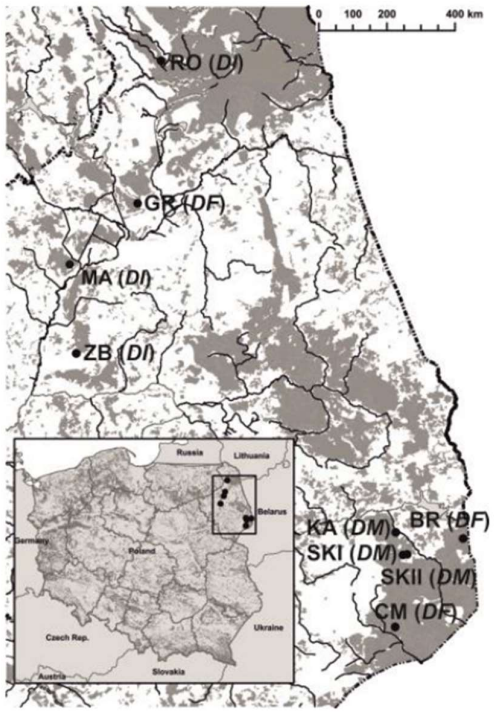

Figure 1. Localities of nine *Dactylorhiza* populations in north-eastern Poland. *D. majalis* (*DM*), KA, SKI, and SKII; *D. incarnata* var. *incarnata* (*DI*), ZB, MR, and RO; *D. fuchsii* (*DF*) CM, BR, and GR.

The study was based on samples comprising 455 individuals representing nine populations of three *Dactylorhiza* taxa (162, 129 and 164 individuals of *DM*, *DI* and *DF*, respectively; Table 1; Figure 1). Even though *Dactylorhiza* rarely regenerates clonally, one leaf sample was taken from a single shoot distance of 1 m from one another within each population to avoid the effects of the population

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substructure. In all populations each sample was mapped in the grid coordinate system, using a hand-held GPS (Garmin GPSMAP 65s) to calculate the distance between samples.

2.2. AFLP analysis

Genomic DNA was extracted from dry leaf tissues with the Genomic Mini AX Plant kit (A & A Biotechnology, Poland), and then samples were genotyped for AFLP markers. The AFLP procedure described by Vos et al. [50] was modified according to the Applied Biosystems protocol (AFLP™ Plant Mapping). First, 12 primer pair combinations were tested on four selected samples from each *Dactylorhiza* taxon. The fluorescence-labeled selective amplification products were mixed with a 500 Liz-labeled size standard (Applied Biosystems) and run on an ABI 3130. From this analysis, we chose seven primer combinations that produced polymorphic, clear, reproducible fragments of homogeneous intensity in three *Dactylorhiza* taxa (*D. majalis* *Eco*R1-ACC/*Mse*I-CAG; *Eco*R1-AGG/*Ms*eI-CAC; *D. incarnata* var. *incarnata* EcoR1-ACA/MseI-CAG; EcoR1-ACA/MseI-CTA; *D. fuchsii* *Eco*R1-AGG/*Mse*I-CAG, *Eco*R1-ACC/*Mse*I-CAT, *Eco*R1-ACC/*Mse*I-CTA). Variable fragments in the 70–500 bp size range were scored as present (1) or absent (0) using Genemapper 4.0 (Applied). To test the repeatability of the AFLP results, three individuals from each population were completely replicated, starting from the restriction/ligation reaction of the AFLP. Potential resampling of clones was checked with the AFLPdat R-script but was insignificant, thus, not corrected for.

To assess the levels of genetic diversity, the proportion of polymorphic fragments (PL_{poly}) and Nei's gene diversity (H) were calculated using the Bayesian method with a nonuniform prior distribution of allele frequencies proposed by Zhivotovsky [51], as implemented in AFLP-Surv ver. 1.0 [52]. The F statistic was determined by analysis of the molecular variance (AMOVA) using the program Arlequin 3.11 [53]. The significance of the variance components was determined using 1,000 independent permutation runs.

The FSGS was described by conducting spatial autocorrelation analysis using the pairwise kinship coefficient F_{ij} for dominant markers [54]. Mean F_{ij} estimates over pairs of individuals for given distance classes were calculated and plotted against distance on a logarithmic scale using the software SPAGeDi 1.4 [54,6]. Separate distance classes (m) were created for each population of *D. majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii* due to different patterns of plant distribution in space. To test the significance of FSGS, the regression slopes (b) of kinship coefficients and the

natural logarithm of distance were compared with the slopes obtained for permutations of individual genotypes (10,000 random permutations). The extent of FSGS was quantified using the S_P statistic proposed by Vekemans and Hardy [6] and calculated as $S_P = -b / (1 - F_1)$, where b is the regression slope and F_1 is the average F_{ij} between individuals. For each spatial distance class, the 99% confidence interval was computed using 1,000 permutations (with SPAGeDi) [55]. The probability value (P) was computed for each spatial distance class and coefficient.

To investigate the F_{IS}, the Metropolis–Gibbs algorithm was applied to I4A software based on dominant markers [56]. The data were run using the prior values of beat-distribution equal to $\alpha = \beta = 1.0$ (corresponding to an 'uninformative' flat distribution) and 60,000 repetitions, including a 10,000 step burn-in.

3. Results

Overall, 193, 215, and 263 polymorphic bands were scored in *D. majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii*, respectively. Considering the error rates (2%, 1.3%, and 1.5%, respectively), none of the samples may have represented clones.

A relatively high proportion of polymorphic fragments (P = 40.4.5–68.4%) and Nei's gene diversity indices (H = 0.140–0.234) were detected among the three orchid species (Table 1). The overall F_{IS} was relatively low-to-moderate and equaled 0.071–0.224 in *D. incarnata* var. *incarnata*, 0.079–0.134 in *D. fuchsii*, and reached the highest values of 0.192–0.312 in *D. majalis*.

Correlograms of the average F_{ij} values for the populations of three *Dactylorhiza* taxa showed significantly positive values, which were observed in the short-distance classes. In *D. majalis* the relatives were noted at a distance from 1 m to 10 m (Figure 2a), and the values were significantly

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negative at the longer distance classes (52–72 m) in the two other populations. Similar observations were made in two of the three populations of *D. incarnata* var. *incarnata* and *D. fuchsii.* Significant positive values were observed at a distance from 2 m to 20 m in *D. incarnata* var. *incarnata* (Figure 2b) and from 2 m to 10 m in *D. fuchsii* (Figure 2c). The b_F values for *D. majalis* (−0.051–0.009), *D. incarnata* var. *incarnata* (−0.055–0.002), and *D. fuchsii* (−0.026–0.002) were almost all significant (permutation test, $p < 0.05$) (Table 1). The highest *Sp* values were observed for *D. incarnata* var. *incarnata* (0.063) and *D. majalis* (0.056) (Table 1).

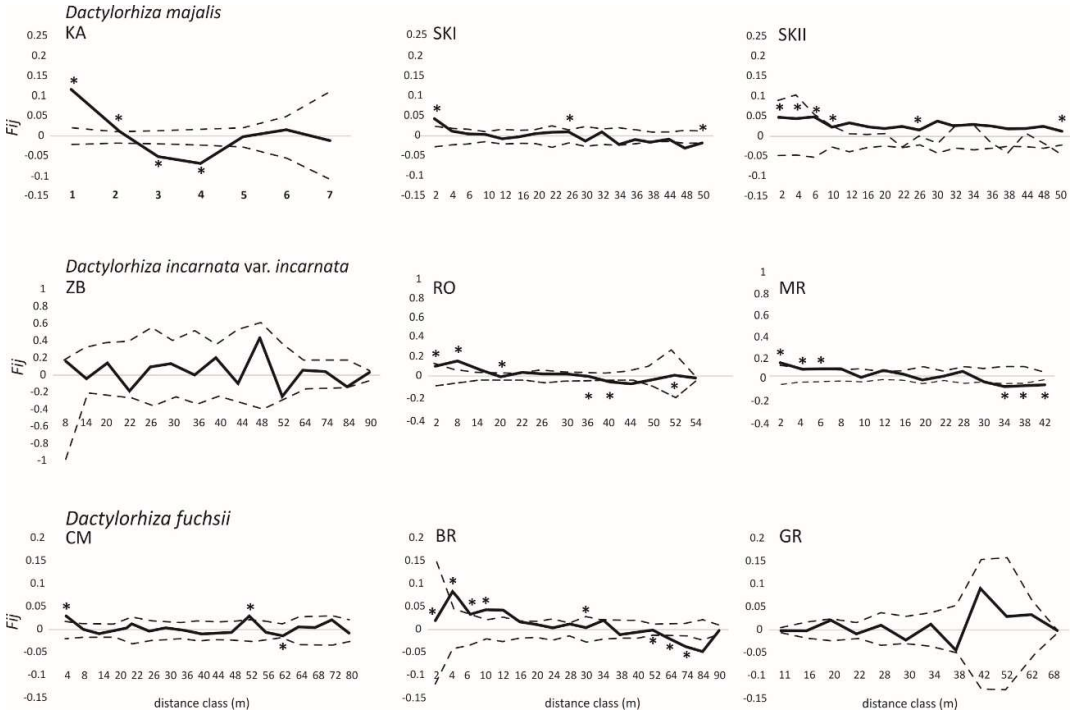

Figure 2. Spatial correlograms for *Dactylorhiza majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii* populations with mean pairwise kinship coefficients (F_{ij}) of distance classes for AFLPs around the hypothesis of random genetic structure obtained by permuting individual spatial locations as implemented in the SPAGeDi 1.4 [6]. The dotted lines indicate the 95% confidence intervals obtained from 10,000 permutations of genotypes). Codes of populations (KA, SKI, SKII, ZA, MR, RO, CM, BR, GR, see Table 1); * $p < 0.05$.

Table 1. Locations of *Dactylorhiza majalis* (*DM*), *D. incarnata* var. *incarnata* (*DI*) and *D. fuchsii* (*DF*) populations in NE Poland and summary statistic of the genetic diversity and spatial genetic structure estimated using SPAGeDi 1.4 (Veckemans and Hardy 2004). *N* – number of AFLP samples, *P*% - frequency of polymorphic loci, *H* –Nei's gene diversity, F_{IS} – inbreeding coefficient, CI - the upper and lower 95% confidence interval values, $F_{ij(1)}$ – mean pairwise kinship coefficient among individuals at the first distance class, b_1 - regression slope of pairwise kinship at the first distance, *Sp* – the intensity of FSGS according Veckemans and Hardy [6]. Vouchers specimens have been collected by Ada Wróblewska and deposited in the herbarium of the Faculty of Biology, University of Bialystok, Poland.

Taxa	Population	*GPS*	*N*	*P*%	*H*	F_{IS} (CI)	$F_{ij\,(1)}$	b_1	*Sp*
DM	KA	52°53'00''N 23°40'29''E	49	62.2	0.205	0.293 (0.000-1.000)	0.095*	-0.051*	0.056
	SKI	52°49'50''N 23°43'10''E	59	59.6	0.205	0.312 (0.000-1.000)	0.038*	-0.009*	0.001
	SKII	52°49'50''N 23°43'10''E	54	40.4	0.140	0.192 (0.000-1.000)	0.071*	-0.021*	0.022

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DI	ZB	53°29’02’’N 22°59’28’’E	48	58.6	0.217	0.179 (0.101-0.284)	0.008	-0.002	0.0002
	RO	53°54’39’’N 22°56’32’’E	48	58.6	0.197	0.071 (0.022-0.149)	0.224*	-0.055*	0.063
	MR	53°47’25’’N 22°57’22’’E	33	58.1	0.206	0.098 (0.032-0.218)	0.092*	-0.037*	0.041
DF	CM	52°41’03’’N 23°39’07’’E	58	68.4	0.234	0.113 (0.034-0.244)	0.078*	-0.021*	0.019
	BR	52°50’59’’N 23°53’40’’E	57	63.9	0.211	0.134 (0.068-0.226)	0.084*	-0.026*	0.028
	GR	53°60’68” E 22°84’68”N	49	56.7	0.197	0.079 (0.024-0.169)	-0.008	0.002	-0.0002

Almost all F_{ST} values were significant in each *Dactylorhiza* taxon, ranging from the lowest values in *D. fuchsii* and *D. majalis* (0.080 and 0.086, $p < 0.05$, permutation test), and the higher value (0.163, $p < 0.05$, permutation test) in *D. incarnata* var. *incarnata* (Table 2). The amount of molecular variance was highest within populations and was maintained at relatively higher and similar levels in *D. majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii* (AMOVA: 76.5%, 85.5%, and 86.6%; $p < 0.001$, respectively).

4. Discussion

The associations of mating system, inbreeding depression and biparental inbreeding with F_{IS} and F_{ST} have been confirmed sparsely in plant surveys ([11], and literature herein). Baskin and Baskin [57]) summarized the effects of inbreeding depression on seed germination in 743 cases of 233 species in 64 families. They demonstrated that in 50.1% of cases, inbred and outbred seeds germinated at a similar frequency, and 8.1% of inbred seeds germinated better than outbred seeds. Notably, the authors did not find a strong relationship between the decrease in germination and an increase in F_{IS} or between an increase in germination and an increase in population genetic diversity. However, we observed the concordant genetic diversity patterns in food-deceptive, allogamous and pollinator-dependent, although also self-compatible with the mix-mating system *D. majalis*, *D. incarnata* var. *incarnata*, and *D. fuchsii*. Genetic diversity within studied *Dactylorhiza* populations was shaped at a relatively high level comparable with the data reported by Naczk et al. [49] as well Hedrén and Nordström [44,58], suggesting that studied *Dactylorhiza* populations can be found by multiple, genetically diverse individuals and/or by gene flow (leptokurtic dispersal) from the surrounding populations. The genetic differentiation among them was low and significant (0.080-0.163), showing that gene flow (historical) in NE Poland was relatively high or population were established from one source. However, the isolation processes of these *Dactylorhiza* population were observed, leading to the formation of substructure.

Furthermore, the inbreed was shaped on moderate to high levels in three *Dactylorhiza* taxa similar to studies Hedrén and Nordström [44], Filippov et al. [59] and Naczk et al. [49]. Meanwhile, in *D. majalis* as the allotetraploid, the F_{IS} has a wide range of values in populations reported by Balao et al. [59], Hedrén and Nordström [44] and Naczk and Ziętara [61]. If we predict that inbreeding is solely the result of mating among neighboring plants, we will expect F_{IS} to be approximately equal to Fij at the smallest distance interval in studied *Dactylorhiza* populations [6]. In our survey, F_{IS} is substantially higher than Fij at the first class of spatial distance in the majority of *Dactylorhiza* populations, suggesting that selfing is at least partially responsible for homozygosity [56,62]. However, the spontaneous autogamy in three *Dactylorhiza* taxa existed until 1% of pollination in the studied populations [47,48]. Hence, the only explanation of selfing in three *Dactylorhiza* taxa is the pollinator behaviour of bumblebees and other pollinators, which were known to promote geitonogamy and/or autogamy, explaining the development of selfed seeds in fruits [40,41]. The important factor shaping F_{IS} was the slightly higher frequency of selfed than outcross seeds germinated *in* vitro treatments in *D. majalis*, and *D. incarnata* var. *incarnata*, while in *D. fuchsii*, the germination pattern was reversed [42]. This phenomenon suggested that inbred and outbred *D. majalis*, and *D. incarnata* var. *incarnata* seeds germinated at a similar or even slightly higher frequency. We stress the careful interpretation of the relationship between seed germination and F_{IS}. This needs to be confirmed by further studies, including the following stages of growth and mortality

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observations of plants germinated from selfed and outcross seeds. However, these data were shown for a single studied *Dactylorhiza* species; we can suppose that in *D. fuchsii,* a similar pattern existed in two out of three populations like in *D. majalis* and *D. incarnata* var. *incarnata*. In the CM and BR populations, high inbreeding and slightly lower kinship coefficient supported the possibility of selfing (geitonogamy). The interesting question is whether biparental inbreeding can exist in food-deceptive *Dactylorhiza* taxa, even though pollinators spend a short time on flowers and inflorescence and learn to avoid the deceptive flowers. They typically visit probe fewer flowers per plant only and/or a few flowers between inflorescences within populations, promoting cross-pollination and skipping more plants between plant visits. In the light of this outcrossing hypothesis [15], the biparental inbreeding is rather unlikely. Ostrowiecka et al. [40] observed using videotaping in *D. majalis* that *A. mellifera* visited three to five flowers on the same inflorescence over a period of 11 s to 40 s contributing to geitonogamy. We observed that *A. mellifera* pollinators never repeatedly returned to the same flowers and never visited all the flowers on the inflorescences. On the other hand, pollinaria bending is a mechanism that prevents geitonogamy and biparental inbreeding between individuals, especially in proximity. In *Dactylorhiza,* the mean bending is 39–54 s, considered a relatively long time for deceptive plants and similar to other deceptive *Dactylorhiza* taxa [63]. The banding time span in each studied *D. majalis* population ranged from 8 s to 2 min 5 s [40]. This short bending time can likely provide an opportunity for geitonogamy. This phenomenon and the bending times in the studied populations support our hypothesis that we cannot completely exclude the possibility of geitonogamy than biparental inbreeding in deceptive orchids. *Dactylorhiza* appears to have a more generalised pollination system and many pollinator were described and studied in detail. These pollinators can spend different time on the flowers promoting geitonogamy.

However, hand pollination treatment with emasculated flowers was also used as the level of apparent selfing in plants [64,65]. A previous fruit set observation from control pollination in three *Dactylorhiza* documented a moderate level of fruit set (35.4-40.5%), while the emasculation experiments in their populations showed a significant decrease in fruiting between these treatments (*D. majalis*, 28.2% fruit set from emasculated flower, paired $t = 2.68$, $df = 8$, $p < 0.002$; *D. incarnata* var. *incarnata*, 14.6% fruit set from emasculated flower, paired $t = 3.46$, $df = 10$, $p < 0.006$; *D. fuchsii*, 28.2% fruit set from emasculated flower, paired $t = 4.83$, $df = 10$, $p < 0.0007$; Wróblewska et al. unpublished data). This study concludes that selfing in three *Dactylorhiza* occurs mainly through geitonogamy. Kropf and Renner [18] have also pointed out the high levels of geitonogamous pollination in *Dactylorhiza*. In this fact, measuring biparental inbreeding can be challenging in deceptive plants.

The F_{IS} observed at the adult stage allowed testing of the impact of inbreeding depression on the population's genetic structure. In long-living plants, F_{IS} reflects inbreeding not only in the current generation but also in previous overlapping generations. However, other factors, such as the long lifespan of plants, can affect inbreeding depression [11]. In *D. majalis*, the selfing (e.g., geitonogamy) and/or progeny, and likely seed dispersal very close to the mother plant, can manifest most strongly in a spatial genetic structure. In the case of *D. majalis*, the results of the present study are inconsistent with those of Husband and Schemske [66], who concluded that purging is a significant evolutionary force in natural populations. Without actually reducing the genetic load, such fixation could reduce inbreeding depression [66,67]. However, inbreeding depression may be lower in long-standing populations with inbreeding than in populations with outcrossing populations where selection may have purged the genome of its genetic load [68,66,67,69]. These two alternative approaches should be tested in a laboratory at a later stage of the life cycle of *D. majalis*, such as in seedlings and adult reproductive individuals. The selfing (meaning of geitonogamy) and the strong fine-scale genetic structure may have additional and unexplored evolutionary consequences in *Dactylorhiza*, and combined with low inbreeding depression may strongly influence to establish the inbred lines in the case of *D. majalis* and *D. incarnata* var. *incarnata*. Today, we cannot state in *Dactylorhiza* that inbreeding depression may be widely viewed as the primary selective factor allowing transition to complete selfing.

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