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Genetic and morphological variation of two local allotetraploid orchids, *Dactylorhiza baltica* and *D. ruthei*

Aleksandra M. Naczk[1,*], Marek S. Ziętara[1], Dan Wołkowycki[2], Marcin Wilhelm[3] & Ekaterina Zheleznaya[4]

[1]Department of Evolutionary Genetics and Biosystematics, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, PL-80308 Gdańsk, Poland; [2]Department of Forest Environment, Institute of Forest Sciences, Białystok Technical University, Wiejska 45A, PL-15351 Białystok, Poland; [3]Institute of Marine and Environmental Sciences, University of Szczecin, Wąska 13, PL-71415 Szczecin, Poland; [4]Dmitrovskiy Proyezd 20-1-151, RU-127206 Moscow, Russia
*corresponding author: aleksandra.naczk@ug.edu.pl

Abstract: *Dactylorhiza baltica* (Klinge) Nevski and *D. ruthei* (M. Schulze ex Ruthe) Soó are taxa of unclear taxonomic status and often distinguished based on a few morphological characters. Their classification as distinct species or infraspecific taxa is problematic and author dependent. More than 300 specimens from nine populations of *D. baltica* and 10 of *D. ruthei* were analysed. Three data sets were used (36 morphological characters, 10 plastid DNA markers and five nuclear microsatellites) to assess the difference between *D. baltica* and *D. ruthei*. Both taxa are distinct young allopolyploids, but share the same genetic inheritance and ploidy level, which means that they are not genetically uniquely defined, but can be identified using a full set of diagnostic morphological traits. They are the result of common evolution from the same pair of parental species with a common evolutionary and post-glacial history, but arose independently at a similar time in the region of the Baltic Sea. Therefore, the observed patterns of genetic variation and quite distinct differences in morphology, as well as the common mechanisms of origin and evolution of these allopolyploid taxa, provide arguments for assigning the same taxonomic status to both *D. baltica* and *D. ruthei*. It is postulated that populations that have integrated within the same allopolyploid gene pool and that appear to be historically related to each other should be considered infraspecific taxa, within the *D. majalis* s.l. group as *D. majalis* subsp. *baltica* (Klinge) H. Sund. and *D. majalis* subsp. *ruthei* (M. Schulze ex Ruthe) H. Kretzschmar, respectively.

Keywords: allotetraploids, cpDNA, *Dactylorhiza baltica*, *Dactylorhiza ruthei*, genetic diversity, haplotype, morphology

Introduction

Most of the commonly known species of *Dactylorhiza* are included in the *Dactylorhiza incarnata/maculata* polyploid complex (Heslop-Harrison 1954, Averyanov 1990, Hedrén 1996). Several processes have modified the patterns shaped by primary polyploidization and have contributed to the high degree of morphological variation observed in this complex (Hedrén et al. 2012b). These processes include multiple origins of single

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species/subspecies from similar sets of parental species, secondary hybridization and backcrossing with parental lineages and hybridization with already existing allopolyploids (Hedrén et al. 2008). Additional processes that have affected the allopolyploid genome include: concerted evolution of nrITS (Devos et al. 2006, Pillon et al. 2007), intergenomic recombination (Hedrén 1996, Otto 2007) and epigenetic modifications (Paun et al. 2007, 2010). The process of allopolyploidization is frequently repeated, but the evolutionary significance of its repetition is not yet fully understood. Particularly interesting are the dynamics of gene flow and the mechanisms that allow young sibling polyploids to remain distinct, but simultaneously sharing the same ploidy level, inheritance and overlapping areas of distribution (Balao et al. 2016). Locally divergent allotetraploids may have arisen in the same area where they occur today and any adaptive modifications that happened after their origin may relate to the different landscapes occurring north and south of the glacial maximum (Bateman 2011).

The most problematic taxa within this complex belong to *D. majalis* (Rchb.) P.F. Hunt & Summerh. group, which has evolved repeatedly by hybridization between two broadly defined parental lineages: *D. incarnata* (L.) Soó s.l. – considered to be the paternal lineage and *D. maculata* (L.) Soó s.l. the maternal lineage (Hedrén 1996, 2001a, b, 2002, 2003, Hedrén et al. 2001, Devos et al. 2003, 2006, Ståhlberg & Hedrén 2008). Within the *D. majalis* group, older and younger allotetraploids can be distinguished (Pillon et al. 2007). Older allotetraploids are inferred to have passed through glacially induced migration bottlenecks in southern Eurasia, e.g. *D. majalis*, *D. elata* (Poir.) Soó, whereas at least some younger allotetraploids currently occurring in northern Europe are inferred to have originated post-glacially and remain sympatric with their parents, e.g. *D. baltica* (Klinge) Nevski, *D. praetermissa* (Druce) Soó, *D. purpurella* (T. et T. A. Stephenson) Soó, *D. traunsteineri* (Saut. ex Rchb.) Soó, *D. sphagnicola* (Höppner ex Soó) Aver. (Pillon et al. 2007, Brandrud et al. 2020). All the above species have unique morphological characters, spatial distributions and ecological preferences (Paun et al. 2010, Hedrén et al. 2011, Wolfe et al. 2023). Moreover, each of these species had a distinct origin and may have inherited some components of somewhat contrasting ecological preferences of their slightly different ancestral lineages (i.e. *D. incarnata* s.l. and *D. maculata* s.l. as parental lines). This situation has contributed to an ongoing taxonomic debate as to whether the numerous allopolyploid lineages should be treated as a single aggregate species, or as subspecies of *D. majalis* s.l., due to the fact that they are all derived from the same parental diploid species (Hedrén 2002, Hedrén et al. 2008), or whether they should be treated as separate species, because each lineage has a distinct and unique evolutionary origin, and these species may have slight or even marked differences in their ecological tolerances (Bateman 2006, 2011, 2022).

Dactylorhiza baltica (Klinge) Nevski and *D. ruthei* (M. Schulze ex Ruthe) Soó are examples of allotetraploids of post-glacial origin that evolved from crosses between two diploid parental taxa – *D. incarnata* s.s. and *D. maculata* subsp. *fuchsii* (Hedrén et al. 2001, 2012b, Shipunov et al. 2005, Brandrud et al. 2020). Both have a chromosome number of 2n = 80 (for *D. ruthei* the only study on its chromosomes is Jagiełło et al. 1989 and for *D. baltica* Averyanov 1983 and Jagiełło et al. 1989) and are not only somewhat morphologically similar, but also grow in similar habitats. Most authors consider *D. baltica* as a separate species (e.g. Senghas 1968, Averyanov 1990, Baumann 2005, Delforge 2006), or subspecies of the aggregate species *D. majalis*, i.e. *D. majalis* subsp. *baltica*

(Klinge) H. Sund. (Pedersen & Faurholdt 1997, Pedersen & Hedrén 2010, Eccarius 2016, Kühn et al. 2019). Morphological characters used to distinguish *D. baltica* differ among authors. However, most descriptions mention long, gradually narrowing leaves, spotted on the upper surface, light purple flowers in a short inflorescence, relatively broad three-lobed lips with a smaller middle lobe, and visible markings on the lip (Supplementary Fig. S1). *Dactylorhiza baltica* usually grows in wet meadows and peat bogs of the *Scheuchzerio-Caricetea nigrae* class, on fertile but calcium-poor soils, often near the coast. It occurs around the Baltic Sea, in the eastern part of Germany, Poland, Estonia, Latvia, Scandinavia and Russia (Bernacki 1990, Shipunov et al. 2005).

In turn, *D. ruthei* is a taxon with a still unclear taxonomic status. It was first described by Rudolf Ruthe from Świnoujście (Swinemünde), on Uznam (Usedom) island (Ruthe 1897). Due to its morphological similarity to *D. baltica*, it is considered to be a subspecies (Bernacki 1989) or local variety (Szlachetko 1993). However, some authors recognize *D. ruthei* as an independent species (Senghas 1968, Bernacki 2001, Baumann 2005, Eccarius 2016) or mention it in the context of similarity to *D. majalis* subsp. *elatior* (Fr.) Hedrén et H. A. Pedersen from Gotland (Kühn et al. 2019), or even indicate it as a subspecies of *D. majalis*, i.e. *D. majalis* subsp. *ruthei* (M. Schulze ex Ruthe) Soó (Kretzschmar 2008). Both taxa are usually distinguished by only a few morphological characters. *Dactylorhiza ruthei* is mainly recognized by having unspotted leaves and paler-coloured flowers than *D. baltica* (Supplementary Fig. S1). It is also characterized by pale green, raised, elongated, and unspotted leaves, pale pink to violet flowers, a clearly three-lobed lip with small spots or a pattern consisting of lines or lack ornamentation. Until the mid-20th century, it was considered endemic to Wolin and Uznam (Pawłowska 1972). The later recording of additional localities in Estonia (Kuusk 1984, 1994), in the west (Szlachetko 1993, Bernacki 2001), as well as in north-eastern Poland (Bernacki 1989, 1998) and Slovakia (Vlčko et al. 2003) changed this status. Its current range includes the coasts and lakeshores of the southeastern Baltic Sea, in Germany, Poland, Latvia and Estonia (Kuusk 1994, Bernacki 1998, Presser 2002), where it grows in wet meadows, coastal rushes, and in fens and peat bogs, mainly in river valleys.

The taxonomic status and classification of these two taxa as distinct species or infraspecific taxa has yet to be clearly established. In this study, different data sets (morphology, plastid DNA markers and nuclear microsatellites) were combined in order to assess whether the differences between the recent allopolyploids sharing the same genetic inheritance and ploidy level, *D. baltica* and *D. ruthei*, is sufficient to be able to distinguish between them.

Material and methods

Plant material

The material was collected from the following areas (with numbers of populations studied in parentheses): Estonia, EST (2); Germany, GER (4); Latvia, LAT (1); Poland, POL (11); and Russia, RUS (1) (Fig. 1). The exact locations of the sites and sample sizes for each data set are given in Supplementary Table S1. All specimens were identified either as *D. baltica* (nine populations, 165 individuals) or *D. ruthei* (10 populations, 157 individuals).

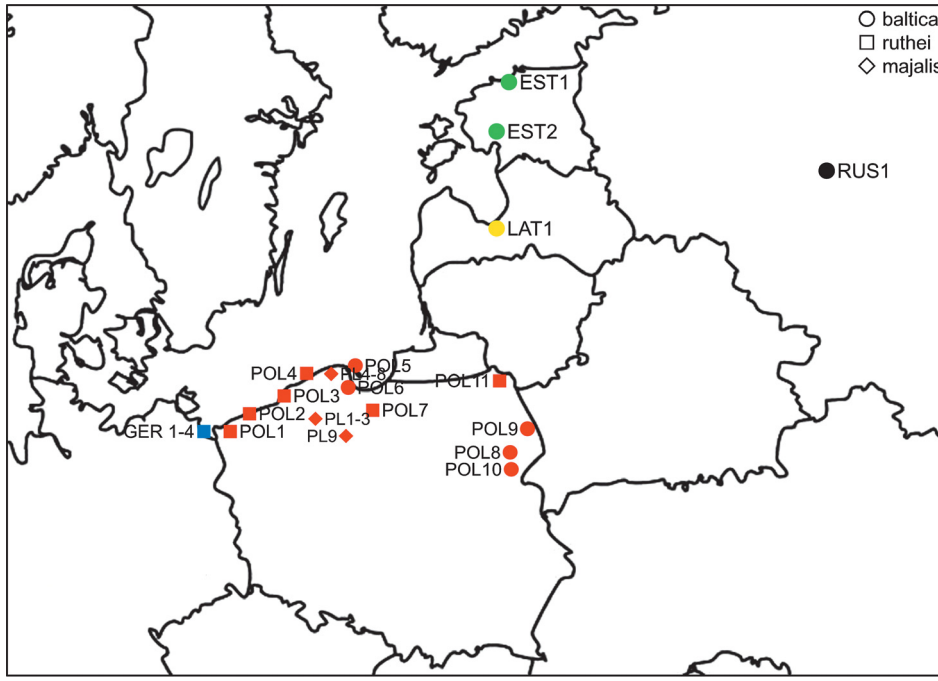


Fig. 1. Geographic distribution of the 19 populations of *Dactylorhiza baltica* and *D. ruthei* studied, as well as additional populations of *D. majalis*. Codes correspond to the localities given in Supplementary Table S1. Symbols: *D. baltica* ○, *D. ruthei* □, *D. majalis* ◇, red colour – Polish, blue – German, yellow – Latvian, green – Estonian, and black – Russian populations.

For the morphometric study, the individuals selected were identified based on vegetative characters (without removing specimens from their sites) and floral characters (for this purpose, a single flower was collected from the central part of the inflorescence of each specimen, dried and its characters measured with the aid of a stereoscopic microscope). For the molecular studies, leaf fragments were mainly collected from specimens that had previously been selected for morphometric studies, as well as from additional samples, which were then dried in silica gel according to the procedure described by Chase & Hills (1991).

Morphology

Morphometric characters used in the present study are listed in Table 1. Twenty-six quantitative and qualitative floral (18) and vegetative (eight) characters and 10 indices were measured. Most of the characters were taken from Bateman & Denholm (1983) and Pedersen & Hedrén (2010). Morphometric data for individual specimens were recorded in terms of their mean values and standard deviations. Both taxa were compared for all the quantitative traits of both taxa (floral and vegetative) were compared using a t-test or Mann-Whitney U test, following the required assumptions of a normal distribution and homogeneity of variance.

Table 1. List of morphological characters and ratios measured in populations of *Dactylorhiza baltica* and *D. ruthei*. Characters slightly modified after Bateman & Denholm (1983) and Pedersen & Hedrén (2010) are marked with an asterisk. Dominant traits are given for characters A17, A18 and B26. The P-values are for the comparison between *baltica* vs *ruthei* revealed by a t-test or Mann-Whitney U test, according to the required assumptions. Significant differences are given in bold.

No.	Morphological character	*D. baltica* (mean±s.d.)	*D. ruthei* (mean±s.d.)	P-values (*baltica* vs *ruthei*)
A	Floral characters			
1	Labellum length from base of spur entrance to apex of central lobe	0.68±0.11	0.72±0.11	**0.008**
2	Labellum length from base of spur entrance to base of sinus	0.46±0.07	0.40±0.10	**<0.001**
3	Labellum length from base of spur entrance to apex of lateral lobe	0.59±0.08	0.51±0.11	**<0.001**
4	Labellum width	0.78±0.13	0.88±0.16	**<0.001**
5	Spur length from entrance to apex	0.72±0.10	0.77±0.10	**<0.001**
6	Spur width at entrance	0.21±0.06	0.23±0.06	0.167
7	Spur width halfway along length	0.18±0.05	0.19±0.05	0.782
8	Ovary length	0.92±0.16	0.94±0.15	0.290
9	Lateral sepal length	0.74±0.11	0.80±0.10	**<0.001**
10	Lateral sepal width	0.29±0.06	0.31±0.05	0.080
11	Dorsal sepal length	0.66±0.09	0.71±0.09	**<0.001**
12	Dorsal sepal width	0.24±0.05	0.29±0.05	**<0.001**
13	Lateral petal length	0.54±0.09	0.61±0.09	**<0.001**
14	Lateral petal width	0.20±0.06	0.25±0.06	**<0.001**
15	Bract length	1.57±0.38	1.62±0.20	**0.046**
16	Bract width	0.24±0.06	0.28±0.06	**<0.001**
17	Labellum colour, on a scale 0–3 (0 = white; 1 = light violet; 2 = violet; 3 = purple)	2–3	1–2	–
18	Labellum markings, on a scale 0–3 (0 = no markings; 1 = spots; 2 = dashes; 3 = spots and dashes)	2–3	0–1	–
B	Vegetative characters			
19	Plant height	47.9±10.8	30.9±7.75	**<0.001**
20	Inflorescence length	9.04±3.15	7.26±2.51	**<0.001**
21	Number of flowers	39.9±20.1	25.6±10.2	**<0.001**
22	Number of sheathing leaves	4.23±0.91	3.75±1.02	**0.002**
23	Number of non-sheathing leaves	1.89±0.96	1.55±0.77	**0.004**
24	Length of longest sheathing leaf	17.6±3.50	13.3±3.13	**<0.001**
25	Width of longest sheathing leaf	2.42±0.65	2.28±0.73	0.067
26	Sheathing leaf markings, on a scale 0–2 (0 = no markings; 1 = bright spotting; 2 = dark spotting)	1–2	0–1	–
C	Indices			
27	Roundness of labellum; A1/(A1+A4)	0.47±0.05	0.45±0.05	**<0.001**
28	Labellum shape index; 2 × A1/(A2+A3)	1.30±0.18	1.63±0.34	**<0.001**
29	Prominence of central lobe; A1–A3	0.09±0.09	0.21±0.13	**<0.001**
30	Spur tapering; A7/(A7+A6)	0.46±0.08	0.45±0.07	**0.011**
31	Length of bract to length of ovary; A15/A8*	1.87±1.37	1.75±0.30	0.474
32	Percentage of stem bearing flowers; 100 × B20/B19	20.5±17.1	23.6±5.79	**<0.001**
33	Inflorescence laxity; B21/B20	4.39±1.65	3.60±1.18	**<0.001**
34	Shape of longest leaf; B25/(B25+B24)	0.12±0.02	0.15±0.04	**<0.001**
35	Length of longest leaf to plant height; B24/B19*	0.38±0.11	0.44±0.10	**<0.001**
36	Length of longest leaf to its width; B24/B25*	7.53±1.60	6.26±2.10	**<0.001**

A symmetric matrix that quantified the similarities of pairs of data sets using the Gower's similarity coefficient was produced (Gower 1971). This matrix was used to calculate the principal coordinates (PCoA) and construct a minimum spanning tree. In order to indicate the relative contribution of each of the original variables to each component, standardization was carried out to normalize the variables studied, which was necessary due to the heterogeneous nature of the set of morphological characters selected for analysis. The principal components that contributed most to the recorded variation were then calculated, in order of decreasing importance. Three separate multivariate analyses (PCoA) were done, the first was based on measurements for individual *D. baltica* and *D. ruthei* plants, the second was done using measurements of specimens, but with additional individuals of *D. majalis* s.s. and the third based on mean values calculated for each analysed variables in each of the 26 populations studied. At his stage, additional data from nine populations of *D. majalis* s.s. were added to the matrix (Supplementary Table S1, S2; see Naczk & Ziętara 2019). *Dactylorhiza majalis* s.s. is an older allotetraploid with stable morphology and distinct features that differ from those of the two taxa studied, especially in the form and length of the leaves and shape of the lip. As expected, its addition to the multivariate analyses strengthened the differences between *D. baltica* and *D. ruthei*. This method of data analysis and interpretation has been repeatedly tested in studies on the genus *Dactylorhiza* (Bateman & Denholm 1983, Bateman 2001, Bateman et al. 2023) and has proved invaluable for assessing relationships among species and populations. A biplot of the linear discriminant analysis (LDA) was also produced, which was used to indicate the potentially diagnostic characters of the putative taxa.

Statistical calculations were performed using STATISTICA 9.1 (StatSoft Inc. 2010) and PAST 4.03 (Hammer et al. 2011).

Molecular methods

Total DNA was extracted from the samples of leaves following the method commonly known as 2× CTAB (Doyle & Doyle 1990). Variation in the nuclear genome was measured in terms of five microsatellite loci (ms3, ms8, ms10, ms11, and ms13), which were developed for *Dactylorhiza* by Nordström & Hedrén (2007). Descriptions of these markers, multiplex reaction and PCR conditions are given by Naczk et al. (2016). Ten size-variable plastid DNA loci (seven microsatellites and three loci with indel variation) were amplified with a set of *Dactylorhiza*-specific primers. Descriptions of these markers are reported by Hedrén et al. (2008) for general studies on plastid DNA variation in *Dactylorhiza*. All fragments were amplified in two multiplex reactions according to protocols described in Naczk et al. (2015) and the combined patterns in variation at all marker sites were designated as haplotypes. The PCR products from each reaction were mixed with appropriately sized standards to determine the exact size of the amplified fragments. All samples of the length-variable fragment analyses were run on a 3130 xl genetic analyzer (Applied Biosystems Inc.), and the size of each fragment was measured using Genescan and Genotyper 3.7 software (Applied Biosystems Inc.).

Analysis of nuclear microsatellites

The following parameters of genetic diversity were calculated: allele frequency, average number of alleles per locus (A), effective number of alleles per locus (A_e) and average gene diversity over loci (H_e). Because sample sizes differed among populations, an added measure of allelic richness (R_A) was computed using the rarefaction method. Fixation indices, F_{IS} (the inbreeding coefficient), were estimated based on Weir & Cockerham (1984) estimators. The statistical significance of F_{IS} was calculated by a permutation test across loci for each population ($P = 0.05$). The aforementioned statistics were calculated using the computer programs: GENEPOP 4.0 (Rousset 2008) and FSTAT 2.9.3.2 (Goudet 2001).

The Bayesian clustering method, implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000), was used to determine the genetic structure of the population and assign individuals to particular taxa (without prior information on population membership). Data were analysed using an admixture model, assuming uncorrelated allele frequencies developed by Falush et al. (2003). Twenty runs were conducted for all possible values of cluster number (K) up to K = 1–17, where all populations of *D. baltica* and *D. ruthei* were included. A burn-in length of 250,000 steps and a run length of 750,000 iterations were used in each run. The results were summarized on the Harvester web platform (Earl & von Holdt 2012), which implements the Evanno's method to estimate the most likely K value for the data (Evanno et al. 2005). An additional analysis was also done with additional individuals of *D. majalis* s.s. as reference material and genetic background (these were the same populations as in the morphological analysis; Supplementary Table S1, S2). As K values higher than three mainly resulted in further subdivision of already defined groups, with only marginal improvements in the test statistic, the data set was not examined for larger K values for the second clustering.

Pairwise-F_{ST} was used to compare the differentiation between all pairs of populations. The calculations were done for each pair of populations as well as the entire sample. The pattern in genetic differentiation between all the populations analysed was illustrated by a principal coordinate analysis (PCoA), which was done on the resulting matrix of genetic distances, using the computer program PAST 4.03 (Hammer et al. 2011).

The program BOTTLENECK 1.2.02 (Cornuet & Luikart 1997) was used to test for a recent reduction of effective population size, because alleles are generally lost faster than heterozygosity. As a consequence, populations that have recently undergone a bottleneck will show an excess of heterozygosity compared to the level expected from the number of alleles found in the sample if the population was in mutation-drift equilibrium. The significance of differences between excess and deficient genetic diversity was determined using the Wilcoxon test (1,000 permutations).

Analysis of plastid haplotypes

Based on the frequency of haplotypes, particular diversity statistics were calculated for the taxa studied: number of haplotypes recorded (A), indices of gene diversity (H) (Nei 1987), and average gene diversity over loci (π) (Tajima 1983, Nei 1987). In addition, haplotype richness (R_H) was calculated, which describes gene diversity, regardless of sample size. The computer programs: Arlequin 3.5 (Excoffier et al. 2005) and Haplotype Analysis 1.05 (Eliades & Eliades 2009) were used to calculate the above statistics.

The relationships between plastid haplotypes were illustrated by means of a median-joining network (Bandelt et al. 1999) in which the variants recognized at 10 loci investigated were treated as ordered characters according to fragment size and number of repeats. The network was developed in NETWORK 10.2 (Fluxus Technology 2020), with all options set as default. To summarize differentiation patterns among the multilocus haplotypes identified in the material studied, the average number of differences between haplotypes in each pair of populations was calculated. Genetic diversity of the populations and haplotypes studied was estimated based on genetic distance, utilizing pairwise-F_{ST} (Slatkin 1995). The resulting distance matrix was used to demonstrate phenetic relationships between haplotypes using non-metric multidimensional scaling (NMDS) and phenetic relationships between populations based on a principal coordinate analysis (PCoA). Data analyses were done using computer programs: Arlequin 3.5 (Excoffier et al. 2005) and PAST 4.03 (Hammer et al. 2011).

AMOVAs and geographic patterns

The hierarchical patterns in the distribution of genetic diversity in the total material, as well as, in each of the taxa studied, was described by means of analysis of molecular variance (AMOVA). Separate analyses were carried out for plastid and nuclear data. Levels of significance for populations were determined using a permutation test (10,000 permutations) and calculations were done in Arlequin 3.5 (Excoffier et al. 2005). The F_{ST} and the F_{IS} estimates derived from the AMOVA of nuclear microsatellites and the F_{ST} estimate derived from the AMOVA of plastid haplotypes were used to calculate the ratio between pollen and seed migration, according to the formula given by Ennos (1994): $[(1/F_{ST(nuclear)} - 1)\ (1 + F_{IS(nuclear)}) - 2(1/F_{ST(plastid)} - 1)] / (1/F_{ST(plastid)} - 1)$.

In addition, the relationships between geographic distances (in km) and pairwise estimates of population differentiation derived from nuclear microsatellites and plastid haplotypes (Supplementary Tables S3, S5) were tested using a Mantel test and PAST 4.03 (Hammer et al. 2011), with significance determined by permutation tests.

Results

Morphological variation

Characters that may be useful to delimit *D. baltica* from *D. ruthei* were identified ($P < 0.05$; Table 1 and Supplementary Table S2). Statistical tests revealed that the taxa differed significantly from each other in most floral traits measured, e.g. in labellum length and width (the lip of *D. ruthei* is wider and has a longer midlobe), and for almost all vegetative traits, they are generally larger in *D. baltica* than *D. ruthei*.

Particular data sets were tested using PCoA analysis, e.g. only quantitative traits (vegetative and floral), only floral characters, quantitative and qualitative traits, but without indices. Finally, it was decided to use all characters together (all quantitative and qualitative traits for floral and vegetative characters, as well as 10 indices), as they best separated the taxa studied. In the PCoA analysis based on measured characters at the individual plant level there was a slight overlap in the morphological variation of the two taxa (Fig. 2A). The first two principal coordinates accounted for 26% of the total variation.

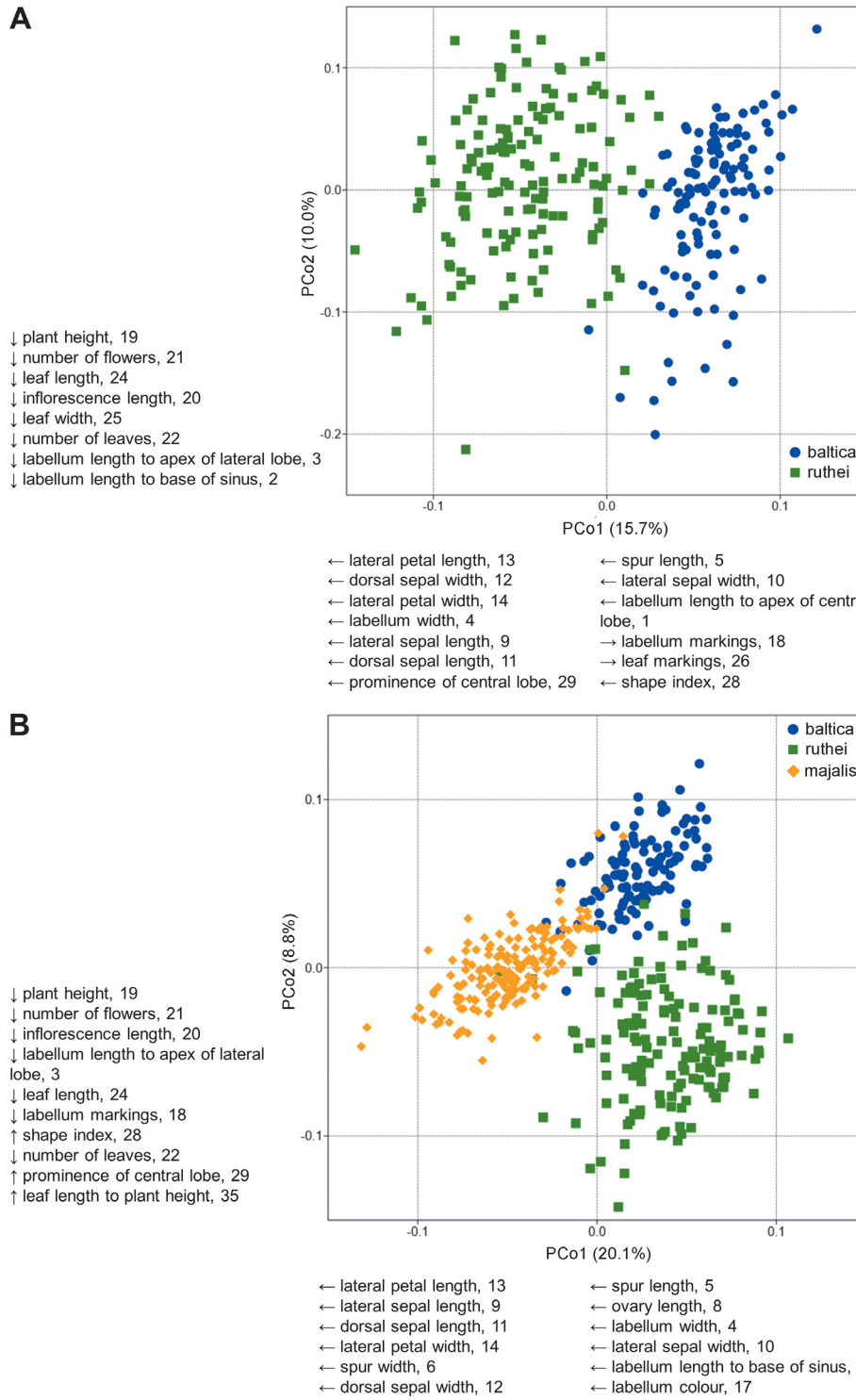


Fig. 2. Plot of the first two principal coordinates (PCoA) based on morphological data of individuals of *Dactylorhiza baltica* and *D. ruthei* (A) and with additional specimens of *D. majalis* s.s. (B). Characters contributing to the coordinates are listed in order of decreasing importance, while arrows indicate the direction of increase in the value of a given traits (see Table 1 for details).

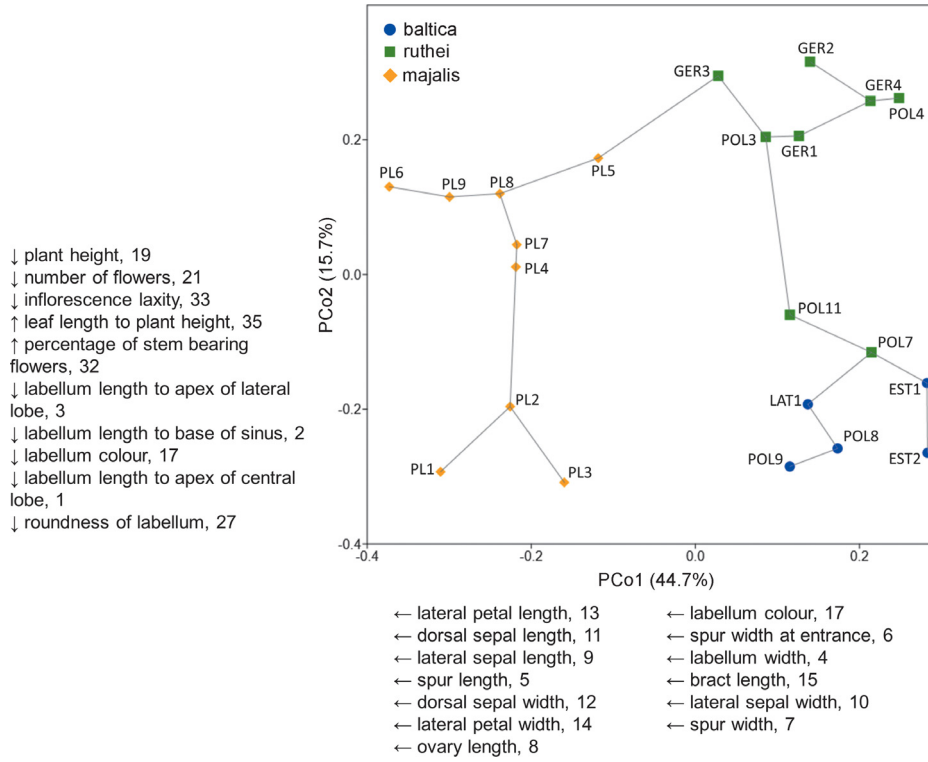


Fig. 3. Plot of the first two principal coordinates (PCoA) for 36 morphological characters measured in 28 populations of *Dactylorhiza baltica*, *D. ruthei* and *D. majalis* s.s., analysed as population mean values (Supplementary Table S2). Characters contributing to the coordinates are listed in order of decreasing importance, while arrows indicate the direction of increase in the value of a given traits. Populations are linked by a minimum spanning-tree.

The first coordinate distinguished the two taxa with a scattering of *D. ruthei* individuals on the left side of the plot and of *D. baltica* individuals on the right. This coordinate (PCo1) reflected the variation between the two taxa mainly in terms of floral traits, where *D. ruthei* has longer and wider petals and sepals, a wider labellum, and a longer spur (arrows show the direction of increase in a given trait). Also, two indices describing the prominence of central lobe and the labellum shape index were indicated as contributing to PCo1. Ancillary variables for this coordinate, which favoured *D. baltica* included anthocyanin markings on both floral and vegetative organs, although these traits are less important than initially thought. In turn, the second coordinate (PCo2) largely reflected the greater vegetative vigour of specimens, mainly in terms of plant height, number of flowers, inflorescence length, and number, length and width of sheathing leaves, with taller plants with a higher number of flowers and longer leaves located in the upper half of the diagram. However, both taxa are more or less equally dispersed in both parts of the diagram.

In a subsequent analysis of individuals of *D. majalis* s.s., the first coordinate (PCo1) separated *D. majalis* on the left side of the plot (Fig. 2B). This coordinate revealed variation in floral traits (length and width of petals, sepals and spurs, ovary length, labellum width) for which *D. ruthei* was intermediate compared to the flowers of *D. baltica* and

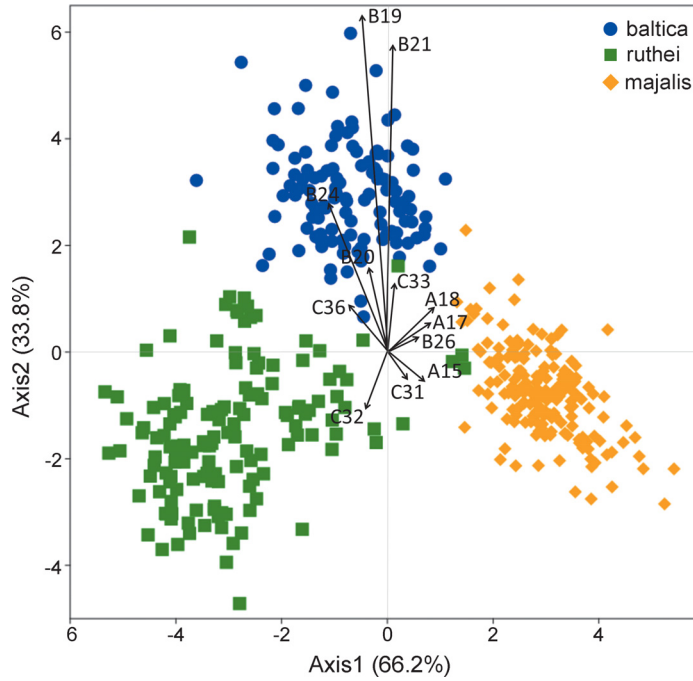


Fig. 4. Biplot of the linear discriminant analysis (LDA) based on 36 morphological characters of individuals of *Dactylorhiza baltica*, *D. ruthei* and *D. majalis* s.s. Shorter vectors of negligible relevance to this analysis were omitted. For a detailed description of the indicated traits see Table 1.

D. majalis s.s. In turn, the second coordinate (PCo2) separated *D. baltica*, which is closer to *D. ruthei* than to *D. majalis* s.s. (Supplementary Table S2).

Restricting the data set to population averages reduced the extent of the data, allowing the first two coordinates to include a larger percentage of the overall variation (in this case 60%; Fig. 3). The two coordinates of the individual-level analyses were very similar at the population level, in which PCo1 is the 'floral' and PCo2 is the 'vigour' coordinates. Together, they include most of the traits that contributed to the distribution of individuals belonging to the three taxa in the above analysis conducted at the individual plant level. Again, the first coordinate distinguished *D. majalis* s.s. from *D. baltica* and *D. ruthei*, in terms of larger petals and sepals, wider labellum, as well as its colouration.

LDA based on the full set of morphological traits was effective in discriminating the three taxa studied (in the plot, only 12 out of 36 traits were retained, which are characterized by the longest vector). The biplot is very similar to the pattern of variation in PCoA for *D. baltica* and *D. ruthei* together with additional specimens of *D. majalis* s.s., with the slight difference that the *D. majalis* grouping is on the right side of the plot (Fig. 4). The first and second linear discriminant axes account for 66.2% and 33.8% of the total variation in morphology, respectively. The first discriminant axis identified the colour and pattern of the labellum (A17 and A18) as the characters with the highest positive coefficient and were associated with *D. majalis* s.s. Alternatively, the second discriminant axis provides a good description of *D. baltica* as taller plants with long leaves and many flowered inflorescences (B19, B21 and B24, respectively).

Patterns in the diversity of nuclear microsatellites

In *D. baltica* the effective number of alleles per locus in a population equaled $A_e = 1.69$ (Table 2). The Tallinn population (EST1) was characterized by the lowest degree of variation, while that from Białystok (POL10) was more variable. The average gene diversity (H_e) ranged from 0.144 to 0.524, with an average of 0.318. The F_{IS} statistic reached an average value of 0.329 and fluctuated between 0.059 (EST1) and 0.563 (LAT1). In *D. ruthei* the number of alleles per locus in a population was $A_e = 1.78$, with the highest value recorded for Karsiborska Kępa (POL1) and the lowest for the Koprowo population (POL2). The same is the case for the statistics describing allele numbers and allelic richness. The average gene diversity (H_e) ranged from 0.067 (POL2) to 0.580 (POL4), with a mean of 0.357. The recorded excess of homozygotes is reflected in the positive inbreeding coefficient values for most populations, except the German population (GER3), where an excess of heterozygotes was recorded compared to that expected at genetic equilibrium. The value of the inbreeding coefficient varied considerably between populations, ranging from –0.060 (GER3) to 0.765 (POL7) with an average value of $F_{IS} = 0.277$.

Table 2. Summary of nuclear microsatellites and plastid haplotypes diversity statistics for the studied populations of *Dactylorhiza baltica* and *D. ruthei*: A/N_h - mean number of alleles per locus/number of haplotypes; A_e = effective number of alleles/effective number of haplotypes; R_A/R_H = allelic/haplotype richness; H_e = average gene diversity over nuclear microsatellite loci; F_{IS} = inbreeding coefficient (*probability of the hypothesis $F_{IS} = 0$, $P < 0.05$); H = mean number of pairwise differences between haplotypes; π = average gene diversity over plastid loci.

Code	Locality	Nuclear microsatellites					Plastid haplotypes				
		A	A_e	R_A	H_e	F_{IS}	N_h	A_e	R_H	H	π
Dactylorhiza baltica											
EST1	Tallinn	2.4	1.23	1.39	0.144	0.059	5	1.68	0.68	0.420	0.174
EST2	Viljandi	2.8	1.61	1.85	0.346	0.561*	1	1.00	0.00	0.000	0.000
LAT1	Mâlpils	3.4	2.16	2.20	0.475	0.563*	9	2.96	1.21	0.689	0.236
POL5	Słone Łąki	–	–	–	–	–	2	1.60	0.75	0.500	0.111
POL6	Mechelińskie Łąki	–	–	–	–	–	3	2.27	1.20	0.700	0.159
POL8	Kamienna Stara	3.4	1.71	1.93	0.310	0.406*	10	6.58	1.67	0.883	0.244
POL9	Nowy Dwór	3.6	1.52	1.83	0.284	0.208*	4	2.33	0.98	0.600	0.260
POL10	Białystok	3.8	2.38	2.50	0.524	0.338*	8	5.16	1.63	0.868	0.305
RUS1	Moscow	2.6	1.22	1.42	0.146	0.171*	4	1.23	0.30	0.193	0.088
Mean		3.4	1.69	2.94	0.318	0.329	5.1	2.76	0.94	0.539	0.175
Dactylorhiza ruthei											
GER1	Rohrschneideplatz	2.8	1.37	1.65	0.229	0.247*	4	1.33	0.41	0.259	0.082
GER2	Knochenberg	3.0	1.27	1.56	0.194	0.217*	2	1.08	0.12	0.080	0.021
GER3	Peenestrom	2.2	1.16	1.31	0.108	–0.060	2	1.10	0.14	0.095	0.004
GER4	Strandwiese	3.4	1.89	2.10	0.418	0.177*	4	2.28	0.93	0.579	0.126
POL1	Karsiborska Kępa	3.8	2.61	2.54	0.533	0.438*	4	3.33	1.38	0.778	0.294
POL2	Koprowo	1.2	1.08	1.20	0.067	0.000	1	1.00	0.00	0.000	0.000
POL3	Włodarka	3.6	2.01	2.24	0.409	0.361*	7	5.83	1.70	0.897	0.196
POL4	Budzistowo	2.8	2.15	2.50	0.580	0.103	4	3.57	1.70	0.900	0.241
POL7	Elbląg	2.6	2.11	2.18	0.532	0.765*	3	2.46	1.13	0.679	0.081
POL11	Pobondzie	3.2	2.19	2.25	0.495	0.522*	3	1.46	0.55	0.345	0.100
Mean		3.1	1.78	1.98	0.357	0.277	3.4	2.34	0.80	0.461	0.115

Table 3. Analysis of the percentage molecular variance (AMOVA) of nuclear microsatellite loci and plastid haplotypes for *Dactylorhiza baltica* and *D. ruthei*. Separate analyses were carried out for different subsets: between populations (A) and between taxa (B). All $P < 0.001$.

A. Source of variation	df	Sum of squares	Variance components	Percentage of variation
Nuclear microsatellites				
Complete material				
Between populations	16	278.20	0.438	36.25
Between individuals within populations	305	316.11	0.267	22.09
Within individuals	322	162.00	0.503	41.67
	$F_{IS} = 0.346$ $F_{ST} = 0.362$ $F_{IT} = 0.583$			
Dactylorhiza baltica				
Between populations	6	67.11	0.225	32.82
Between individuals within populations	158	98.79	0.164	23.92
Within individuals	165	49.00	0.297	43.27
	$F_{IS} = 0.356$ $F_{ST} = 0.328$ $F_{IT} = 0.567$			
Dactylorhiza ruthei				
Between populations	9	91.90	0.305	27.61
Between individuals within populations	147	153.23	0.243	21.95
Within individuals	157	87.50	0.557	50.44
	$F_{IS} = 0.303$ $F_{ST} = 0.276$ $F_{IT} = 0.496$			
Plastid haplotypes				
Complete material				
Between populations	18	261.08	0.739	28.60
Within populations	312	575.76	1.845	71.40
	$F_{ST} = 0.286$			
Dactylorhiza baltica				
Between populations	9	70.48	0.434	24.83
Within populations	147	193.02	1.313	75.17
	$F_{ST} = 0.248$			
Dactylorhiza ruthei				
Between populations	8	143.75	0.832	26.39
Within populations	165	382.74	2.320	73.61
	$F_{ST} = 0.264$			

B. Source of variation	df	Sum of squares	Variance components	Percentage of variation
Nuclear microsatellites				
Between taxa	1	65.77	0.150	11.74
Between populations within taxa	15	212.43	0.364	28.53
Between individuals	627	478.11	0.762	59.73
	$F_{ST} = 0.403$			
Plastid haplotypes				
Between taxa	1	46.85	0.185	6.93
Between populations within taxa	17	214.23	0.640	23.97
Between individuals	312	575.76	1.845	69.10
	$F_{ST} = 0.309$			

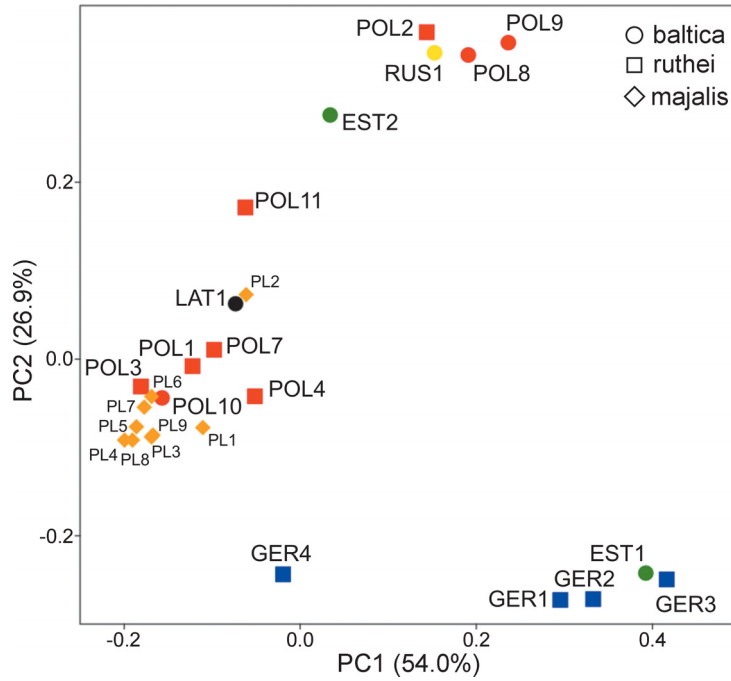


Fig. 5. Principal coordinates analysis (PCoA) showing differentiation between populations of *Dactylorhiza baltica D. ruthei* and additional *D. majalis* s.s. for nuclear microsatellites. The analysis was based on pairwise-F_{ST}. The percentages of variance accounted for by the first two axes were 54.0 and 26.9%, respectively. Populations colours correspond to their geographical distribution.

The genetic differentiation for *D. baltica* was $F_{ST} = 0.328$ and for *D. ruthei* $F_{ST} = 0.276$ (Table 3). Pairwise genetic differentiation between the *D. baltica* populations ranged from 0.002 (POL8–POL9) to 0.731 (EST1–RUS1), while that for the *D. ruthei* populations ranged from 0.008 (GER1–GER2) to 0.877 (GER3–POL2) (Supplementary Table S3). Differentiation between populations is summarized by the PCoA analysis (Fig. 5). There were no clear groupings of populations according to presumed taxon affiliation. However, the percentage of total variation accounted for by the first two components was quite high (81%). German *D. ruthei* populations (GER1–GER4) and one Estonian population of *D. baltica* (EST1) were placed at the lower right, populations from EST2, RUS1, POL2, POL8, POL9, and POL11 were separated in the upper part of the plot, and *D. majalis* s.s. populations formed a tight group to the left, with the other Polish populations and one Latvian population. Furthermore, the STRUCTURE analysis of the data set including all individuals of three taxa with no information on population origin, indicated a clear mode for ΔK. The most likely number of clusters was determined to be K = 3 (Fig. 6). The first *baltica* cluster was quite coherent, except for one Estonian EST1 population, which indicated genetic similarity to the second *ruthei* cluster. In the aforementioned cluster, all German populations had a low degree of admixture with almost homogeneous genetic backgrounds, in contrast to Polish populations, which had a high degree of admixture and similarity to the other two species (e.g. POL3 – *D. majalis*, POL11 – *D. baltica*). The third *majalis* cluster was the most coherent and genetically homogeneous.

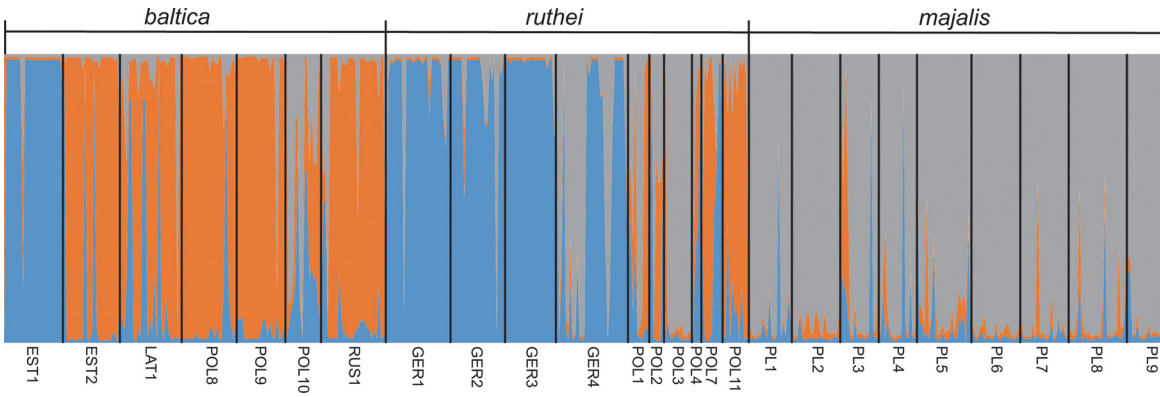


Fig. 6. Clustering results for K = 3 obtained by STRUCTURE under admixture model. Each population is represented by a single chart, showing the relative proportion of membership to the different clusters, where population codes correspond to the localities given in Supplementary Table S1. The analysis was done for the data set comprising all the populations of *Dactylorhiza baltica* and *D. ruthei* studied, together with additional individuals of *D. majalis* s.s. as reference material.

Some of the low-frequency alleles were only recorded in single populations, that is they are private alleles. Several populations (POL1, POL3, POL10, POL11, LAT1, RUS1, EST1) had at most one or two such alleles. At each of the five microsatellite loci, low-frequency alleles were identified in *D. baltica* and *D. ruthei* that were previously identified in the *Dactylorhiza incarnata/maculata* complex in northern Poland (Naczk et al. 2016, Naczk & Ziętara 2019), and also at a low frequency in the diploid parental species (*D. incarnata* var. *incarnata* – paternal lineage and *D. maculata* subsp. *fuchsii* – maternal lineage). At the ms3 locus, the 151-bp allele with a frequency of 0.003 was detected in *D. baltica* (LAT1), but not in *D. ruthei*. The opposite situation was recorded for loci ms11 and ms13, as the 160-bp and 76-bp alleles were present in *D. ruthei* (0.083 and 0.019, respectively), but not in *D. baltica*. At the ms13 locus, a new 79-bp allele was recorded in the POL10 population (0.006; *D. baltica*), which differs by one step of three base pairs in the repeat motif from the 76-bp allele previously identified in *D. incarnata* var. *incarnata*, and recorded in this study in *D. ruthei*.

A significant excess of expected heterozygosity was recorded in *D. baltica* and *D. ruthei* populations (data not shown). Analysis of the distribution of allele frequency indicated that all the populations studied had recently experienced a recent reduction in the number of individuals (bottleneck effect).

Characterization and grouping of plastid haplotypes

A total of 42 fragment size variants at 10 loci were recorded (Supplementary Table S4). For a single locus, the number of alleles ranged from one (locus 18) to 11 (locus 10B). Further markers revealed the occurrence of 36 different haplotypes in the total material (annotated HPL1–36), 29 of which were private haplotypes: 17 for *D. baltica* and 12 for *D. ruthei.* Only seven haplotypes were characterized by a broad taxonomic distribution, and their presence was noted in 74% of the individuals (total number of specimens = 331). Sixteen haplotypes were recorded only once, and the most common haplotypes

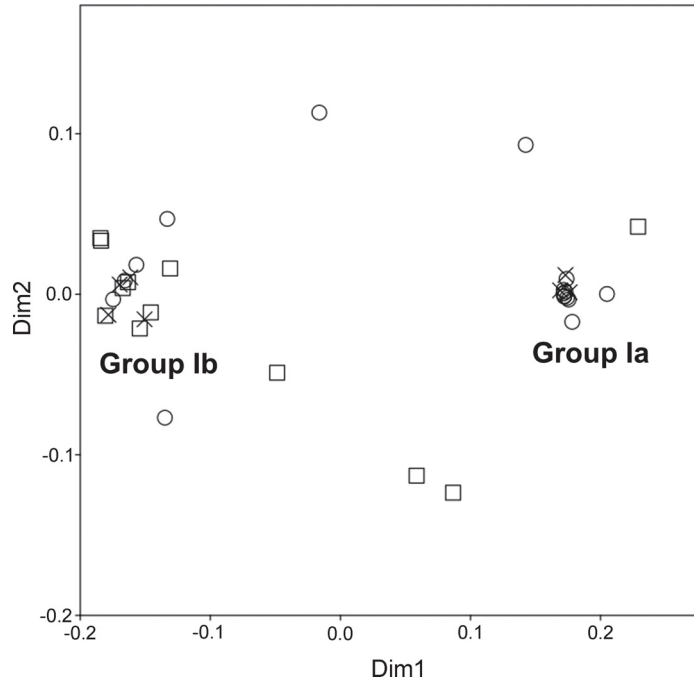


Fig. 7. Non-metric multidimensional scaling analysis of the 36 plastid haplotypes identified in the populations of *Dactylorhiza baltica* and *D. ruthei* studied. Two recognized groups of haplotypes are indicated. For each haplotype, its taxonomic affiliation was defined: *D. baltica* ○, *D. ruthei* □, haplotypes present in two taxa ×.

were recorded in 38 and 179 specimens (HPL4 and HPL6), respectively. Some rare haplotypes may have originated in the region where they were recorded (e.g. for *D. ruthei*: HPL5, HPL12, HPL13 in POL3; HPL15, HPL19, HPL21 in POL4; HPL2 – GER4; HPL16 – GER3; HPL20 – GER2; for *D. baltica*: HPL1 – EST1; HPL7, HPL9, HPL31 in LAT1; HPL18, HPL35 – POL8; HPL22–25 in POL10) (Supplementary Tables S4, S6). Six haplotypes described here were previously reported by Naczk et al. (2015) in northern Poland (given as a subscript in brackets in Supplementary Table S4). Four of them were characterized by a broad affiliation with the *Dactylorhiza incarnata/maculata* complex, whereas in this study they were assigned to *D. baltica* (HPL3 and HPL17) or could not be assigned to either of the main haplotype groups (HPL6 and HPL10). The other two haplotypes were previously described for the *incarnata* group, whereas in the current study, HPL28 occurred widely in the specimens studied and HPL36 was only recorded in *D. ruthei*.

NMDS analysis revealed a clear pattern of grouping for most haplotypes, with two distinct haplotype groups, which are mixed groups and not characteristic of the taxa studied (Fig. 7). The relatively low-stress value (S = 0.074) indicates that the order of distances between haplotypes in the original distance matrix is well represented in the resulting two-dimensional plot. Furthermore, six haplotypes (marked with crosses in the diagram) were identified that are typical for both taxa and are grouped into the two haplotype groups described below. Seventeen haplotypes, which together occur in 17% of the samples, were classified in Group Ib on the left side of the diagram. On the opposite side,

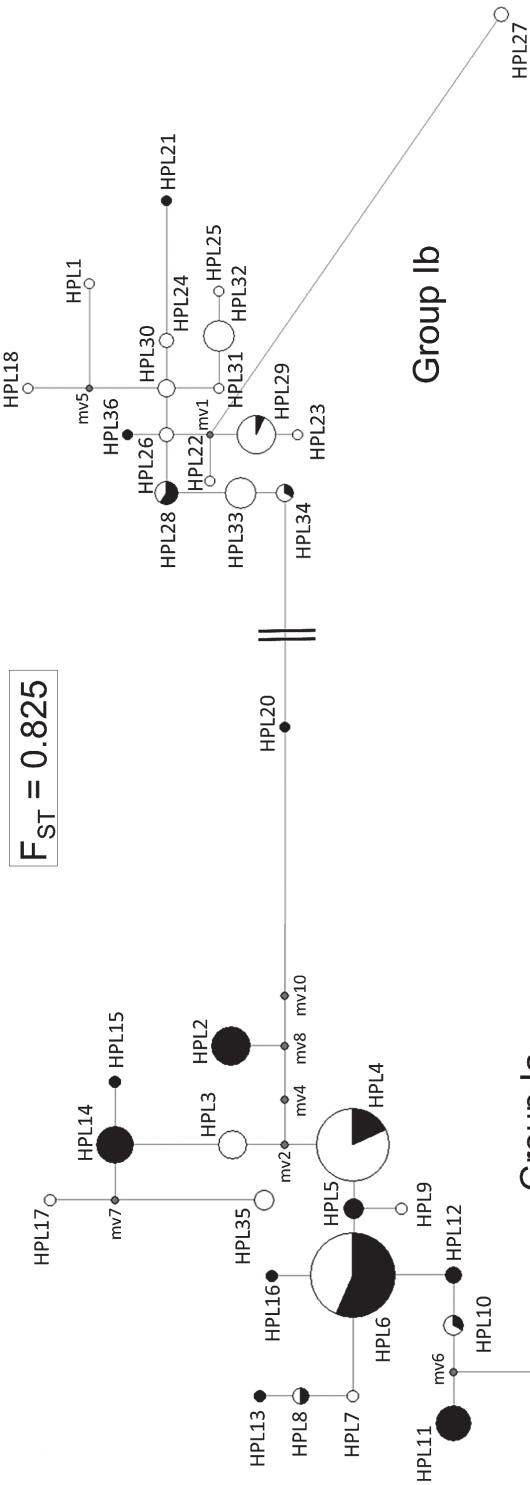


Fig. 8. Median-joining network of the plastid haplotypes encountered in the analysed material of *Dactylorhiza baltica* and *D. ruthei*. The size of each symbol is proportional to the number of individuals with a particular haplotype, and the sectors are proportional to the number of different taxa in which the haplotype was recorded. Marker sites and mutational steps separating haplotypes are indicated along with numbers of mutational steps at each site. Branch lengths are approximately proportional to the numbers of mutational steps. Small diamonds denote median vectors. Black sectors, percentage of haplotypes recorded in *D. ruthei*; white sectors, percentages of haplotypes recorded in *D. baltica*.

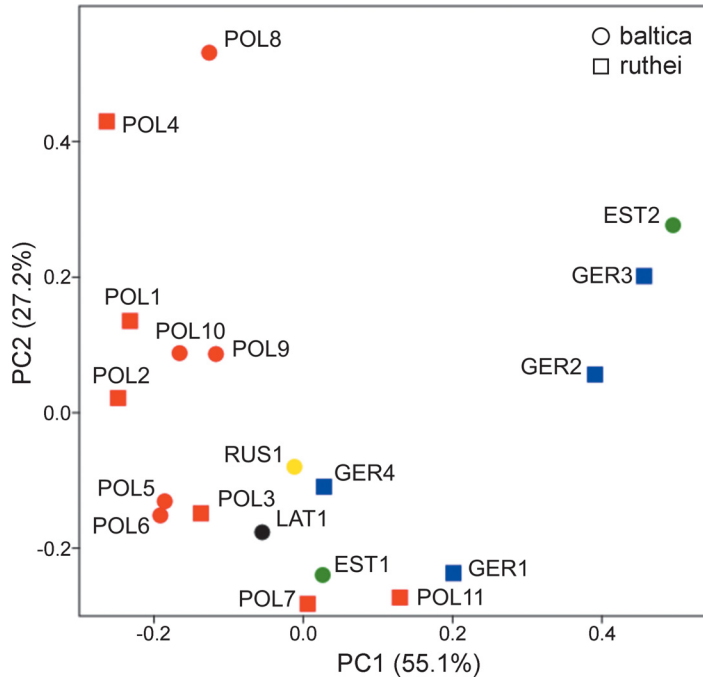


Fig. 9. Principal coordinates analysis (PCoA) showing differentiation between the populations of *Dactylorhiza baltica* and *D. ruthei* in plastid haplotypes. The analysis was based on the matrix of mean pairwise haplotype differences between populations. The first two axes account for 55.1 and 27.2%, of the total variation, respectively. Populations colours correspond to their geographical distribution.

Group Ia includes 19 haplotypes and 83% of all the samples (274 specimens) belonged to this group. Moreover, none of the haplotypes occurred only in particular geographical regions.

Median-joining network illustrating the relationships between the recorded haplotypes for *D. baltica* and *D. ruthei* is presented in Fig. 8. The resulting haplotype grouping is similar to that revealed by the NMDS analysis. There are two distinct haplotype groups in this network, for which the genetic distance was determined as F_{ST} = 0.825. Group Ia, located on the left side of the network, is separated from Group Ib by 15 mutational steps at eight marker sites, together with the intermediate HPL20 haplotype located almost in the middle (this is a private haplotype for *D. ruthei* from the GER2 population). There were 19 haplotypes in Group Ia, with a high frequency of HPL4 and HPL6 haplotypes. Most of the specimens classified as *D. ruthei* were in the above group, in contrast to the *D. baltica* specimens, which mainly occurred in Group Ib.

Population differentiation in plastid haplotypes was also revealed by pairwise-F_{ST} (Supplementary Table S5) and summarized in the PCoA analysis (Fig. 9). In the resulting diagram the populations are not clearly separated, which may be due to the absence of taxon-specific haplotypes, as most populations contained a mixture of haplotypes (Supplementary Table S6). The first ordination axis (PC1) divided populations into two groups (below and above the axis), with six Polish populations located in the upper left and one Estonian EST2 and two German populations (GER2 and GER3) on the right side

of the plot. The F_{ST} fixation index revealed a high degree of differentiation between populations in total material (0.286), which was also high between populations for individual taxa (*D. baltica* – 0.248 and *D. ruthei* – 0.264) (Table 3).

Hierarchical distribution of genetic diversity

Hierarchical structure of genetic diversity was calculated for all the material, as well as separately for *D. baltica* and *D. ruthei*. The results of the AMOVA analyses are summarized in Table 3. The distribution of genetic variation at particular levels of differentiation had fairly similar percentages both for the taxa studied and the complete material. For nuclear microsatellites, AMOVA revealed that 36% of the variation was due to differentiation between populations and for all the material 42% was due to differentiation within individuals. Similar values of intra-population variation were obtained for the taxa studied, which were slightly more variable but similar to the other two levels of variation (between populations – *baltica* vs *ruthei*: 33 vs 28% and within individuals, 43 vs 50%). For plastid haplotypes, the highest percentage of genetic variation was recorded within populations (over 70%). In the subsequent analysis at the taxa level, a low percentage of genetic diversity was attributed to differentiation between them: 12% and 7% for nuclear and plastid genomes, respectively. All estimates were significantly higher than zero ($P < 0.001$).

Correlation between data sets

Genetic distances based on both nuclear and plastid data correlated non-significantly with geographic distances for the *D. baltica* populations ($r = 0.142$ and 0.250, respectively; $P > 0.05$) (Table 4). In contrast, for populations of *D. ruthei*, there was no correlation between differentiation in plastid markers and geographic distances ($r = –0.132$; $P > 0.05$), but there was a correlation between diversity of nuclear markers and geographic distances ($r = 0.331$; $P < 0.05$). Moreover, comparing the pairwise-F_{ST} distance matrices between populations obtained for nuclear microsatellites with data derived from plastid haplotypes, weak and statistically insignificant correlations were recorded for *D. baltica* and *D. ruthei* ($r = –0.152$ and $–0.011$; $P > 0.05$).

The pollen-to-seed flow ratio calculated for all the total material was 0.49, while for the taxa studied it was 0.48 for *D. baltica* and 0.68 for *D. ruthei*.

Table 4. Results of Mantel tests for associations between molecular marker data sets and geographical distances of populations. Separate analyses were performed for the entire material, for *Dactylorhiza baltica*, as well as, for the *D. ruthei* populations. Significance levels: ns $P > 0.05$; * $P < 0.05$.

	Geographic distances (km)	Nuclear microstallites (F_{ST})
Nuclear microsatellites (F_{ST})	$r = –0.030^{ns}$ (complete material) $r = 0.142^{ns}$ (*baltica*) $r = 0.331^{*}$ (*ruthei*)	
Plastid haplotypes (F_{ST})	$r = 0.130^{ns}$ (complete material) $r = 0.250^{ns}$ (*baltica*) $r = –0.132^{ns}$ (*ruthei*)	$r = –0.090^{ns}$ (complete material) $r = –0.152^{ns}$ (*baltica*) $r = –0.011^{ns}$ (*ruthei*)

Discussion

Morphological variation

For most floral traits, *D. ruthei* is larger in terms of labellum length and width (the lip of *D. ruthei* is wider and has a longer midlobe than *D. baltica*; characters A1–5, A9, A11–16, Table 1). In contrast, for almost all vegetative traits, *D. baltica* is generally larger than *D. ruthei* (characters B19–24). The main characters that distinguish *D. baltica* from *D. ruthei* are the shape, length, and width of the labellum, as well as the other vegetative traits measured, with the exception of the width of the longest leaf. These features are consistent with those cited by Shipunov et al. (2005) for *D. baltica*, where leaf spots, length of the longest leaf and length of lateral lip lobes are listed as diagnostic. A similar range of variation accounted for by the first two coordinates was obtained as that reported in other studies on the *D. majalis* aggregate (Bateman & Denholm 1983, 2012), i.e. 26% when analysing only *D. baltica* and *D. ruthei* and 29% when *D. majalis* s.s. was included. This value is low because the numerous taxa included in the *D. majalis* group are a consequence of repeated allopolyploidization between different ecotypes of the same two parental lineages (Paun et al. 2011, Balao et al. 2017, Brandrud et al. 2020).

Dactylorhiza baltica and *D. ruthei* are not genetically unique (i.e. they are a result of common evolution from the same pair of parental species), but they can each be identified by morphological characters (Table 1). As expected, the addition of the morphologically well-distinguished *D. majalis* to the analysis increased the differences between *D. ruthei* and *D. baltica*. The morphological analyses resulted in quite good separation of the two taxa at the individual level. *Dactylorhiza ruthei* and *D. baltica* are similar morphologically, with some overlap in morphology between individual plants, suggesting that these taxa would be better treated as subspecies of *D. majalis*, rather than as separate species (cf. Bateman 2001, Bateman et al. 2011). Care must be taken not to overestimate the value of anthocyanin-based characters as indicated by the PCoA analysis (Fig. 2). However, these traits are important for distinguishing infraspecific taxa within *Dactylorhiza*. On the other hand, a comparison of the extensive morphometric and molecular data for *Dactylorhiza* reveals that the most common type of taxonomic error has been to overestimate the systematic importance of anthocyanin-based characters. This pigmentation of traits is very obvious and can involve the whole plant, as it not only influences flower colour and markings, but also the markings of the above-ground vegetative organs of the plant (Bateman & Denholm 2012). Thus, despite the problematic and continuous nature of the features this pigmentation should not be overlooked in studies on the morphology and taxonomy of taxa in the *D. majalis* group.

The *Dactylorhiza incarnata/maculata* complex is morphologically very variable and as a consequence individual taxa differ very little in their morphological features (Bateman & Denholm 1983, 1985, 1988, 1989, Ståhlberg & Hedrén 2008, Naczk et al. 2015). Individual taxa contain numerous morphotypes and are not sharply separated from each other, so distinguishing cryptic taxa (especially at the rank of species) does not seem justified. In such a situation, combining critical taxa into collective species seems to be an appropriate way of dealing with this situation, especially in the group of *D. maculata* s.l. and *D. majalis* s.l. (e.g. Hedrén 2002, 2003, Devos et al. 2003, Shipunov et al. 2004, Hedrén et al. 2008). A taxonomic system for ranking inconsistent taxa of *Dactylorhiza* was proposed by Pedersen (1998), and is widely accepted (e.g. Hedrén et al. 2008, 2012b,

Ståhlberg & Hedrén 2008). In this system, 'species' correspond to the biological (Mayr 1940, Jonsell 1984), 'subspecies' to ecological (Van Valen 1976), and 'varieties' to phenetic (Sneath 1976) concept of species. In turn, Bateman defined 'species' as aggregates of populations whose individual members are distinguishable from members of all other comparable populations (in multivariate ordinations such taxa are separated by morphological discontinuities), and 'subspecies' and 'varieties' mainly separated by morphometric comparison of populations using individuals as the basic entities in multivariate ordinations of such taxa reveal morphological overlap (Bateman & Denholm 1989, 1995).

The current results indicate that *D. ruthei* is morphologically more similar to *D. baltica* than to *D. majalis* s.s. This is not always reflected in its taxonomic treatment, as *D. baltica* is sometimes merged with *D. majalis*, whereas *D. ruthei* is separated. It is worth recalling that most authors consider *D. baltica* a separate species (e.g. Senghas 1968, Averyanov 1990, Baumann 2005, Delforge 2006) or a subspecies of the aggregate species *D. majalis* (i.e. *D. majalis* subsp. *baltica*; Pedersen & Faurholdt 1997, Pedersen & Hedrén 2010, Eccarius 2016, Kühn et al. 2019). In turn, *D. ruthei* is considered to be a subspecies of *D. baltica* (Bernacki 1989), but some authors recognize *D. ruthei* as an independent species (Senghas 1968, Bernacki 2001, Baumann 2005, Eccarius 2016), or even a subspecies of *D. majalis* (Kretzschmar 2008). The above sheds new light on the ongoing taxonomic discussion about the taxonomic rank of the two taxa, as well as whether the numerous allopolyploid lineages should be treated as single aggregate species of *D. majalis* s.l. (Hedrén 2002, Hedrén et al. 2008), or as a separate species (Bateman 2006, 2011, 2022).

Nuclear genome

Morphologically divergent populations of *Dactylorhiza* may arise in different parts of its range and have different habitat preferences. These parapatric populations are usually the consequence of genetic bottlenecks, followed by local adaptation and/or drift. In northern regions, these genetic bottlenecks have resulted in a number of genetically, as well as morphologically well-characterized lineages within the *Dactylorhiza majalis* group (Hedrén et al. 2011). *Dactylorhiza baltica* and *D. ruthei* populations have undergone genetic bottlenecks in their recent history, likely due to the colonization of areas available shortly after the retreat of the ice sheet and/or by environmental changes caused by anthropogenic pressure. Genetic drift appears to play an important role in the populations studied, leading to a high probability of inbreeding (Ellstrand & Elam 1993). The current study also detected a tendency for inbreeding, and a high and positive fixation index may indicate limited gene flow and spatial genetic structure within populations (Brzosko et al. 2002, Wells & Young 2002). If pollen dispersal is restricted, this will result in inbreeding, reinforcing genetic structure and subdividing the population over time, contributing to the process of isolation by distance (Wright 1943, Sokal & Wartenberg 1983).

Moreover, when gene flow is limited or absent, populations undergo genetic differentiation, either by natural selection or genetic drift. Forrest et al. (2004) and Phillips et al. (2012) compiled data on population differentiation in orchid taxa and calculated averages of F_{ST}/G_{ST} = 0.187/0.146. The nuclear F_{ST} values obtained in this study (0.328 for *D. baltica* and 0.276 for *D. ruthei* populations) do not differ significantly from the mean values reported for other European subspecies of the *D. majalis* group (e.g. *D. majalis*

subsp. *elatior*, *D. majalis* subsp. *sphagnicola*, *D. majalis* subsp. *purpurella*) (Hedrén et al. 2011, 2012a, b). Geographic isolation, habitat fragmentation and restricted seed dispersal further decrease gene flow in *D. baltica* and *D. ruthei* populations, which was also confirmed by the Bayesian cluster analysis. The first *baltica* cluster was well defined, except for one Estonian EST1 population. In the *ruthei* cluster, all German populations had a low degree of admixture and had almost homogeneous genetic backgrounds, in contrast to the Polish populations that belong in this group, which have a high degree of admixture and are similar to the other species. Estonian EST1 population, which is genetically similar to the second *ruthei* cluster, was morphologically classified as *D. baltica* and grouped with individuals of this taxon in the multivariate analysis. This indicates that the taxonomic affiliation to *D. ruthei* is incorrect. Despite efforts not to overestimate the anthocyanin-based traits, this failed in the EST1 population. Individuals from this population have few markings on the labellum and spotted leaves. On the other hand, such traits are often polymorphic within populations and show broadly gradual changes in frequency (Bateman & Denholm 2012). Thus, only the accurate measurement of a complete set of quantitative characters and the assignment of qualitative traits to individuals, guarantees the determination of the correct taxonomic affiliation of *D. ruthei* and *D. baltica*. In contrast, a high degree of admixture was observed in almost all Polish populations, suggesting some dispersal and gene flow at these sites, as opposed to populations in which there was only one gene pool.

Allotetraploid lineages of *Dactylorhiza* vary greatly in the degree of anthocyanin pigmentation, in terms of leaf spotting, degree of colouration of stem and bracts and the intensity of both background colour and flower pattern (Hedrén et al. 2011). Anthocyanin pigmentation is darker in the north and west than in the south and east (Bateman 2006, 2011, Hedrén et al. 2011). *Dactylorhiza majalis* subsp. *calcifugiens* H. A. Pedersen and *D. majalis* subsp. *sphagnicola* may be an example of this, as they passed through a genetic bottleneck as siblings when they first colonized northern Jutland. As a result of the fixation of certain alleles, they may also have lost the ability to produce red coloured flowers (subsp. *sphagnicola* trait) and produce light cream flowers (subsp. *calcifugiens*) (Hedrén et al. 2011, 2012b). A similar situation is reported in *D. incarnata* var. *ochroleuca* (Boll) Hyl. in which creamish-yellow flowers appear to be associated with a single recessive allele (Hedrén & Nordström 2009). Different degrees of anthocyanin pigmentation also occurred in *D. ruthei* and *D. baltica*. Both taxa differ from each other in terms of unspotted/spotted leaves and lighter/darker coloured flowers. Because the pigment in leaf spots is anthocyanin, spotted plants might be either homozygous or heterozygous for genes that synthesise or express this pigment.

Paun et al. (2011) suggest that allotetraploid populations of *Dactylorhiza* have more variable expression of the same genes than their parental species, and that selection for different gene variants in different allotetraploids may be adaptive and promote the survival of these plants in specific habitats. Studies on patterns in gene expression (Paun et al. 2011) and DNA methylation (Paun et al. 2010) in *D. majalis* subsp. *majalis* and *D. majalis* subsp. *traunsteineri* have revealed that they were shaped by strong directional environmental selection, particularly that related to water availability (associated with rainfall) and temperature differences in the microhabitats they inhabit. In this context, adaptation to climate has been the main driver of the modest but nevertheless significant differences in ecological tolerance of different allotetraploid marsh-orchid species (Paun

et al. 2010, 2011), especially species with a recent post-glacial origin. Moreover, morphological differences are widely recognized as adaptive (Westoby & Wright 2006, DeWoody et al. 2015). To maintain phenotypic differentiation, natural selection within populations must be strong enough to overcome gene flow from morphologically divergent populations (Kremer et al. 2002). On the other hand, local adaptation requires a species to express multiple morphotypes, each with a higher fitness in its microhabitat than in others (Kawecki & Evert 2004, Savolainen et al. 2013).

The positive and significant correlation between genetic and geographic distance suggests that seed dispersal follows the stepping-stone model (Kimura & Weiss 1964), and furthermore, founders of new populations are more likely to originate from neighbouring than from distant populations, probably as a result of the founder effect. For the taxa studied here, a significant correlation between genetic and geographic distance was only obtained for *D. ruthei* ($r = 0.331$; $P < 0.05$). This may reflect the relatively recent origin of the populations studied or the high level of gene flow between populations. The average gene diversity over nuclear microsatellite loci was similar for the *D. baltica* and *D. ruthei* populations (Table 2). Assuming that genetic diversity in the nuclear genome accumulates over time, it is possible to estimate the age of allotetraploid taxa (Hedrén et al. 2012a). The resulting RAD-seq study revealed the following succession of allopolyploid formation within the *Dactylorhiza incarnata/maculata* complex, where *D. baltica* and *D. purpurella* were identified as the youngest allopolyploids (Brandrud et al. 2020). Unfortunately, *D. ruthei* was not analysed separately for RAD-seq, but was included in the *D. baltica* samples, so it is difficult to comment on the results in this situation (the study included only three specimens and described them as '*D. baltica* incl. *D. ruthei*'). However, it seems that *D. baltica* and *D. ruthei* may be of similar age and could be classified as younger allotetraploids within the *D. majalis* group. On the other hand, the question of whether *D. ruthei* may have arisen relatively recently from *D. baltica*, or alternatively represents an independent polyploid event, is still unresolved and requires further research.

Plastid genome

The same processes occur in *D. baltica* and *D. ruthei* as in the *D. majalis* s.l. group (e.g. Hedrén et al. 2008, 2011, 2012a, b, Nordström & Hedrén 2009). Thus, these closely related taxa had multiple origins and backcrossed with their parental lineages. These processes contributed to the geographic differentiation that occurred in both taxa, but part of the variation may have resulted from mutations that happened shortly after the origin of these allotetraploids. Some of the known allotetraploids, like both of the taxa studied, have a restricted distribution and are probably of postglacial origin, although others may be older and carry plastid haplotypes not found in present-day representatives of the parental lineages. Introgression and hybridization between diploids and allotetraploids, as well as between different independently derived allotetraploids, could have contributed to genetic differentiation at the tetraploid level (Hedrén 2003).

Six haplotypes described here were previously reported by Naczk et al. (2015) in northern Poland. Four of them were characterized by broad affiliation within the *D. incarnata/maculata* complex, whereas in the present study they were assigned to *D. baltica* HPL3 and HPL17 or could not be assigned to any of the main haplotype groups. A further two haplotypes are reported in the *incarnata* group, whereas in the present study, HPL28

had a wide distribution in the specimens studied, and HPL36 was a haplotype of *D. ruthei* (Supplementary Table S4). *Dactylorhiza baltica* and *D. ruthei* are likely the result of several recent and mainly local hybridization events between the diploids *D. maculata* subsp. *fuchsii* and *D. incarnata* s.s. (Brandrud et al. 2020). Furthermore, introgression from *D. incarnata* to *D. baltica* may be relatively frequent (Shipunov & Bateman 2005 and Shipunov et al. 2005). It can be assumed that young allotetraploids hybridize with their parental lineages more frequently than older ones (Hedrén et al. 2012a). For example, in *D. baltica* (Shipunov et al. 2005) and in *D. majalis* subsp. *purpurella* (Hedrén et al. 2011), which are considered to be young allopolyploids, a high percentage of the plastid haplotypes in the populations are *incarnata* haplotypes, which must have been introduced by secondary hybridization with local forms of *D. incarnata*. On the other hand, in older taxa, i.e. *D. majalis* subsp. *majalis* and *D. majalis* subsp. *lapponica* (Laest.) H. Sund., secondary introgression from *D. incarnata* is less common (Nordström & Hedrén 2009, Hedrén et al. 2012b).

Two groups of plastid haplotypes were recorded, with a slight dominance of Group Ia in *D. ruthei* and Group Ib in *D. baltica*. It is likely that *D. ruthei* may have originated in the south-western part of the Baltic Sea coast, whereas *D. baltica* arose several times along the eastern coast. There are quite a few cases in which haplotypes occurring in the same population differ by only a single mutational step at one of the variable sites. At least some of these events are likely to be mutations that occurred in local populations. Several distinct haplotypes are common in both allotetraploids and are widely distributed (e.g. HPL4, HPL6, HPL28, HPL29), indicating gene flow between these closely related taxa (at least historically), or that they evolved from a genetically polymorphic ancestral taxon.

For most of the haplotypes, no pattern in their geographic distribution was detected in the area studied, with high differentiation between the two described haplogroups (F_{ST} = 0.825). Both haplogroups (Group Ia and Ib) are dominated by several widespread plastid haplotypes (HPL4, HPL6, HPL28, HPL29) and a large number of rare and geographically restricted haplotypes. However, the possibility that *D. ruthei* is derived from *D. baltica* as a result of long-distance dispersal cannot be completely dismissed and genetic variation reduced by the bottleneck associated with colonization of the Baltic region. *Dactylorhiza baltica* and *D. ruthei* are not known from other parts of Europe, and it is likely they originated postglacially in the Baltic region (with *D. baltica* having a slightly wider range towards the east than *D. ruthei*). Local origins have already been inferred for some other young allotetraploids recognized as subspecies of *D. majalis* (e.g. *D. majalis* subsp. *elatior*, *D. majalis* subsp. *sphagnicola*), indicating that they evolved in situ in northern regions as a result of long-distance seed dispersal (Hedrén et al. 2001, 2012a, Hedrén 2002). Interestingly, the HPL2 haplotype was only recorded in the German population, suggesting that it may have evolved in this area, whereas HPL4, HPL28 and HPL29, which are widely distributed, are not present there.

Geographic structure

In plants, the genetic structure depends on gene transfer via seed and pollen (Petit et al. 2003, 2005). In orchids the dispersal of a large proportion of their genes occurs mainly by seed, which means that they tend to have a low pollen-to-seed gene dispersal ratio (Squirrel et al. 2001, Cozzolino et al. 2003). Two reasons for such low values may be due to the fact

that orchid seed is very light and therefore easily dispersed (Arditti & Ghani 2000) and the pollen is packed in pollinia and thus is dispersed over relatively short distances by insects. The pollen-to-seed flow coefficient calculated for *D. baltica* was 0.48 and for *D. ruthei* 0.67. These values are similar to or slightly lower than those reported for other *Dactylorhiza* subspecies that have also experienced recent and postglacial range expansion (e.g. Gotland populations of *D. majalis* subsp. *lapponica* 0.96; Hedrén et al. 2018). The values recorded indicate that pollen dispersal between populations is even more limited, with most genes spreading between populations by seed (Hamrick & Godt 1996, Nybom & Bartish 2000).

Comparisons of the geographic distances and molecular data sets were mostly non-significant, except for the correlation between geographical distances and nuclear microsatellites for *D. ruthei* (Table 4). However, there is a weak correlation between genetic distances calculated from plastid and nuclear markers. This may support the hypothesis that the geographic structure of *D. ruthei* is primarily based on seed dispersal (it cannot be ruled out for *D. baltica* as well, given that is is likely they have a common evolutionary history). The taxa studied had low pollen-to-seed dispersal ratios and no clear correlations between genetic differentiation and geographic distance. This may not only be due to isolation by distance, but also reflect historical patterns in seed dispersal and colonization (Hedrén et al. 2007, Ray & Excoffier 2010).

The recorded pattern of variation in cpDNA haplotypes may reflect the colonization history of local populations. The separation of the resulting haplotypes into two distinct lineages indicate a postglacial migration from at least two different directions and/or their local origin. It is also possible that long-distance seed dispersal were ancestral populations. In the case of orchids, long-distance seed dispersal by wind over distances of up to 2,000 km is repeatedly reported (Arditti & Ghani 2000, Jersáková & Malinová 2007, Vandepitte et al. 2012). The prevailing westerly and south-westerly winds along the southern and eastern Baltic coasts greatly facilitate dispersal over considerable distances by seed (Tarnowska 2011, Bierstedt et al. 2015). Therefore, the recorded genetic structure of these two taxa based on the haplotype network may be related to their place of origin or wind direction. For *D. ruthei* (Group Ia), the oldest haplotypes should have easily reached many eastern locations (large circles), a the direction of the wind is westerly. *Dactylorhiza baltica* (Group Ib) is expected to have more difficulty spreading westwards (‘upwind’). This suggests that the older haplotypes should be more local (represented by small circles). In addition, the Estonian population (EST1) may be exceptional, but it confirms long-distance connections by seed, as the distance between Tallinn and the *D. ruthei* populations in Usedom (GER1–3) is 1,700 km.

Thus, the apparent separation of haplotypes into two distinct lineages probably indicates long-distance dispersal and that genetic variation was reduced by the bottleneck associated with colonization of the Baltic Sea region. The Baltic Sea region was completely covered by the Weichselian ice sheet, so either these taxa survived the ice age elsewhere and migrated to the area, or they evolved within it during the Holocene, which seems more likely. *Dactylorhiza baltica* and *D. ruthei* are not known from other parts of Europe, and a postglacial origin in the Baltic region is quite likely.

Conclusions

Allotetraploid taxa within the *Dactylorhiza incarnata/maculata* complex differ slightly, both morphologically and genetically. This was most likely due to their independent and multiple origins by hybridization between the same two diploid lineages (*D. incarnata* s.l. and *D. maculata* subsp. *fuchsii*). *Dactylorhiza baltica* and *D. ruthei* as allotetraploids that share a common evolutionary history, but maintain phenotypic distinctness and private alleles with similar genetic backgrounds. It seems possible that the two taxa arose independently but at a similar time. The recorded patterns of genetic variation are similar to those presented in other subspecies belonging to the *D. majalis* group. Therefore, it is concluded that taxa that have integrated within the same ancestral allopolyploid gene pool with limited gene flow and appear to be historically related to each other should be considered to be infraspecific taxa within the *D. majalis* group. They deserve to be treated as independent taxa at the same taxonomic level and according to our results as subspecies.

Supplementary materials

Fig. S1. Flowers and habit of *Dactylorhiza baltica* and *D. ruthei*.
Table S1. Locality data for the populations of *Dactylorhiza baltica* and *D. ruthei* studied.
Table S2. List of morphological characters and ratios recorded for populations of *Dactylorhiza baltica*, *D. ruthei* and *D. majalis* s.s.
Table S3. Geographical distance and F_{ST}-values for nuclear microsatellites between pairs of the populations of *Dactylorhiza baltica* and *D. ruthei* studied.
Table S4. Characterization of plastid haplotypes in the *Dactylorhiza baltica* and *D. ruthei* populations based on using the primer pairs.
Table S5. Geographical distance and F_{ST}-values for plastid haplotypes between pairs of the populations of *Dactylorhiza baltica* and *D. ruthei* studied.
Table S6. Distribution of plastid haplotypes within the populations of *Dactylorhiza baltica* and *D. ruthei* studied.

Supplementary materials are available at https://www.preslia.cz

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Genetická a geografická variabilita dvou regionálních alotetraploidních orchidejí *Dactylorhiza baltica* a *D. ruthei*

Dactylorhiza baltica (Klinge) Nevski a *D. ruthei* (M. Schulze ex Ruthe) Soó jsou taxony s nejasným taxonomickým statusem, rozlišované na základě několika morfologických znaků. Jejich hodnocení jako samostatné druhy nebo infraspecifické taxony zůstává problematické, přičemž názory jednotlivých autorů se liší. Analyzovali jsme více než 300 exemplářů z devíti populací *D. baltica* a desíti *D. ruthei*. K posouzení diferenciace *D. baltica* a *D. ruthei* jsme použili tři soubory dat (36 morfologických znaků, 10 úseků plastidové DNA a 5 jaderných mikrosatelitů). Oba taxony jsou odlišné mladé alopolyploidy, ale sdílejí stejný genetický základ a ploidní úroveň, což znamená, že nejsou geneticky unikátní, lze je však odlišit na základě souboru diagnostických morfologických znaků. Jsou výsledkem vývoje hybridizace stejného páru rodičovských druhů, mají podobnou evoluční a postglaciální historií, ale vznikly v Pobaltí nezávisle na sobě. Pozorovaná genetická variabilita a poměrně výrazné rozdíly v morfologii, stejně jako společné mechanismy vzniku a evoluce těchto alopolyploidních taxonů, poskytují argumenty pro přisouzení stejného taxonomického statusu jak *D. baltica*, tak *D. ruthei*. Navrhujeme, že by tyto příbuzné populace se společným alopolyploidním genofondem měly být hodnoceny jako infraspecifické taxony v rámci *D. majalis* s.l., a to jako *D. majalis* subsp. *baltica* (Klinge) H. Sund. a *D. majalis* subsp. *ruthei* (M. Schulze ex Ruthe) H. Kretzschmar.

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