



Dactylorhiza maculata agg. (Orchidaceae) in Central Europe: Intricate Patterns in Morphological Variability, Cytotype Diversity and Ecology Support the Single-Species Concept

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Received: 25 May 2023 / Revised: 12 January 2024 / Accepted: 13 January 2024
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Abstract Effective protection of endangered species is often limited by taxonomic discrepancies across state borders. This is also the case of the *Dactylorhiza maculata* agg. in Central Europe, where one to three species and several infraspecific taxa are recognized in various countries. Based on an extensive analysis of morphological variation, ploidy levels, environmental traits and habitats of 64 populations in Central Europe and adjacent regions, we aimed to propose a unified taxonomic concept applicable throughout the study area. Multivariate analysis of morphological traits revealed continuous variation at the individual level and only minor differences between

particular clusters of populations. Four DNA-ploidy levels were detected using flow cytometry. Diploids ($2n = 40$) and tetraploids ($2n = 80$) were the most abundant and usually formed single-cytotype populations whereas DNA-triploids and DNA-hexaploids occurred only sporadically as minority cytotypes. The inferred patterns of morphological and ploidy variation were not congruent with traditional taxonomic treatment regarding diploid *D. fuchsii* and tetraploid *D. maculata* as two species with several infraspecific taxa. Instead, all taxa analysed in the current study are best treated at the subspecies level within *D. maculata* s. lat. due to somewhat continuous morphological variation between morphotypes. A total of eight *D. maculata* subspecies may be recognized in Central Europe, of which one is newly described here as *D. maculata* subsp. *arcana*, subsp. nov. Some

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12224-024-09441-0>.

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nomenclatural riddles have been resolved, and the threat status of the recognized taxa is discussed.

Keywords Chromosome numbers · Endangered species · Habitat protection · Morphometrics · Ploidy levels · Orchids · Red List · Taxonomic revision

Introduction

The terrestrial orchid genus *Dactylorhiza* Neck. ex Nevski, distributed from the temperate to the boreal belt of the Northern Hemisphere with a centre of genetic diversity in the Mediterranean Basin and the Caucasus Mts, is one of the most taxonomically challenging groups of the orchid family (Pedersen 1998; Delforge 2006; Pillon et al. 2006; Eccarius 2016). With the exceptions of *D. sambucina* (L.) Soó and *D. viridis* (L.) R. M. Bateman, Pridgeon et M. W. Chase, all Central European members of the genus belong to the so-called *D. incarnata* / *maculata* polyploid complex. Within this complex, three groups can be recognized: the *D. incarnata* agg. (diploid only), the *D. maculata* agg. (comprising diploids and autopolyploids) and the *D. majalis* / *traunsteineri* complex, which includes allopolyploid derivatives of the previous two groups (Hedré 2001; Pillon et al. 2007; Devos et al. 2005; Hedré et al. 2008; Nordström and Hedré 2009; Balao et al. 2016; Brandrud et al. 2020).

The evolutionary history and phylogeny of the *D. maculata* agg. has been explored using allozymes (Hedré 1996), AFLP (Hedré et al. 2001), nuclear and plastid markers (Hedré 2003; Devos et al. 2003, 2005; Ståhlberg and Hedré 2008, 2010; Nacz et al. 2015), and, most recently, RADseq data analyses (Brandrud et al. 2020). In general, all these methods revealed a similar pattern, dividing the *D. maculata* agg. into two major groups or clades, corresponding to two widely distributed taxa, namely *D. *maculata* and *D. *fuchsii* (the asterisk here and further on is used when dealing with taxa regardless of their taxonomic rank). The *fuchsii* group is considerably variable, but its genetic variation lacks any geographical structure. The *maculata* group, on the other hand, consists of two major evolutionary lineages with only a small contact zone between the southwestern and northeastern European lineage (Ståhlberg and Hedré 2008, 2010). However, contradictory results have been obtained for some other taxa. For example, diploid *D. *foliosa* is

either positioned as an early diverging group within the *D. maculata* agg. (Ståhlberg and Hedré 2010), or it is nested within the *maculata* clade (Brandrud et al. 2020). The southeastern European diploid *D. *saccifera* is usually considered close to *D. *fuchsii* but may alternatively represent an early diverging clade of the whole group (Brandrud et al. 2020; Bateman 2021). Several other taxa with more regional distributions are sometimes included in large-scale phylogenetic studies, for example *D. *caramulensis*, *D. *ericetorum*, *D. *islandica*, *D. *kolaënsis*, *D. *savogiensis* or *D. *transsilvanica*, and they usually appear to be segregates of the *maculata* clade. However, because they are almost constantly under-represented, little is known about their genetic variation and phylogenetic position. Moreover, hybridization between members of particular groups / clades has been suggested to occur (e.g. Ståhlberg and Hedré 2010; Nacz et al. 2015; Brandrud et al. 2020). The Madeiran endemic *D. foliosa* (Soland. ex Lowe) Soó is almost constantly recognized as a separate species, while the rest of the group may be treated as (i) a single species *D. maculata* (L.) Soó with three subspecies, namely subsp. *maculata*, subsp. *fuchsii* (Druce) Hyl. and subsp. *saccifera* (Brongn.) Diklić; (ii) two or more species, including *D. maculata* and *D. fuchsii* (Druce) Soó as the most frequent representatives; or (iii) a complex system of taxa recognized at the species, subspecies and variety levels.

These discrepancies are also apparent in the recent Central European taxonomic literature and regional floras with significant differences in the numbers of recognized taxa, their circumscription and, eventually, their taxonomic status (Table 1). A traditional concept of two species is applied in Hungary, where only *D. maculata* subsp. *transsilvanica* (Schur) Soó and *D. fuchsii* are recognized (Molnár and Csábi 2021), the latter alternatively including var. *sooana* ined. (Molnár 2011). A similar approach is applied in Germany (Müller et al. 2021), where a total of five taxa are recognized: *D. maculata* subsp. *maculata*, *D. maculata* subsp. *elodes* (Griseb) Soó, *D. fuchsii* subsp. *fuchsii*, *D. fuchsii* var. *sudetica* (Rchb.f.) H. Baumann, Künkele et R. Lorenz, and *D. fuchsii* subsp. *psychrophila* (Schltr.) Holub. However, the last has been recently rejected by Hassler and Muer (2022). Only *D. maculata* s. lat. is mentioned in the field guide to Austrian flora because of the unresolved taxonomy of the group (Fischer et al. 2008), but Redl (2003)

Table 1 List of groups recognized in this study, their abbreviations and final classification following the taxonomic concept accepted here. An overview of names used for these groups / taxa in most recent monographs, national orchid floras and other relevant taxonomic literature. En dashes (–) mark taxa not occurring in the area of interest of the particular work; question marks (?) denote taxa occurring in the respective area but not resolved by the author. Populations we surveyed that did not fall into any of these groups are referred to in this paper as ‘aggregate’, abbreviated as ‘agg’

This work – analysed groups (abbreviation)	This work – final clas- sification	Redl 2003	Vlčko et al. 2003	Kreutz 2004	Delforge 2006	Eccarius 2016	Ponert 2019	Mirek et al. 2020	Müller et al. 2021	Molnár and Csábi 2021
<i>maculata</i> (mac)	<i>D. maculata</i> subsp. <i>maculata</i>	<i>D. maculata</i>	<i>D. maculata</i> subsp. <i>maculata</i>	<i>D. maculata</i> subsp. <i>maculata</i>	<i>D. maculata</i>	<i>D. maculata</i> subsp. <i>maculata</i>	<i>D. maculata</i> subsp. <i>maculata</i>	<i>D. maculata</i>	<i>D. maculata</i> subsp. <i>maculata</i>	–
<i>fuchsii</i> (fuc)	<i>D. maculata</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i> (incl. subsp. <i>psychrophila</i>)	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i>	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i>	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i>
<i>sooana</i> (soo)	<i>D. maculata</i> subsp. <i>sooana</i>	–	<i>D. fuchsii</i> subsp. <i>sooana</i>	<i>D. fuchsii</i> var. <i>sooana</i>	?	<i>D. fuchsii</i> subsp. <i>fuchsii</i>	<i>D. fuchsii</i> subsp. <i>sooana</i>	–	–	?
<i>elodes</i> -WE (e-WE)	<i>D. maculata</i> subsp. <i>elodes</i>	–	–	<i>D. maculata</i> subsp. <i>elodes</i>	<i>D. maculata</i> var. <i>elodes</i>	<i>D. maculata</i> subsp. <i>maculata</i> ' <i>elodes</i> '	–	–	<i>D. maculata</i> subsp. <i>elodes</i>	–
<i>elodes</i> -BM (e-BM)	<i>D. maculata</i> subsp. <i>averyanovii</i>	–	–	<i>D. maculata</i> subsp. <i>maculata</i>	?	?	<i>D. maculata</i> subsp. <i>elodes</i> ' <i>averyanovii</i> '	?	–	–
<i>elodes</i> -CA (e-CA)	<i>D. maculata</i> subsp. <i>arcana</i>	–	<i>D. maculata</i> subsp. <i>elodes</i>	?	?	?	–	?	–	–
<i>ericetorum</i> (eri)	<i>D. maculata</i> subsp. <i>averyanovii</i>	–	<i>D. ericetorum</i>	?	?	?	–	–	–	–
<i>transsilvanica</i> (tra)	<i>D. maculata</i> subsp. trans- <i>silvanica</i>	–	<i>D. maculata</i> subsp. trans- <i>silvanica</i>	<i>D. maculata</i> subsp. trans- <i>silvanica</i>	<i>D. maculata</i> var. trans- <i>silvanica</i>	<i>D. maculata</i> subsp. trans- <i>silvanica</i>	<i>D. maculata</i> subsp. trans- <i>silvanica</i>	–	<i>D. fuchsii</i> var. <i>sudetica</i> (unclear)	<i>D. maculata</i> subsp. trans- <i>silvanica</i>
<i>psychrophila</i> (psy)	<i>D. maculata</i> subsp. <i>sudetica</i>	<i>D. sudetica</i>	–	<i>D. fuchsii</i> subsp. <i>sudetica</i>	<i>D. sudetica</i>	<i>D. maculata</i> subsp. <i>sudetica</i>	<i>D. fuchsii</i> subsp. psy- <i>chrophila</i>	<i>D. sudetica</i>	<i>D. fuchsii</i> subsp. psy- <i>chrophila</i>	–

recognized as many as three species in this country, namely *D. maculata*, *D. sudetica* (Rchb.f.) Averyanov, and *D. fuchsii* (incl. subsp. *psychrophila*). In Czechia, *D. maculata* is reported to consist of subsp. *maculata*, subsp. *transsilvanica* and subsp. *elodes* whereas *D. fuchsii* is divided into subsp. *fuchsii*, subsp. *sooana* ined. and subsp. *psychrophila* (Ponert 2019). The latter subspecies is treated at the species level by Mirek et al. (2020), who thus recognized a total of three species in Poland, *D. maculata*, *D. fuchsii* and *D. psychrophila* (Schltr.) Aver. The most intricate taxonomic concept is applied in Slovakia, where *D. maculata*, *D. fuchsii* and *D. ericetorum* (Linton) Aver. are recognized at the species level. *Dactylorhiza maculata* is further divided into three subspecies, namely subsp. *maculata*, subsp. *transsilvanica* and subsp. *elodes*, while *D. fuchsii* includes subsp. *fuchsii* and subsp. *sooana* (as '*sooiana*'); Vlčko et al. 2003).

The taxonomic concept used in a given country is mirrored in its national checklist, red lists, and legislation. It is thus crucial for the evaluation of the threat status of taxa recognized within any group (e.g. Bate-man and Denholm 2003; Pillon et al. 2006; Joffard et al. 2022). A unification of these concepts across national borders, based on a thorough examination of the variation of the *D. maculata* agg., is therefore needed for the effective protection of its members at a European level. In this study, we analyse the morphological variability, cytotype diversity and habitat conditions of *D. maculata* agg. populations throughout Central European countries. Our aims for this study were to re-evaluate the morphological variation, cytotype diversity and ecological differentiation between particular taxa of this group. To this end, we have attempted to resolve some taxonomic and nomenclatorial ambiguities and to provide a unified taxonomic concept and determination key for the group that would be applicable throughout the study area. Finally, we assess the Red List categories of particular taxa in Czechia, for which thorough distribution data are available.

Material and Methods

Plant material and Designation of Taxonomic Groups

Data were sampled primarily in populations of *D. maculata* agg. in Central European countries

(Austria, Czechia, Germany, Hungary, Poland and Slovakia). Additional populational samples were collected also in other parts of Europe, namely in Bulgaria, the Netherlands, Romania and Slovenia. For the purposes of the analyses detailed below, the populations were classified into several groups corresponding to taxonomic treatments used in the respective country (Vlčko et al. 2003; Molnár and Csábi 2021; Ponert 2019; Müller et al. 2021; Hassler and Muer 2022). Ambiguities were addressed as follows: (i) Because the taxonomic homogeneity of *D. *elodes* has been questioned (Vermeulen 1968; Szczepanski 2006; Kubát 2010), its populations from particular regions were analysed separately, distinguishing among *elodes*-WE (West Europe), *elodes*-BM (Bohemian Massif) and *elodes*-CA (Carpathians); (ii) A preliminary analysis of *D. *transsilvanica* (Taraška 2014) revealed a homogeneity of populations composed of typical plants and sympatric individuals with similar characters (morphological, karyological, ecological, and phenological), yet possessing flower and leaf pigmentation; all such plants were thus classified as *D. *transsilvanica*; (iii) Due to unsatisfactory treatment of the *D. maculata* agg. in Austrian and Polish literature, local populations were classified following the criteria used in neighbouring countries. In Poland, populations from the Bohemian Massif were determined following Ponert (2019), while those from the Carpathians and their foothills were classified according to Vlčko et al. (2003). In total, we recognized nine groups (Table 1): *elodes*-BM, *elodes*-CA, *elodes*-WE, *ericetorum*, *fuchsii*, *maculata*, *psychrophila*, *sooana* and *transsilvanica*. Several populations did not allow for unequivocal classification using the literature, so they were designated as 'aggregate' (also abbreviated as 'agg' in figures and tables). A total of 64 populations were used in the analyses; their list together with locality details is provided in Table S1 of the electronic supplementary material.

Morphometric Analysis

Morphological variability was assessed using univariate and multivariate morphometric analyses based on a total of 1,195 individuals originating from 58 populations (Table S1 in the electronic supplementary material), including 474 individuals from 25 populations of *D. *fuchsii* and *D. *sooana* used in a previous study (Taraška et al. 2021). The

morphological characters under study included those that are traditionally used in determination keys and special taxonomic literature for the delimitation of various *Dactylorhiza* taxa as well as characters identified in our preliminary screening of Central European populations of the *D. maculata* agg. Altogether, 17 quantitative and 5 qualitative traits

were measured or scored on living plants or on scans of flower lips; subsequently, 11 ratios were computed (Table 2; for a schematic illustration of the quantitative characters measured on examined plants, see Table S2a in the electronic supplementary material).

Six datasets were used for morphometric analyses. Pearson correlation coefficients were calculated for

Table 2 List of morphological traits measured or scored for *D. maculata* agg. and their abbreviations. For schematic illustration of quantitative traits, see Table S2a in the electronic supplementary material

No.	Character abbreviation [unit]	Numerical characters
1.	hPl [mm]	plant height
2.	nrL [count]	number of leaves
3.	IL1 [mm]	length of the 1st leaf
4.	wL1 [mm]	width of the 1st leaf
5.	aL1 [°]	angle between the stem and the 1st leaf
6.	IL2 [mm]	length of the 2nd leaf
7.	wL2 [mm]	width of the 2nd leaf
8.	mL2 [mm]	distance between the base of the 2nd leaf and its widest part
9.	aL2 [°]	angle between the stem and the 2nd leaf
10.	A [mm]	flower trait (see Table S2a in the electronic supplementary material)
11.	B [mm]	flower trait (see Table S2a)
12.	C [mm]	flower trait (see Table S2a)
13.	E [mm]	flower trait (see Table S2a)
14.	F [mm]	flower trait (see Table S2a)
15.	lSp [mm]	length of the spur
16.	wSp [mm]	width of the spur in the middle of its length
17.	ipInf	intensity of pigmentation of the inflorescence (3–9); sum of values for axis, bracts and ovaries, each classified as: 1 – green, 2 – purple, 3 – dark purple
Categorical characters		
18.	sLA1a, sLA1s, sLA1o	shape of the 1st leaf apex: a – absent, s – subacute, o – obtuse
19.	sLA2a, sLA2s, sLA2o	shape of the 2nd leaf apex: a – absent, s – subacute, o – obtuse
20.	cLBw, cLBp, cLBd	colour of the labellum: w – white, p – pale, d – dark
21.	mLBa, mLBp, mLBb	marking of the labellum: a – absent, p – pale, b – bold
22.	spLa, spLp, spLb	spots on the leaves: a – absent, p – pale, b – bold
Derived numerical characters – formulas		
23.	lSp/wSp	lSp/wSp
24.	lSp/A	lSp/A
25.	hPl/IL1	$hPl/IL1$
26.	hPl/IL2	$hPl/IL2$
27.	hPl/nrL	hPl/nrL
28.	IL1/wL1	$IL1/wL1$
29.	IL2/mL2	$IL2/mL2$
30.	HH; Heslop-Harrison index	$2A/(B + C)$
31.	AD	$A/(A - C)$
32.	FE	F/E
33.	BBC	$B/(B - C)$

all datasets prior to all multivariate analyses to check for highly correlated pairs of quantitative characters ($|r| \geq 0.9$). Whenever a pair of characters was highly correlated, one character from the pair was excluded. Multicollinearity in categorical characters was examined using Cramer's V (Legendre and Legendre 1998), but no pair of characters showed high association coefficients. An overview of the datasets, the types of OTUs used, groups and characters, and analyses performed is presented in Table 3.

Agglomerative hierarchical clustering (Ward's and UPGMA methods) and principal component analysis (PCA), using Euclidean distance and standardization of traits to a zero mean and unit variance, were carried out using populations as operational taxonomic units (OTUs). The relative frequency of each state of particular categorical variable was considered as a quantitative variable. Principal coordinate analysis (PCoA) using Gower's dissimilarity coefficient (Legendre and Legendre 1998) was used to obtain insight into the phenetic relationships among individuals of all groups studied and with the aggregate group excluded.

To test the morphological differentiation among a reduced set of seven groups and to identify the traits contributing the most to the differentiation among groups, partial least-squares discriminant analysis (PLS-DA; Barker and Rayens 2003; Scott and Crone 2021) was employed. The *fuchsii* and *sooana* groups, whose variability was previously studied by Taraška et al. (2021), were excluded from this reduced dataset in order to obtain more detailed insight into the variability of the other groups. Populations of the aggregate group were excluded

as well, because they do not represent a coherent taxonomic unit. This reduced dataset was randomly divided into a training set (i.e. about 75% of the dataset) and a validation set (25%) balanced across the groups. Ten-fold cross-validation was used to estimate the number of components required for the best performance of PLS-DA. The area under the curve (*AUC*) was calculated from training cross-validation sets to complement the performance of PLS-DA and averaged across one-vs-all group comparisons. Using the final tuned model, variable importance in the projection (*VIP*), which is an indicator of the modelling power of a predictor in PLS, was calculated for each analysed morphological variable. Confusion matrices were constructed for the final model which summarizes the success of the reclassification / prediction of the observations for the training and validation samples, respectively.

To estimate whether a priori unclassified populations (the aggregate group) are really morphologically transient, they were passively projected into the ordination space in the PCA of populations, and an additional PCoA was carried out with all individuals as OTUs, including those of aggregate populations.

For each study group, descriptive data analysis was carried out to obtain basic statistics of quantitative traits and ratios (minimum, mean, maximum and standard deviation). For qualitative traits, the frequencies of particular states of character were calculated. To illustrate the variation in selected traits, box-and-whisker or stacked bar plots were used. The Kruskal–Wallis test was used for the comparison of quantitative characters and their ratios. Differences in qualitative characters were analysed by the χ^2 test.

Table 3 An overview of the datasets, types of OTUs, set of groups and characters excluded, and analyses employed in this study

Dataset	Number of populations	Number of individuals	OTU used	Groups excluded	Characters excluded	Descriptive statistics	Clustering analyses	Ordination analyses	PLS Discriminant analysis
Dataset 1	58	1,195	individuals	–	–	DS_1	–	–	–
Dataset 2a	51	1,018	individuals	agg	IL2/wL2	–	–	PCoA_1	–
Dataset 2b	58	1,195	individuals	–	IL2/wL2	–	–	PCoA_2	–
Dataset 3	28	544	individuals	agg, <i>fuchsii</i> , <i>sooana</i>	–	–	–	–	PLS-DA_1
Dataset 4	51	–	population	agg	IL2, wL2, C	–	CLUST_1, CLUST_2	PCA_1, PCA_2	–
Dataset 5	26	–	population	agg, <i>fuchsii</i> , <i>sooana</i>	IL2, wL2, C	–	–	PCA_3	–

Most statistical analyses were performed using R 4.0.4 (R Core Team 2022). PCA and PLS-DA were computed using the mixOmics 3.15 package (Rohart et al. 2017) and the software x1stat (Addinsoft 2022), hierarchical clustering and descriptive statistics using the MorphoTools package (Koutecký 2015). PCoA was computed using Canoco 5.12 (ter Braak and Šmilauer 2012), ANOVAs, and log-linear models were run using the NCSS 9 software (NCSS 2013).

Ploidy Level Determination

DNA ploidy level was estimated by flow cytometry (FCM) following the protocol of Doležel et al. (2007). In total, 989 individuals from 64 populations were analysed (Table S1 in the electronic supplementary material). Plant material collected in the field was stored in a wet paper tissue at 4°C until processed, usually within 1–5 days. One or two ovaries of *Dactylorhiza* were analysed together with leaf tissue of the internal standard *Pisum sativum* cv. Ctírad (2C = 9.09 pg; Doležel et al. 1998). For triploids, the analysis was repeated with *Zea mays* cv. CE-777 (2C = 5.43 pg; Lysák and Doležel 1998). The nuclei solution was prepared by co-chopping the sample and standard tissue (Galbraith et al. 1983) in LB01 buffer with polyvinylpyrrolidone (PVP, 20 mg/ml; Doležel et al. 2007) in a Petri dish and subsequent filtration through a 40-µm nylon mesh. Before analysis, 30–50 µl of the respective fluorescent dye (depending on the laboratory and the type of flow cytometer) was added, which was either 4,6-diamidino-2-phenylindole (DAPI, 4 µg/ml) or propidium iodide (PI, 50 µg/ml). The samples stained with PI were also supplemented with 30 µl of RNase to digest RNA.

Four flow cytometers were used: BD Accuri C6 (BD Biosciences, San Jose, CA, USA) and Partec CyFlow ML (Partec GmbH, Münster, Germany) at the Department of Botany, Palacký University Olomouc; Partec CyFlow ML at the Department of Botany and Biodiversity Research, University of Vienna; and Partec CyFlow ML at the Institute of Experimental Botany, Olomouc. Individual plants were analysed as separate samples and the fluorescence of at least 3,000 particles was recorded in each run. FCM histograms were analysed in BD Accuri software or Partec FloMax software. Relative fluorescence was calculated for each plant as the ratio of the mean position of G₀/G₁ peak (cf.

2C-peak; Trávníček et al. 2015) of *Dactylorhiza* and the mean position of the G₀/G₁ peak of the internal standard. The ratios obtained from analyses with *Z. mays* were recalculated to *P. sativum* using a coefficient 2.25 (value obtained from several simultaneous measurements of *Zea* and *Pisum*). A subset of fourteen individuals were analysed with both fluorescent dyes (i.e. DAPI and PI) to assure compatibility between results obtained by different staining methods. These measurements were then used for the calculation of the ratio between DAPI and PI. The value of 0.88 was used to recalculate the standard:sample ratio of PI-stained samples. For the *fuchsii* and *sooana* groups, the same data were employed as in our previous study (Taraška et al. 2021).

Chromosome Counts

Gametophytic chromosome numbers (*n*) were established in immature pollinaria. Flower buds were collected ca 5–10 days before flowering, fixed in an ethanol : acetic acid (3 : 1) solution and stored at –20°C until use. The chromosomal spreads were made following the standard protocol of Feulgen staining (Weiss et al. 2003). Briefly, flower buds were hydrolysed in 5 N HCl for 30 min at room temperature, washed with water and stained with Schiff's reagent (Sigma, Vienna, Austria) for 1–2 hours. Afterwards, pollinaria were extracted from the buds and squashed in 60% acetic acid. Chromosome spreads were observed under 1,000× magnification using an Olympus BX60 microscope equipped with an Olympus DP72 digital camera (both Olympus, Tokyo, Japan) and Axioplan light microscope (Carl Zeiss, Jena, Germany). Chromosomes were counted in at least ten cells per individual.

Environmental Differentiation Between Groups

To test associations of groups with environmental conditions, values for 19 bioclimatic variables and mean annual solar radiation, and 24 physical and chemical soil variables for each population were obtained from WorldClim 2.1 (Fick and Hijmans 2017) and SoilGrid 2.0 (Hengl et al. 2017), respectively. Bioclimatic and soil variables had a spatial resolution of ca 1 km and 250 m, respectively. Prior to the analyses, the variance inflation factor (VIF) was calculated for a set of

variables and the highly correlated variables with biologically less meaningful importance were excluded from the set through a stepwise procedure using the ‘vifstep’ ($th = 15$) function from the usdm package (Naimi et al. 2014). Elevation as well as six bioclimatic and eight soil variables (from the top 5 cm soil layer) were preselected and analysed by discriminant analysis (DA) using Canoco 5.12. The significance of the first and all discriminant axes was evaluated by a Monte Carlo permutation test with 499 permutations. Additionally, the vegetation type of each population was recorded in the field and later reclassified into the phytosociological syntaxa using the level of phytosociological order according to the Hierarchical floristic classification system of European vegetation (Mucina et al. 2016). One habitat category was classified separately as forest roadside ditches because it was impossible to assign this habitat to any syntaxon. The frequency distribution of vegetation types for the groups studied was visualized as a mosaic plot. The aggregate group was excluded from the DA but included in the boxplot and mosaic plot.

Estimation of the IUCN Red List Categories

All members of the *D. maculata* agg. occurring in Czechia were evaluated against the Red List criteria following the methodology of IUCN (2012a, b). Data on their recent and former distribution were obtained from our current research, critically evaluated floristic records (Kaplan et al. 2017) and the Pladias database (Wild et al. 2019), with regard to differences in nomenclature and the circumscription of some taxa. The categories presented here substitute the categories previously published by Grulich (2017). The threat status was not estimated for other Central European countries because of a lack of data on geographic distribution and population abundance.

Results

Population-Level Morphometrics

Cluster analysis of populations as OTUs (CLUST_1 analysis; Ward’s method; Table 3) resulted in two main clusters (‘a’ and ‘b’). Cluster ‘a’ included populations of the *fuchsii* and *sooana* groups, and cluster ‘b’ consisted of the rest of the groups (Fig. 1a). Using

slice at a distance of 15, cluster analysis recognized seven clusters that mostly corresponded to the groups under study. The only exceptions were the *elodes*-BM and *ericetorum* groups and populations RUD and JES of the *maculata* group that were grouped together into one cluster, as well as population PBZ of the *maculata* group and SMU of the *fuchsii* group that were clustered with populations of the *transsilvanica* group (Fig. 1a). Cluster analysis using the UPGMA method (CLUST_2 analysis) also revealed clusters mostly corresponding to the groups studied using a smaller distance slice width (Table S3a in the electronic supplementary material), but the clustering pattern did not recognize two main clusters (‘a’, ‘b’) found by the CLUST_1 analysis (Fig. 1a).

The main gradient revealed by the first axis of the PCA (PCA_1, Fig. 1b) corresponded to the differentiation between the *fuchsii*, *sooana* and partially also *transsilvanica* groups on the right-hand side and all other groups on the left-hand side. Populations of the respective groups usually tended to occur in close proximity, but no apparent discontinuities between clusters of neighbouring groups were identifiable in the ordination diagram. Populations of the *elodes*-BM and *ericetorum* groups clumped together. The first PCA axis was positively correlated mainly with leaf width (wL1), plant height (hPl), the ratio of plant height to the length and number of leaves (hPl/IL1, hPl/IL2, hPl/nrL) and some flower size/shape traits (E, HH). It was negatively correlated mainly with some flower size traits and their ratios (B, AD, BBC) and leaf shape (IL1/wL1). The shape of the leaf apex (sLA) was mostly obtuse on the right and most acute on the left of the first PCA axis. The second PCA axis was mostly related to the pigmentation of vegetative and flower parts of the plants. Along the second PCA axis, the frequency of populations with a pink to purple labellum (cLBp) with bold markings (mLBb) and darker parts of inflorescence (ipInf) decreased, and the frequency of populations with a white labellum (cLBw) with absent markings (mLBa) increased (Fig. 1c). No morphological differentiation between diploid and tetraploid populations of the *fuchsii* group was identifiable from the PCA (Fig. 1b). Passively projected aggregate populations within the PCA diagram (PCA_2 analysis, Table S3b in the electronic supplementary material) filled the ordination space in-between several groups, namely the *fuchsii*, *maculata*, *psychrophila* and *elodes*-CA groups.

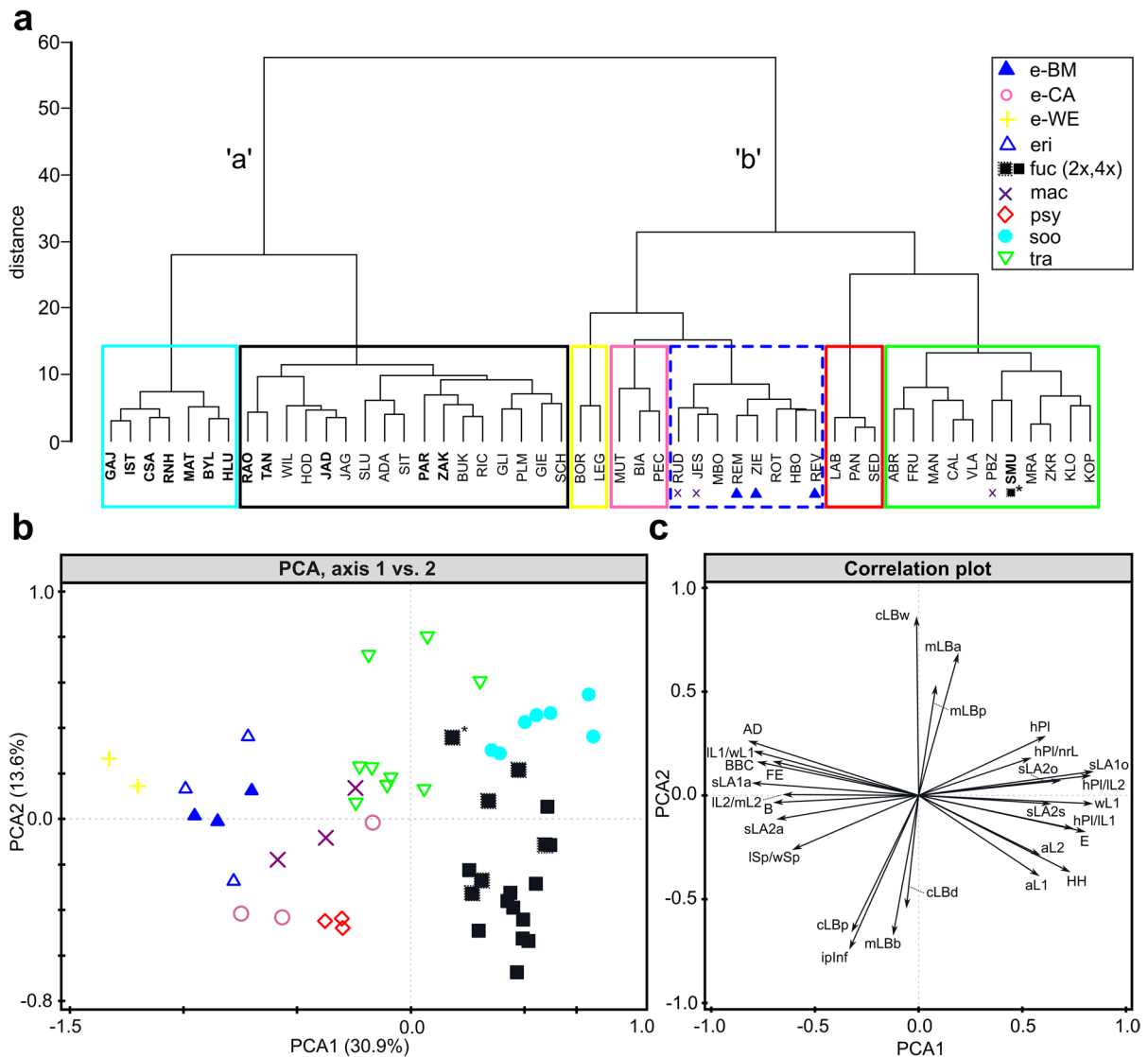


Fig. 1 Multivariate analyses of morphological traits of *D. maculata* agg. populations as OTUs. Groups are identified by different colours and symbols. **a** – Results of hierarchical cluster analysis using Ward’s method (CLUST_1 analysis, Table 3) with resulting clusters ‘a’ and ‘b’. Boxes demarcate clustered populations at the respective distance ($d=15$). Codes of populations in bold and normal styles represent (predominantly) diploid and tetraploid populations, respectively. Symbols below some population codes denote their group identity. * – SMU population of the *fuchsii* group misclassified into the cluster predominated by the *transsilvanica* group.

Population codes are explained in Table S1 in the electronic supplementary material. **b** – Sample plot of the first two axes (PCA1, PCA2) of the PCA (PCA_1 analysis, Table 3). Variation explained by each axis is within parentheses. Predominantly diploid and tetraploid populations of the *fuchsii* group are distinguished by different symbols. **c** – PCA correlation plot of analysed characters. Only variables whose correlations exceed |0.50| with at least one axis are displayed in the plot. Group abbreviations are explained in Table 1 and character abbreviations in Table 2.

Because the relationships between populations within cluster ‘a’ have already been studied by us in another paper (Taraška et al. 2021), we conducted

further multivariate analyses with populations of cluster ‘b’ (dataset 5; Table 3). The ordination space of the first three PCA ordination axes (PCA_3

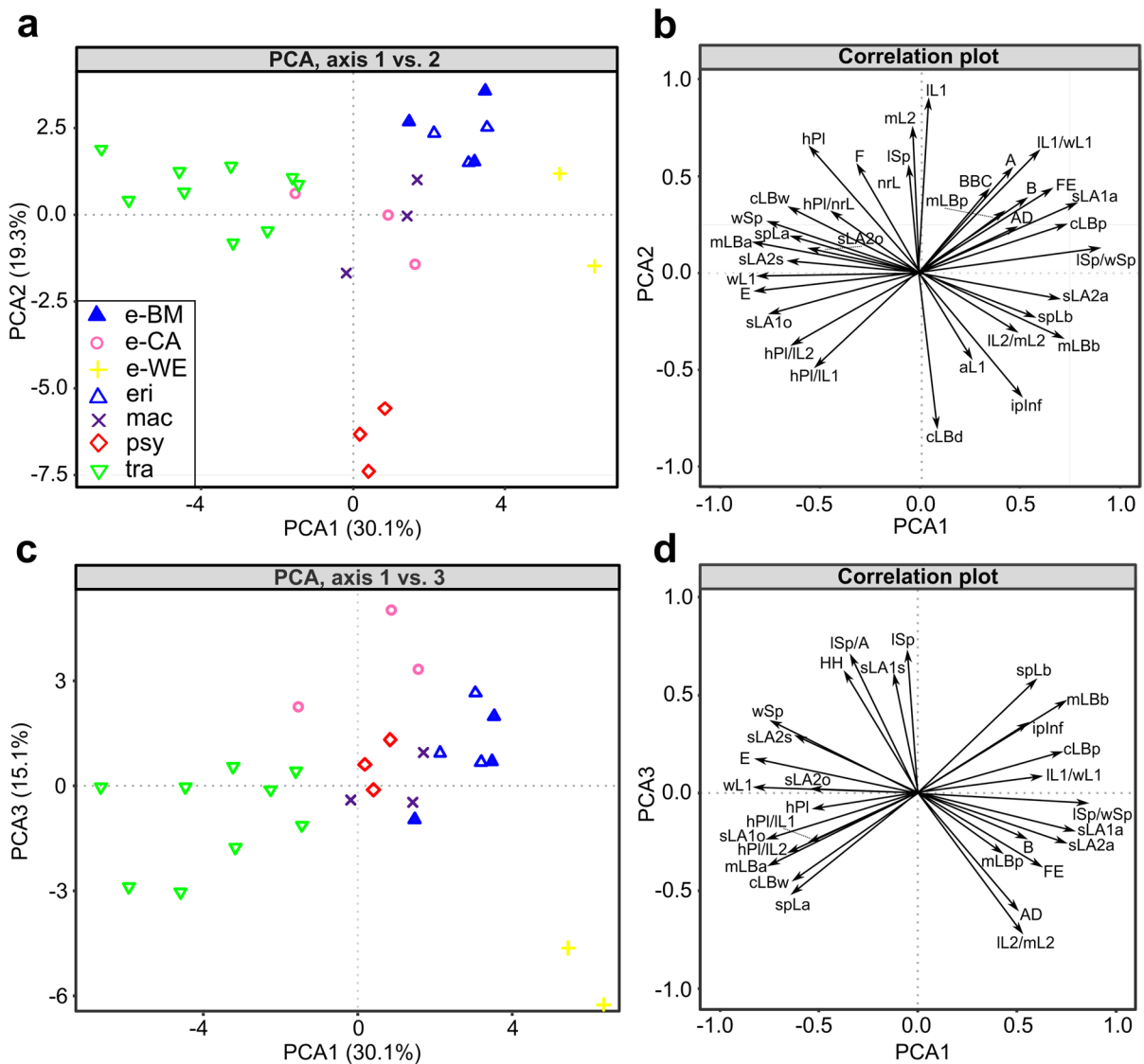


Fig. 2 Multivariate analyses of morphological traits of *D. maculata* agg. populations as OTUs, with populations of the *fuchsii* and the *sooana* groups excluded (dataset 5, Table 3). Results of PCA_3 with (a, b) axes 1 and 2 and (c, d) axes 1 and 3, with sample plots and correlation plots. Vari-

ation explained by each axis is within parentheses. Only variables whose correlations exceeded |0.50| with at least one axis are displayed in the plot. Group abbreviations are explained in Table 1 and character abbreviations in Table 2.

analysis, Fig. 2a, c) showed the clustering of populations of each studied group, but the *elodes*-BM and *ericetorum* groups clustered together. Characters correlated with the first PCA axis indicated that plants of the *elodes*-WE group typically had a high spur length / width ratio (ISp/wSp), an acute leaf apex (sLA1a, sLA2a) and a narrow middle lobe of the lip (F/E). On the opposite side of the first PCA axis,

plants of the *transsilvanica* group were typically taller (hPl), with subacute to obtuse apices of the leaves (sLA2s, sLA2o), and flowers often having a white labellum (cLBw) without markings (mLBa; Fig. 2b). The *psychrophila* populations strongly separated from the other groups along the second PCA axis (Fig. 2a), mainly due to intensive pigmentation of their lips (cLBd) as well as other parts of the inflorescence

(ipInf), and several traits related to plant height and stature (Fig. 2b). The third PCA axis (Fig. 2c) separated populations of the *elodes*-WE group with the lowest scores and the *elodes*-CA group with the highest scores from the populations of other groups with intermediate scores. Plants of the *elodes*-WE group had flowers with a relatively short spur (ISp/A) and low Heslop-Harrison index (HH) and their leaves were widest in the basal part (IL2/mL2), while plants of the *elodes*-CA group had flowers with both absolutely and relatively long spur (ISp, ISp/A), and rather intensely pigmented both inflorescence (mLBb, ipInf) and leaves (spLb; Fig. 2d).

Individual-Level Morphometrics

The first two axes of the PCoA of individuals as OTUs (PCoA_1 analysis, Fig. 3a, Table 3) revealed an almost identical pattern as that found in the PCA of populations as OTUs (PCA_1 analysis, Fig. 1b) but with marked overlap among groups. While the first PCoA axis represented a composite gradient of both quantitative and qualitative characters, the

second PCoA axis was primarily correlated with qualitative characters related to the colour of the labellum (cLB) and spots on the leaves (spL), separating plants with a white labellum (cLBw) with absent or pale markings (mLBa, mLBp) and leaves without spots (spLa) in the upper part from the plants with darker flowers (cLBp) and intensely pigmented inflorescences (ipInf) in the bottom part of the ordination diagram (Fig. 3b).

Plants of the aggregate group included in the PCoA (PCoA_2 analysis, Table 3) were spread over most parts of the ordination diagram, but most of them were placed in its bottom part, where they overlapped with marginal parts of the morphospaces of several other groups, namely the *fuchsii*, *elodes*-BM, *ericetorum*, *elodes*-CA, and *psychrophila* groups (Table S3c in the electronic supplementary material).

Univariate descriptive statistics are presented in Table S2b, c, d in the electronic supplementary material. Box-and-whisker plots or stacked bar plots of the traits studied for each group (DS_1 analysis; Table 3) are presented in Table S3f, g in the electronic supplementary material.

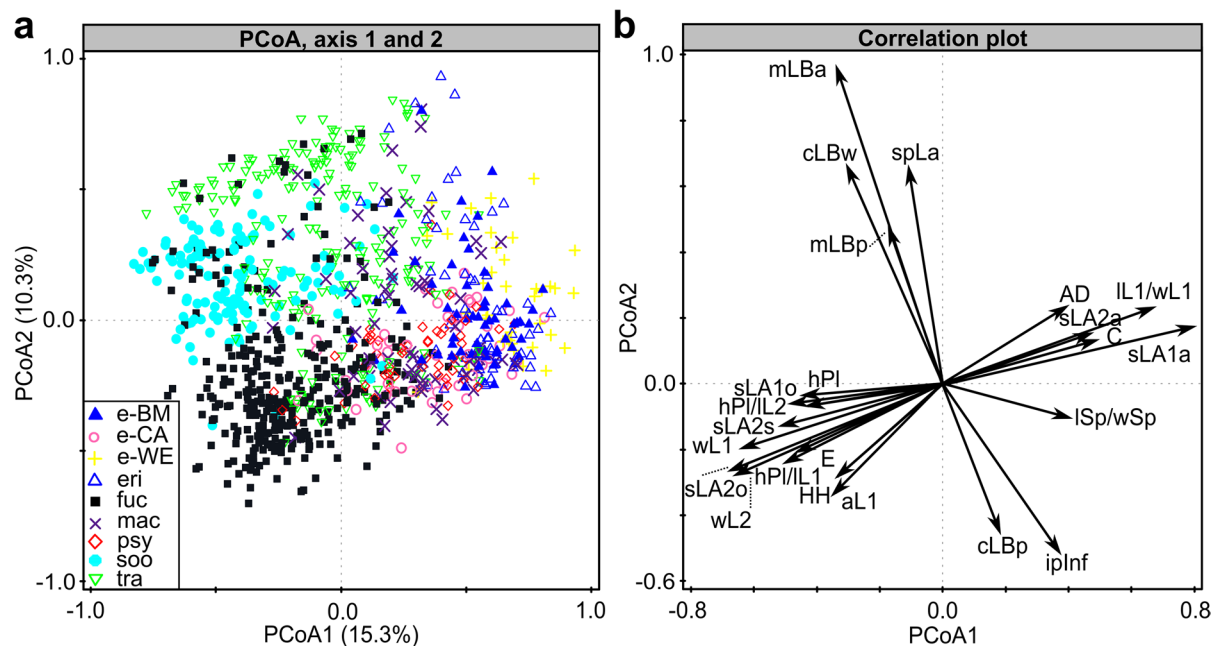
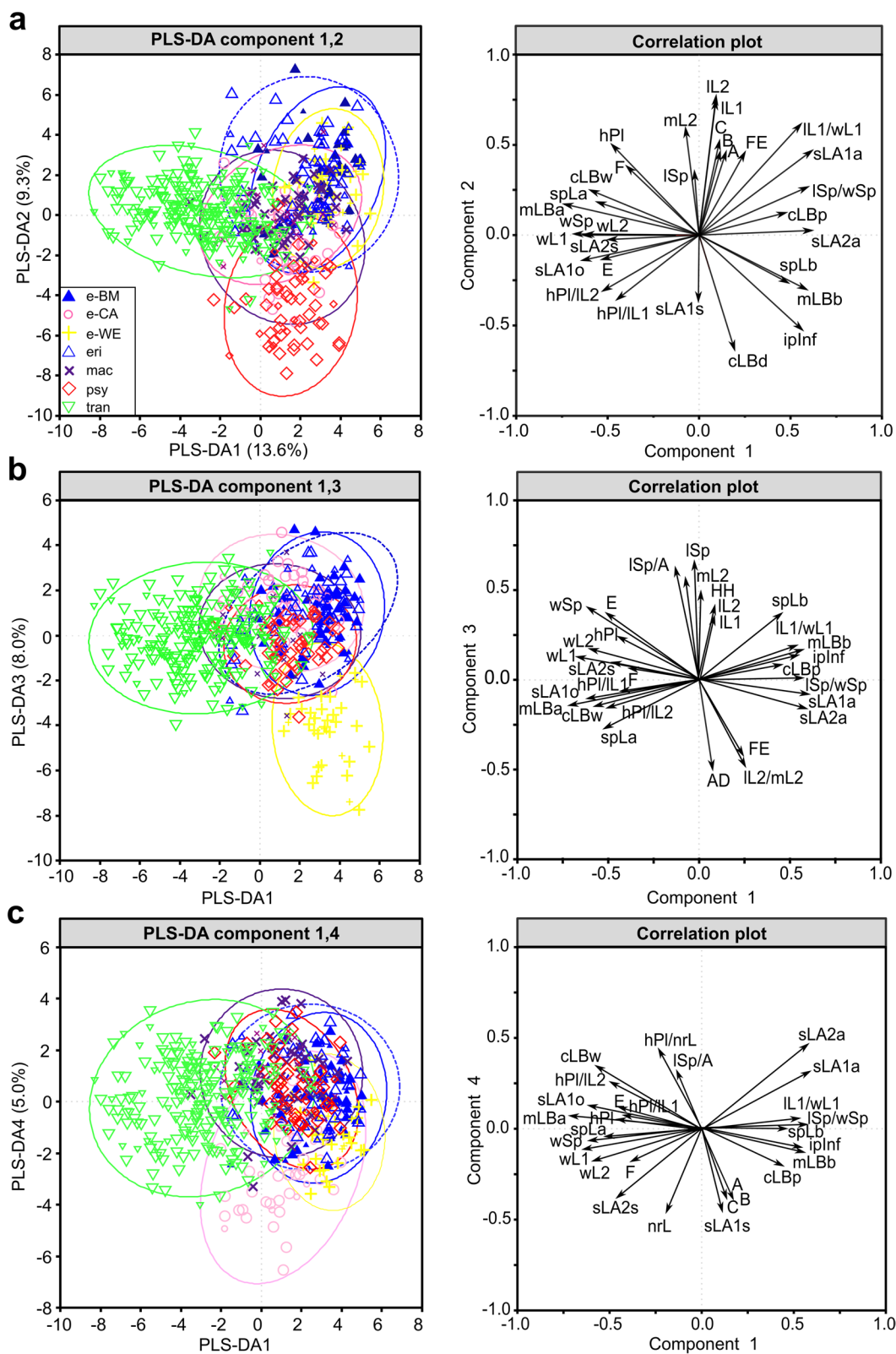


Fig. 3 Multivariate analyses of morphological traits of *D. maculata* agg. individuals as OTUs, with populations of the aggregate group excluded (dataset 2a, Table 3). **a** – Sample plot of the first two axes (PCoA1, PCoA2) of PCoA_1 (Table 3). Variation explained by each axis is within paren-

theses. **b** – PCoA correlation plot of characters analysed. Only variables whose correlations exceed 0.401 with at least one axis are displayed in the plot. Group abbreviations are explained in Table 1 and character abbreviations in Table 2.



◀**Fig. 4** Sample plots and correlation plots from partial least squares discriminant analysis (PLS-DA₁) of individuals of seven taxonomic groups of *D. maculata* agg. (dataset 3, Table 3), divided into training (75% of dataset) and validation (25%) samples and balanced across the groups. The first four predictive components as axes are visualized. **a** – PLS-DA1 vs PLS-DA2, **b** – PLS-DA1 vs PLS-DA3, **c** – PLS-DA1 vs PLS-DA4. Ellipses are drawn for each group representing 95% quantile of the approximated bivariate normal density distribution. Only variables with Pearson correlations $> |0.30|$ with at least one predictive component within each plot are displayed in the respective correlation plot. Variability of the Y matrix (intergroup variability) explained by respective predictive components (in %) are displayed within parentheses. Group abbreviations are explained in Table 1 and character abbreviations in Table 2. Large-sized symbols represent training samples, and small-sized symbols represent validation samples passively projected into the plots.

PLS Discriminant Analysis

PLS discriminant analysis of individuals (PLS-DA₁ analysis, Table 3) estimated the number of 8 predictive components to be optimal for the final model, with $R^2X_{\text{cum}} = 0.570$, $R^2Y_{\text{cum}} = 0.451$, and $Q^2 \text{ cum} = 0.394$. This suggests a rather complex structure of the dataset. Seventeen variables (or their categories) had a $VIP > 1$ and could be considered important for discrimination between groups (Table S3d in the electronic supplementary material), with two qualitative (cLB, mLB) and four quantitative variables or ratios (ISp, wSp, IL1/wL1, ISp/A) having the highest VIP . The distribution of individuals of groups in the space of the first four components showed satisfactory discrimination of the *transsilvanica* group from the *elodes*-BM and the *elodes*-WE groups on the first component, and the *psychrophila* group vs. most other groups on the second component (Fig. 4a). Adding the third and fourth components differentiated the *elodes*-WE and the *elodes*-CA groups from most other groups (Fig. 4b, c). Only the *maculata* group was difficult to discriminate from the other groups, which is also clear from the cumulative AUC values (Table S3e in the electronic supplementary material) and the confusion matrices (Table 4). The analysis revealed that 81.6%/77.2% of the individuals could be correctly reclassified / predicted in the training / validation subsets. The *maculata* and *ericetorum* groups resulted in the lowest classification accuracy, approaching 51.9%/52.4% and 59.1%/38.9% (training / validation subset), respectively. The *elodes*-CA and *elodes*-BM groups showed an intermediate percentage of correctly classified individuals (73.7%/70.0%;

68.3%/68.8%). More than 95% of individuals in other groups were correctly reclassified / predicted in both training and validation subsets. The largest morphological overlap was found between the *maculata* and the *transsilvanica* groups and between the *elodes*-BM and the *ericetorum* groups (Table 4).

Chromosome Numbers and Ploidy Level Screening

Chromosome numbers were established for ten individuals representing five groups. Two different gametophytic chromosome counts were encountered among the plants analysed: $n = 20$ and $n = 40$, corresponding to diploids and tetraploids, respectively. Diploid chromosome numbers were found in the *sooana* group and one individual of the *fuchsii* group (see also Taraška et al. 2021), while tetraploid plants belonged to the *elodes*-CA, *elodes*-BM, *elodes*-WE and *fuchsii* groups. These counts were used to calibrate the results of the flow cytometry analyses (Table S4 in the electronic supplementary material).

Two major ploidy levels were found: diploids and tetraploids. Furthermore, two minority cytotypes were detected, for which chromosome numbers were not established, with relative fluorescence corresponding to DNA-triploids and DNA-hexaploids. Diploids were confined to the *sooana* group and about one-third of analysed individuals of the *fuchsii* group, while relative fluorescence corresponding to tetraploids was detected in some individuals of the *fuchsii* group, the majority of individuals of the *elodes*-CA and *transsilvanica* groups, and in all individuals of the *elodes*-BM, *elodes*-WE, *maculata* and *psychrophila* groups (Table 5). DNA-triploids were detected only in the *fuchsii* group, and DNA-hexaploids were found within the *elodes*-CA and *transsilvanica* groups (Table 5). These cytotypes always co-occurred in mixed-ploidy populations with some of the major cytotypes.

Environmental Differentiation Between Groups

Discriminant analysis of environmental variables produced eight discriminant axes (1. DA: pseudo- $F = 0.3$, $P = 0.002$, all DA: pseudo- $F = 2.7$, $P = 0.002$) and showed that the populations of the *elodes*-WE and *psychrophila* groups were the most distinct in terms of environmental conditions (Fig. 5a). Populations of the *elodes*-WE group were situated at the lowest elevations,

Table 4 Results of partial least squares discriminant analysis (PLS-DA_1) of individuals of dataset 3 (see Table 3) with six taxonomic groups of *D. maculata* agg. Confusion matrices for the training (408 individuals in total) and the validation (136 individuals in total, numbers in parentheses) samples

From / to	<i>elodes</i> -BM	<i>elodes</i> -CA	<i>elodes</i> -WE	<i>ericetorum</i>	<i>maculata</i>	<i>psychrophila</i>	<i>transsilvanica</i>	Total	% Correct (training)	% Correct (validation)
<i>elodes</i> -BM	28 (11)	1 (0)	0 (1)	2 (2)	2 (1)	1 (0)	7 (1)	41 (16)	68.29	68.75
<i>elodes</i> -CA	1 (1)	28 (7)	0 (0)	3 (0)	1 (0)	1 (0)	4 (2)	38 (10)	73.68	70.00
<i>elodes</i> -WE	0 (0)	0 (0)	29 (8)	0 (0)	0 (0)	0 (0)	0 (0)	29 (8)	100.00	100.00
<i>ericetorum</i>	7 (4)	1 (2)	2 (0)	26 (7)	2 (1)	0 (1)	6 (3)	44 (18)	59.09	38.89
<i>maculata</i>	1 (1)	1 (1)	1 (1)	5 (1)	27 (11)	1 (0)	16 (6)	52 (21)	51.92	52.38
<i>psychrophila</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	41 (13)	2 (0)	43 (13)	95.35	100.00
<i>transsilvanica</i>	1 (0)	0 (0)	0 (1)	1 (1)	2 (0)	3 (0)	154 (48)	161 (50)	95.65	96.00
Total	38 (17)	31 (10)	32 (11)	37 (11)	34 (13)	47 (14)	189 (60)	408 (136)	81.62	77.21

Table 5 Ploidy level variation in the studied groups of *D. maculata* agg. *N* – number of individuals analysed; % – proportion of detected cytotype in the group; Mean – mean sample : standard ratio for DAPI staining and *Pisum sativum* cv. Ctirad as an internal standard. As several flow cytometers were used for the analysis, sample : standard ratios are shown here only for the purpose of DNA-ploidy level estimation

Group	<i>N</i>	%	Mean	<i>SD</i>	Inferred ploidy
<i>elodes</i> -BM	38	100.00	1.270	0.062	4x
<i>elodes</i> -CA	58	98.31	1.192	0.027	4x
	1	1.69	1.750	–	6x
<i>elodes</i> -WE	32	100.00	1.167	0.016	4x
<i>ericetorum</i>	32	100.00	1.241	0.043	4x
<i>fuchsii</i>	83	32.68	0.691	0.023	2x
	5	1.97	0.998	0.014	3x
	166	65.35	1.212	0.041	4x
<i>maculata</i>	54	100.00	1.215	0.044	4x
<i>psychrophila</i>	31	100.00	1.234	0.033	4x
<i>sooana</i>	121	100.00	0.679	0.016	2x
<i>transsilvanica</i>	220	99.55	1.194	0.049	4x
	1	0.45	1.848	–	6x
agg	143	100.00	1.225	0.042	4x

having the lowest amount of solar radiation (Srad), the lowest values of temperature (Bio4) and precipitation seasonalities (Bio15), and the highest mean annual temperature (Bio1). Populations of the *psychrophila* group occupied the highest elevations above 1,100 m a.s.l., with the lowest mean annual temperature (Bio1) and isothermality (Bio3), high cation exchange capacity (CECSOL) and the highest soil organic matter content (ORCDRC) (Fig. 5a, Table S5 in the electronic supplementary material).

Discriminant analysis of the reduced dataset (without the *elodes*-WE and *psychrophila* groups) produced six discriminant axes (1. DA: pseudo- $F = 0.3$, $P = 0.004$, all DA: pseudo- $F = 1.8$, $P = 0.006$) and revealed that the populations of the *sooana* and *transsilvanica* groups and some populations of the *fuchsii* group situated on the right side of the diagram occupied sites with higher temperature seasonality (Bio4) and amount of solar radiation (Srad) and soils with higher pH and proportion of clay particles (CLYPPT), lower participation of soil organic matter (ORCDRC), lower probability of histosol occurrence (HISTPR) and smaller available soil water capacity (AWCh2) (Fig. 5b, Table S5 in the electronic

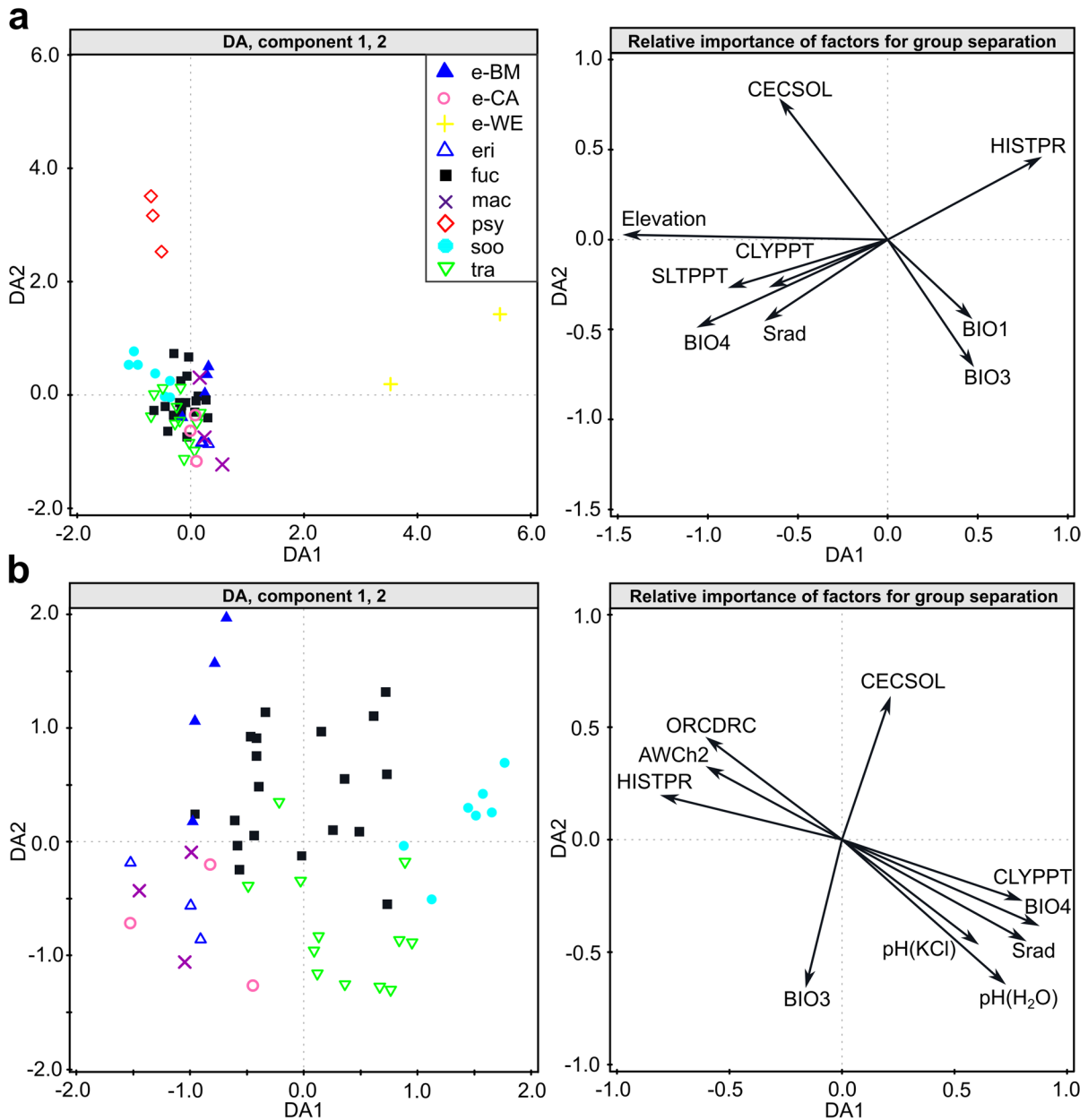


Fig. 5 Sample plots and plots of relative importance of factors for group separation from discriminant analysis (DA) of environmental conditions extracted from the WorldClim and SoilGrid databases for the sites of groups of *D. maculata* agg. studied (abbreviations explained in Table 1). The first two components are visualized in each diagram. **a** – DA of nine groups with aggregate group excluded, **b** – DA of seven groups with the aggregate, *elodes*-WE and *psychrophila* groups excluded. The proportion of intergroup variability explained by the respective discriminant axis (in %) is displayed within parentheses. Explanations of variables (for details see Fick and Hijmans 2017; Hengl et al. 2017): Elevation – elevation;

Srad – mean annual solar radiation; BIO1 – mean annual temperature; BIO3 – isothermality (BIO2/BIO7) ($\times 100$); BIO4 – temperature seasonality (standard deviation $\times 100$); pH(KCl) – soil pH measured in KCl solution; pH(H₂O) – soil pH measured in water solution; SLTPPT – weight percentage of the silt particles (0.0002–0.05 mm); CLYPPT – weight percentage of the clay particles (< 0.0002 mm); ORCDRC – soil organic carbon content; CECSOL – cation exchange capacity of soil; AWCh2 – available soil water capacity (volumetric fraction) with $FC = pF\ 2.3$; HISTPR – Histosols probability cumulative. Only the best discriminating variables are shown in the diagrams.

supplementary material). Populations of the *maculata*, *elodes*-BM, *ericetorum* and *elodes*-CA groups were situated on the opposite side of the diagram, preferring sites with a lower amount of solar radiation (Srad) and temperature seasonality (Bio4), and with more acidic soils (pH) containing higher amounts of organic matter (ORCDRC) and available soil water capacity (AWCh2). Populations of the *fuchsii* group were intermediate in climatic and soil variables between the groups mentioned above. Boxplots of selected bioclimatic and soil variables for each group are available in Table S5 in the electronic supplementary material.

However, being aware of the different sample sizes between the study groups, it is possible to observe habitat differences between them (Fig. 6). The *elodes*-WE and *psychrophila* groups each inhabited one specific vegetation type, only recorded in these groups. On the other hand, populations of the *fuchsii* group inhabited the widest range of vegetation types, including semi-anthropogenic habitats (forest road ditches). Populations of the *elodes*-BM and *ericetorum* groups occupied a narrower but mutually similar spectrum of vegetation types (predominantly *Caricetalia fuscae*, *Vaccinio uliginosi*-*Pinetalia sylvestris*), differing from the rest. Mesic, subxerothermic and *Nardus*

grasslands were important components of the vegetation harbouring members of the *sooana* and *transsilvanica* groups, while these vegetation types were only rarely recorded in connection with some of the other groups.

Evaluation of the Red List categories in Czechia

All taxonomically recognized groups have been successfully evaluated against the Red List criteria at the national level in Czechia. Only the *fuchsii* group was deemed near-threatened (NT), while the other five groups met the criteria of being under some level of threat. Four groups, namely *maculata*, *sooana*, *psychrophila* and *transsilvanica*, were classified as endangered (EN). They are threatened mostly because of their fragmented occurrence, declining area of occupancy, number of locations, and both the extent and quality of their habitats (criterion B). The *sooana* and *transsilvanica* groups also evince a low and declining number of individuals (criterion C). The category of critically endangered (CR) was inferred for the *elodes*-BM group, which grows at a single locality (with a few subpopulations) in Czechia, and it is confined to vanishing habitats (criterion B). For details on the evaluation see Table 6.

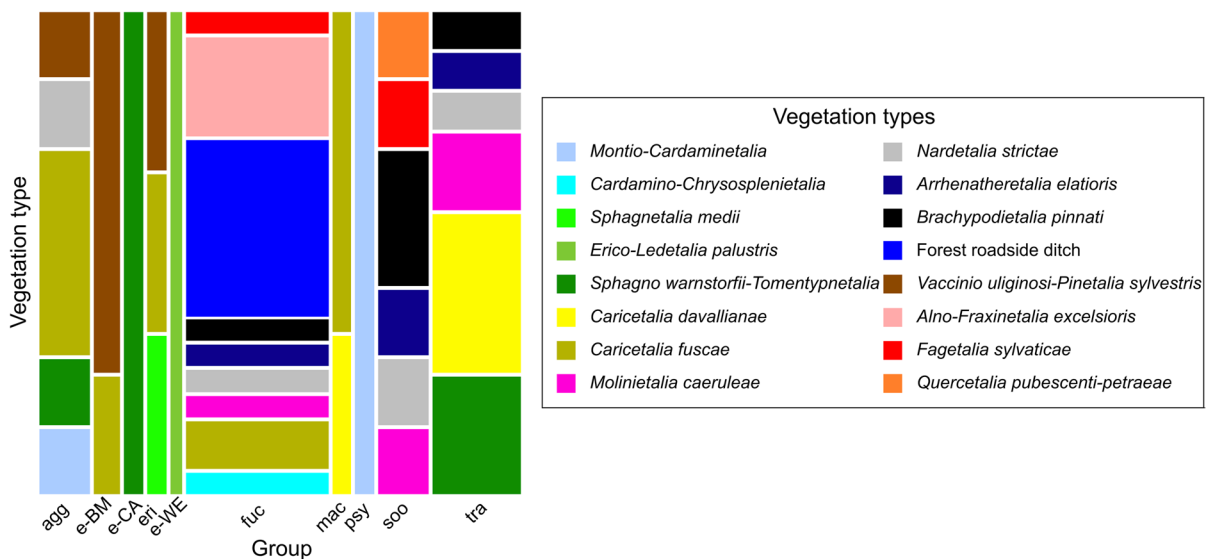


Fig. 6 Mosaic plot of the frequencies of vegetation types (phytosociological orders *sensu* Mucina et al. 2016 plus an additional type 'forest roadside ditch') in ten study groups of *D. maculata* agg. (abbreviations are explained in Table 1).

Table 6 The IUCN Red List categories for *D. maculata* agg. taxa occurring in Czechia

Taxon	IUCN Red List Category for Czechia
<i>D. maculata</i> subsp. <i>averyanovii</i>	CR B1ab(iii)+2ab(iii)
<i>D. maculata</i> subsp. <i>fuchsii</i>	NT
<i>D. maculata</i> subsp. <i>maculata</i>	EN B1ab(ii,iii,iv)+2ab(ii,iii,iv)
<i>D. maculata</i> subsp. <i>sooana</i>	EN B1ab(ii,iii,iv)+2ab(ii,iii,iv); C2a(i)
<i>D. maculata</i> subsp. <i>sudetica</i>	EN B1ab(iii)+2ab(iii)
<i>D. maculata</i> subsp. <i>transsilvanica</i>	EN B1ab(ii,iii,iv)+2ab(ii,iii,iv); C2a(i)

Discussion

A high level of morphological variability was observed among Central European populations of *D. maculata* agg. They could be assigned to several morphotypes which were, however, weakly separated at both the individual and the population level. Diploids formed a coherent group but were morphologically indistinguishable from some tetraploids. Furthermore, the occasional occurrence of DNA-triploids and DNA-hexaploids pointed to recurrent polyploidization and/or hybridization between major cytotypes. Such a pattern challenges taxonomic concepts which recognize two or more distinct species within the *D. maculata* agg. in the study area. Despite that, a total of eight morphotypes with particular geographical, ecological or karyological attributes were inferred to exist and were circumscribed for Central Europe. These can be evaluated taxonomically.

Morphological Variability and Ploidy Level Diversity

Leaf morphology, lip shape and flower colouration are generally used for the delimitation of particular taxa within the *D. maculata* agg. (e.g. Vermeulen 1947; Heslop-Harrison 1951; Bateman and Denholm 1988; Dufrêne et al. 1991; Ståhlberg and Hedrén 2008), and they were also crucial in this study. The main gradient of morphological variability stretched from broad-leaved plants with a deeply three-lobed lip, corresponding to the *fuchsii* and *sooana* groups, to narrow-leaved plants with a nearly-entire lip, representing the *elodes*-WE, *elodes*-BM and *ericetorum* groups. Still, these extreme morphotypes were interconnected by the other groups (*elodes*-CA, *maculata*, *psychrophila*, *transsilvanica*). The other important gradient was related to flower pigmentation. This was crucial for the separation of the *sooana* from the *fuchsii* group, the *elodes*-CA and *psychrophila* groups

from the *maculata* group, but also the *transsilvanica* group from the rest of the populations.

With the exceptions of the *ericetorum* and *elodes*-BM groups, each group represented a more or less coherent assemblage of populations, representing unique morphotypes. Populations of the *ericetorum* and *elodes*-BM groups formed a single coherent cluster, obviously assembling taxonomically identical plants, for which different names are used in various countries, specifically *D. ericetorum* in Slovakia (Vlčko et al. 2003) and *D. maculata* subsp. *elodes* in Czechia (Ponert 2019). Populations of the *maculata* group were morphologically coherent, but they alternately clustered with other groups, which stemmed from their intermediate morphological characteristics and difficult delimitation from other groups. Despite these ambiguities, the *maculata* group could not be unambiguously merged with any other group. Moreover, the unsatisfactory segregation of the *maculata* group from the *elodes*-CA, *psychrophila* and *transsilvanica* groups was likely to be caused by poor population sampling of these taxa, which reflects their overall rarity in the study area (cf. Vlčko et al. 2003; Kaplan et al. 2017).

Although it was usually possible to delimit individual groups in the analysis of populations, the analysis based on individuals revealed serious overlaps between pairs of morphologically similar groups, which points to fully continuous morphological variability within the *D. maculata* agg. (see also Naczek et al. 2015). Morphologically ambiguous individuals belonging to the *D. maculata* agg. are usually considered primary hybrids between particular taxa, most often *D. *maculata* and *D. *fuchsii* (e.g. Druce 1915; Heslop-Harrison 1948; Ståhlberg 2009). However, not only single individuals, but whole morphologically transitional populations occur in Central Europe, disrupting the discontinuities even at the population level. The overall variation of the *D. maculata* agg.

in Central Europe thus seems to be more complicated than reported from Western and Northern Europe (e.g. Heslop-Harrison 1951; Tyteca and Gathoye 2003; Ståhlberg and Hedrén 2008).

The polyploid system of the *D. maculata* agg., too, is more complex than previously believed (e.g. Heslop-Harrison 1968; Vöth and Greilhuber 1980; Delforge 2006; Kubát 2010), as indicated by several studies (Jagiełło and Lankosz-Mróz 1988; Ståhlberg and Hedrén 2008, 2010). Four DNA-ploidy levels were detected in our FCM analysis, corresponding to diploids, DNA-triploids, tetraploids and DNA-hexaploids. Only diploids and tetraploids formed single-cytotype populations whereas DNA-triploids and DNA-hexaploids always occurred as minority cytotypes within mixed-ploidy populations. The frequency of polyploidization and ploidy level diversity within the *D. maculata* agg. thus resembles that of *Gymnadenia conopsea* (Trávníček et al. 2011, 2012), which is a representative of the phylogenetically closest genus (Bateman et al. 2003, 2018).

Diploid populations were strictly concentrated within the *sooana* and *fuchsii* groups whereas the other groups, including unclassified (aggregate) plants, comprised only tetraploids (with sporadic DNA-hexaploid individuals). Moreover, a considerable number of tetraploid individuals, morphologically indistinguishable from diploids, were found in the *fuchsii* group, which also assembled all DNA-triploids. Two processes may be involved in the formation of minority cytotypes: heteroploid hybridization and polyploidization via unreduced gamete formation (Kolář et al. 2017). Triploids are mostly regarded as hybrids between diploid and tetraploid individuals of the genus *Dactylorhiza* (e.g. Heslop-Harrison 1968; Lord and Richards 1977; Pedersen 2006; Ståhlberg 2009), which is also a common way of triploid formation in vascular plants (cf. Popelka et al. 2019; Koutecký et al. 2022). Hexaploids are more likely to originate as a result of unreduced gamete formation within tetraploid populations (Ståhlberg and Hedrén 2008), which may also be the case with some triploids found in diploid populations (cf. Koblrová et al. 2022; Gajdošová et al. 2023; Vojtěchová et al. 2023).

The evolutionary and taxonomic significance of ploidy level variation within the *D. maculata* agg. has been a matter of dispute. Differences in chromosome numbers have long been held to represent a strong reproductive barrier and a good predictor of

morphological characters in Northern and Western Europe (e.g. Heslop-Harrison 1951; Tyteca and Gathoye 2003). Also in Central Europe, the ploidy level has traditionally been believed to be the most important character distinguishing between *D. fuchsii* (diploid) and *D. maculata* (tetraploid), despite their morphological similarity (e.g. Borsos 1961; Vöth 1978; Procházka 1979; Kubát 2010). Nonetheless, reproductive barriers between cytotypes are sometimes bypassed, resulting in gene flow across ploidy levels (Hülber et al. 2015; Kolář et al. 2017; Hanušová et al. 2019). The tetraploidy of Central European populations of *D. *fuchsii* may further facilitate its hybridization with other taxa of the group (Ståhlberg and Hedrén 2010; Naczek et al. 2015; Brandrud et al. 2020). This might have led not only to the establishment of primary hybrids between distinct tetraploid lineages, but also to the origin of morphologically transitional populations (here referred to as the aggregate group). This hypothesis should be tested further by molecular methods focused on population genetics.

Habitat and Environmental Differentiation Among Groups

Diploids and tetraploids of the *D. maculata* agg. have been reported to occupy different (micro)habitats, mainly depending on light conditions and soil pH (Heslop-Harrison 1951; Vaucher 1966; Dufrêne et al. 1991; Tyteca and Gathoye 2003; Ståhlberg 2009), which was sometimes thought to support their separation into two species, namely *D. fuchsii* growing in more shaded (forest) habitats on base-rich soils and *D. maculata* found in open peat bogs and meadows on acidic soils (e.g. Heslop-Harrison 1951). However, our analysis revealed a more complex pattern. We partially confirmed the observations of Jagiełło (1988) that there is a correlation between leaf shape and soil pH, as some narrow-leaved groups (e.g. the *elodes*-BM, *elodes*-WE groups) were associated with extremely acidic soils whereas groups characterized by broad leaves (e.g. the *fuchsii*, *sooana* groups) were found on just slightly acidic soils. However, the rather narrow-leaved *transsilvanica* and broad-leaved *sooana* groups had almost the same soil pH requirements and shared some habitat types. In addition, the environmental niche of the *sooana* group was clearly distinct from that of the *fuchsii* group

despite their morphological similarity. Furthermore, the *fuchsii* group, regardless of its ploidy level, was found to grow in a wide range of habitats, including woodlands, forests and meadows, with different environmental conditions, for example soils with a wide range of pH. Such a diversity of habitats occupied by *D. *fuchsii* has also been reported by Kirillova et al. (2022) from the Ural Mts.

Consistently with the general ecological pattern of niche breadth and geographic range size (Slatyer et al. 2013), groups with larger distribution areas, such as *fuchsii* or *transsilvanica*, occupied a wider range of habitats and tolerated more diverse environmental conditions whereas groups with local distributions (e.g. *elodes*-CA, *elodes*-BM, *ericetorum*, *psychrophila*) were usually confined to specific habitats (e.g. open coniferous woods in oligotrophic mires, subalpine water-springs) that have a very sparse, patchy distribution pattern across Central Europe. The morphological distinctions of the latter groups may thus be partly explained by the habitat-island effect (Mendez-Castro et al. 2021) and the gradual morphological and ecological differentiation of isolated populations (cf. Majeský et al. 2022). In addition, also quaternary climatic oscillations (Roy et al. 1996) may have facilitated contacts between distinct lineages, resulting in the establishment of locally distributed hybridogenous populations that later became ecologically and geographically isolated from their parents (Kadereit 2015).

It remains unclear to what extent morphology can be affected by the environment and whether some local morphotypes do not in fact represent ecotypes rather than taxa (cf. Lowry 2012). On the other hand, environment-induced adaptive changes in *Dactylorhiza* may be stabilized by epigenetic changes, which are hardly detectable even by conventional molecular methods but enable the ecological separation of taxa with similar genomes (Paun et al. 2011). Our observations suggest that the environment may shape individual phenotypes only to some extent and that similar habitats can be occupied by different morphotypes, which may be obviously attributed to different (epi)genotypes. For example, both the *elodes*-BM and *maculata* groups can colonize transitional mires; the *elodes*-CA and *transsilvanica* groups can colonize fen meadows; the *fuchsii* and *sooana* groups can colonize beech woodlands or forests, etc. However, the resolution of our environmental data

is rather coarse and these limitations must be taken into account when interpreting environmental differences between the groups. Whereas our soil data have a spatial resolution of 250 m, habitat differentiation between distinct cytotypes may be apparent at much finer spatial scales (Ståhlberg 2009; Šafářová and Duchoslav 2010).

An Intricate Pattern of Morphological, Cytogenetic and Ecological Variability Supports the Concept of a Single Species

A total of four distinct groups were recognized in a recent phylogenetic study among European *D. maculata* agg. taxa (Brandrud et al. 2020): *D. *saccifera* clade, *D. *gervasiana* clade, *D. *fuchsii* clade and the substantially heterogeneous *D. *maculata* clade, which included representatives of several taxa, among others *D. *foliosa* and *D. *transsilvanica*, but also plants termed as *D. *ericetorum*. However, their topology (reviewed by Bateman 2021) was unstable and with low bootstrap values, especially with respect to the *D. *fuchsii* and *D. *maculata* clades. Moreover, some taxa (e.g. *D. *fuchsii* and *D. *transsilvanica*) were rather undersampled regarding their variability and geographical distribution area. Despite these ambiguities, Bateman (2021) argued for a taxonomic concept treating the four clades resolved by Brandrud et al. (2020) as separate species. Still, however, he allowed for the Madeiran endemic *D. foliosa* to be recognized at the species level because of its morphological divergence from *D. maculata* s. str., which was thus rendered paraphyletic. An alternative taxonomic concept which complies with the phylogeny elucidated by Brandrud et al. (2020) is considering the whole *D. maculata* agg. as one species with multiple infraspecific taxa, typically subspecies (Baumann et al. 2002; Ströhle 2003; Conti et al. 2005; Ståhlberg and Hedrén 2010; Naczek et al. 2015; Průša 2019; Taraška et al. 2021). This rather conservative treatment was rejected by Bateman (2021) because it lacks a hierarchical framework of classification.

The most discussed ambiguities in *D. maculata* agg. relate to the delimitation of *D. maculata* s. str. and *D. *fuchsii*. In Western and Northern Europe, they seem to be well distinguishable based on morphology and ploidy level (e.g. Heslop-Harrison 1951; Bateman and Denholm 2003; Tyteca and Gathoye 2003; Ståhlberg and Hedrén 2008). The traits used for

discrimination, however, often fail in Central Europe, where tetraploids of both taxa occur and boundaries between them are weakened by reciprocal gene flow (Ståhlberg and Hedrén 2010; Naczek et al. 2015; Brandrud et al. 2020). This was also apparent in our data. Clustering using Ward's method was found to be the most congruent with classifications based on molecular data (e.g. Ståhlberg and Hedrén 2010; Bateman 2021), dividing the dataset into two main clusters corresponding to *D. *fuchsii* clade and *D. *maculata* clade as recognized by Brandrud et al. (2020). However, other methods did not show such a clear pattern, as the clustering of groups was highly unstable. In other words, some groups could not be unequivocally subordinated either to *D. *maculata* or *D. *fuchsii*. Previously, this was manifested by the unstable taxonomic treatment of taxa represented by these groups. For example, populations of the *psychrophila* group have been alternately incorporated into *D. maculata* (Jagiello 1988; Eccarius 2016) or *D. fuchsii* (Baumann et al. 2004; Kreutz 2004; Kubát 2010), or set aside as a separate species (Redl 2003; Delforge 2006; Mirek et al. 2020; see also Table 1). Serious difficulties have also been reported with regard to distinguishing between *D. *fuchsii* and *D. *transsilvanica*, traditionally subordinated to *D. *maculata* (cf. Borsos 1961; Bernátová et al. 1993; Baumann et al. 2002; Kubát 2010), but sometimes also to *D. *fuchsii* (e.g. Baumann et al. 2004; Jäger and Werner 2006). After all, misidentifications and confusions are frequent even between *D. *fuchsii* and *D. *maculata* (Kaplan et al. 2017). Unlike in Atlantic and Nordic Europe, where *D. *maculata* is reported to be clearly distinct from other taxa, it occupies a central position within the overall, more or less continuous, morphological variability of the *D. maculata* agg. in Central Europe. In this area, it may be considered a transitional morphotype between broad-leaved *fuchsii* and narrow-leaved groups of *ericetorum* and *elodes*-BM. It is also morphologically close to the *elodes*-CA, *psychrophila* and *transsilvanica* groups, which, however, differ by a set of quantitative and, above all, qualitative traits.

The observed patterns of morphological variability, cytotype diversity and eco-sociological attributes do not allow for a hierarchical classification of the *D. maculata* agg., which is here treated as a single species – *D. maculata*. Some of its Central European members with a limited distribution area and distinctive morphological and ecological properties

may be derived from widely distributed lineages of the *D. *maculata* clade and the *D. *fuchsii* clade, which would make them analogous to *D. *foliosa* in Brandrud et al. (2020). By contrast, some other taxa are likely to represent introgressions between these two clades, particularly the *psychrophila* and *transsilvanica* groups. Moreover, transitional populations (here referred to as the aggregate group) were recorded between the *fuchsii/maculata* (53, Suché kopce; 61, Zinnwald), *fuchsii/psychrophila* (55, Velká kotlina), *ericetorum/maculata* (35, Pavlová) or even *fuchsii/maculata/psychrophila* (17, Horská louka u Háje) groups.

Therefore, the rank of subspecies seems to be most appropriate for all these taxa. It is also congruent with the taxonomic treatment applied to the allopolyploid taxa of the *D. majalis / traunsteineri* complex subordinated to the species *D. majalis* despite their multiple origins (Bateman and Denholm 1983; Pedersen et al. 2003; Nordström and Hedrén 2008, 2009).

Overview of *D. maculata* subspecies in Central Europe

Analysis of taxonomic concepts used in the regional literature (see Table 1) revealed that the circumscription of some taxa needed to be re-evaluated. Thus, a total of eight taxa may be recognized in the region (Fig. 7).

The *fuchsii* group represents *D. maculata* subsp. *fuchsii* (Druce) Hyl. (Fig. 8), the most widespread taxon of the *D. maculata* agg. in Central Europe. It is generally considered morphologically, karyologically and ecologically distinct from *D. maculata* s. str. and all its subordinated taxa. The morphological distinctiveness of the *fuchsii* group was partially observed also in our data, despite overlaps with other groups, mainly the *transsilvanica* and *sooana* groups. The separation of the *fuchsii* group became less clear after adding some unclassifiable tetraploid populations to the dataset, representing morphological transitions to the *maculata* or *psychrophila* groups (see above). It may be hypothesized that morphologically transitional populations arose from repeated hybridization between various tetraploid taxa, including *D. *fuchsii*. Simultaneously, gene flow between diploids and tetraploids of *D. *fuchsii* can be facilitated by recurrent polyploidization (Taraška et al. 2021). High

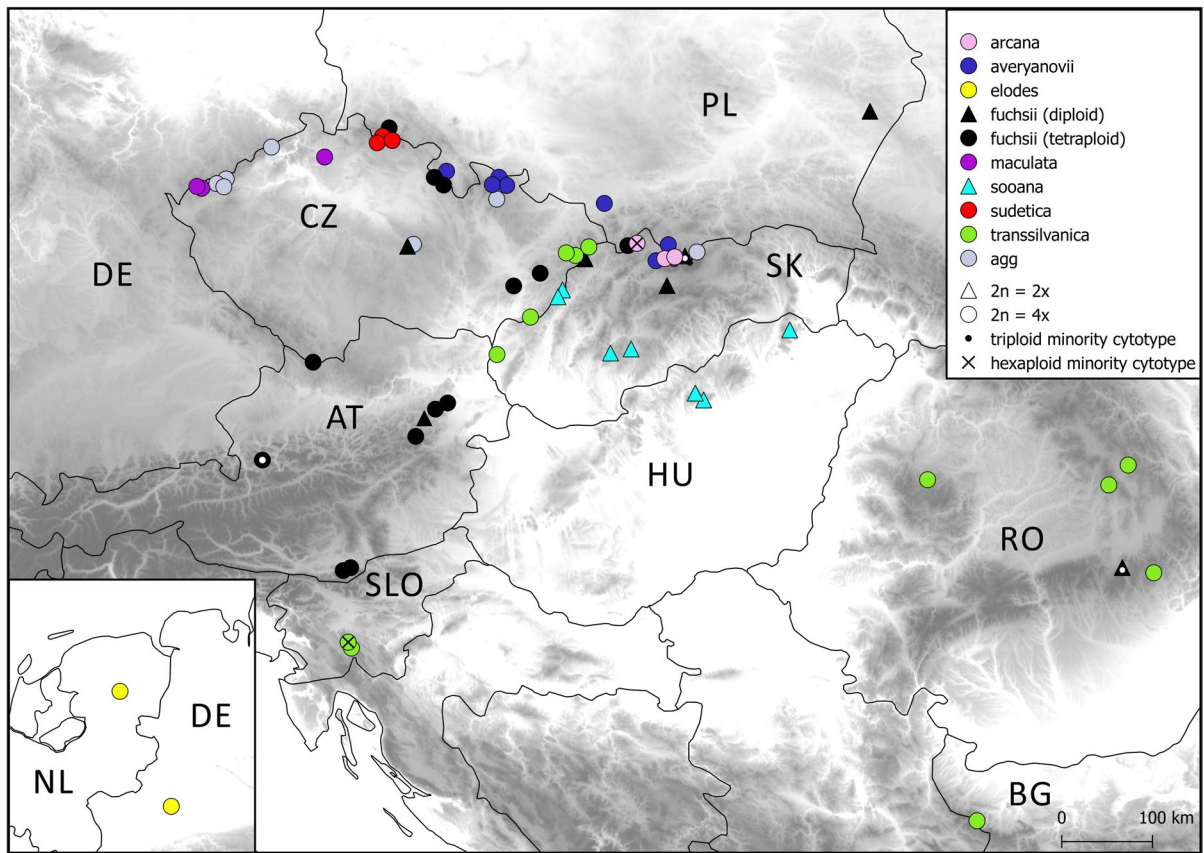


Fig. 7 Map of the locations of the sample populations, classified as subspecies following the here accepted taxonomic concept. The symbol shapes indicate the ploidy levels, and the colours indicate subspecies identity.

genetic variation (Ståhlberg and Hedrén 2010; Naczek et al. 2015) may allow *D. *fuchsii* to grow in a number of environmental conditions and range of habitats, which results in relatively frequent co-occurrence with other taxa of the group. Thus, *D. *fuchsii* is likely to be involved in gene exchange with other taxa of the *D. maculata* agg. and it seems inappropriate to treat it as a separate species.

The *sooana* group has been identified as *D. maculata* subsp. *sooana* Batoušek, Taraška et Trávn. (Fig. 9). This taxon was first recognized by Borsos (1959) and validly described by Taraška et al. (2021). It is confirmed to occur only in the West Carpathians, with one plausible report on its occurrence in Transcarpathian Ukraine (Loya 2015); records from other areas are likely misidentifications. It is a regional vicariant of *D. maculata* subsp. *fuchsii*, from which it differs in having a strictly diploid chromosome number and a distinct pattern of pigmentation, always

having white anther caps and, simultaneously, spotted leaves, but also in its occurrence in more mesic and thermophilous habitats. A detailed analysis of this taxon and its relations to *D. maculata* subsp. *fuchsii* has been provided elsewhere (Taraška et al. 2021).

Various taxa used to be recognized as *D. *elodes* in different European regions (Vermeulen 1968; Sczepanski 2006). Three geographically distinct groups of this taxon were therefore established for the purpose of our analysis, namely *elodes*-WE, *elodes*-BM and *elodes*-CA. *Dactylorhiza maculata* subsp. *elodes* (Griseb.) Soó was described by Grisebach (1845) as *Orchis elodes* Griseb. from the Atlantic wet heaths in the border area of Germany and Netherlands. This name should therefore be primarily applied to populations represented by the *elodes*-WE group in our study (Fig. 10). They were clearly morphologically separated from all other groups in our analysis, including the *elodes*-BM and *elodes*-CA groups.



Fig. 8 *Dactylorhiza maculata* subsp. *fuchsii*: **a** – habitat, loc. 41, Ranský brook; **b** – inflorescence, loc. 41, Ranský brook; **c** – leaves, loc. 34, Pârâul Rece; **d** – whole plant, loc. 41, Ranský brook.

Also, the environmental conditions differ between the stands of the *elodes*-WE populations and populations from Central Europe. Moreover, differences were also found between both Central European *elodes* groups. Populations of the *elodes*-BM group appeared to be morphologically indistinguishable from those of the *ericetorum* group, which allowed us to amalgamate these two groups into one. By contrast, populations of the *elodes*-CA group were morphologically close to the *maculata* group, from which they differed by the number of stem leaves, the shape of the leaves, darker flowers, and flower lips with a more robust spur (Fig. 11). Because of these characters, the *elodes*-CA group may to some extent resemble plants of the *D. majalis*/*traunsteineri* complex, especially *D. traunsteineri* s. str. Other morphological traits as well as genome size integrate the *elodes*-CA group

into the *D. maculata* agg., but introgression from other taxa cannot be ruled out. Moreover, populations of the *elodes*-CA group could not be reliably merged with any other group nor any taxon recognized in the area, and a new name *D. maculata* subsp. *arcana*, subsp. nov. is therefore proposed here (see below).

Populations assigned to the *ericetorum* and *elodes*-BM groups (Fig. 12) were characterized by extremely narrow leaves, up to 10–14× longer than wide, they represented a distinctive morphotype among all Central European plants, and they also typically occupied a specific habitat, namely open coniferous forests on mires. In Czechia, they are called *D. maculata* subsp. *elodes* (Ponert 2019), but this name should be applied to a different taxon (see above). The names based on the basionym *Orchis maculata* subsp. *ericetorum* E. F. Linton do



Fig. 9 *Dactylorhiza maculata* subsp. *sooana*: **a** – habitat, loc. 18, István-kút; **b** – inflorescence, loc. 18, István-kút; **c** – leaves, loc. 11, Gajdošovo; **d** – whole plant, loc. 11, Gajdošovo.

not seem to be appropriate either. Linton (1900) characterized *O. *ericetorum* as plants with narrower leaves compared to typical '*O. maculata*', but he misapplied the latter name to *D. *fuchsii*, which has relatively broader leaves (Vermeulen 1947; Szczepanski 2006). Vermeulen (1968) regarded *O. *ericetorum* as a variety of *D. maculata* (= *D. maculata* subsp. *maculata*) growing on heaths, and the names based on the epithet '*ericetorum*' are also regarded as synonyms of *D. maculata* subsp. *maculata* in most of recent works (e.g. Bateman and Denholm 2003; Eccarius 2016). Anyway, the *elodes*-BM group also contained the population from the *locus classicus* of *D. maculata* subsp. *averyanovii* Jagiełło, described by Jagiełło (1990) from Zieleniec, Poland (loc. 60). This seems to be the only valid name for plants of the *elodes*-BM and *ericetorum* groups. Whether it applies also to the

West European narrow-leaved populations, sometimes referred to as *D. *ericetorum*, must be scrutinized further.

The *transsilvanica* group corresponds to *D. maculata* subsp. *transsilvanica* (Schur) Soó (Fig. 13), which was described as *Orchis transsilvanica* by Schur (1853) and typified by his collection from Romania (Klein and Deutsch 2005). Plants from Slovenia and Bulgaria were reported to be tetraploids (Klein and Deutsch 2005; Petrova et al. 2009), but the ploidy level of plants in other parts of the subspecies' distribution range was long uncertain (e.g. Kubát 2010). Our data confirmed tetraploidy in all studied populations, but one DNA-hexaploid plant was found in Slovenia. *Dactylorhiza *transsilvanica* is usually characterized by white flowers and unspotted leaves (e.g. Soó 1980; Delforge 2006; Eccarius 2016), which



Fig. 10 *Dactylorhiza maculata* subsp. *elodes*: **a** – habitat, loc. 4, Borkenberge; **b** – inflorescence, loc. 4, Borkenberge; **c** – leaves, loc. 26, Leggelderveld; **d** – whole plant, loc. 4, Borkenberge

corresponds with the original description (Schur 1853). Sympatrically growing plants with different patterns of pigmentation, but the same morphological, karyological and habitat attributes, were usually determined as different taxa, typically *D. *maculata* or *D. *fuchsii*. However, such individuals were observed in all visited localities in Transylvania, that is, in the broad *area classica*. The situation at the type locality is unknown, as it has probably ceased to exist (V. Taraška and B. Trávníček, pers. observ.). These variable populations seem to be common in the Carpathians whereas populations of almost exclusively ‘pure’ (i.e. non-pigmented) *D. *transsilvanica* plants were only found in certain parts of its distribution range (Bílé Karpaty Mts, Dinarides and Stara Planina Mts). Such a pattern is analogous to that observed in *D. sambucina* with two flower-colour morphs,

intermediate individuals and rarely occurring ‘pure’ populations of uniform flower colouration (Gigord et al. 2001; Jersáková et al. 2006). The generally accepted circumscription of *D. *transsilvanica* therefore needs to be extended so that it includes both its colour morphs and transitional individuals.

The *psychrophila* group aggregated populations of dwarf plants growing in subalpine habitats, usually recognized as *D. fuchsii* subsp. / var. *psychrophila* (e.g. Procházka 1979; Kubát 2010; Ponert 2019). *Dactylorhiza *psychrophila* was described by Schlechter (in Keller and Schlechter 1928:183) as ‘*Orchis maculata* var. *psychrophila*’, and its neotype comes from Lapland (Vermeulen 1947). Some authors (e.g. Averyanov 1990; Devillers and Devillers-Terschuren 2000; Baumann et al. 2002; Tyteca and Gathoye 2003; Delforge 2006; Eccarius 2016) suppose

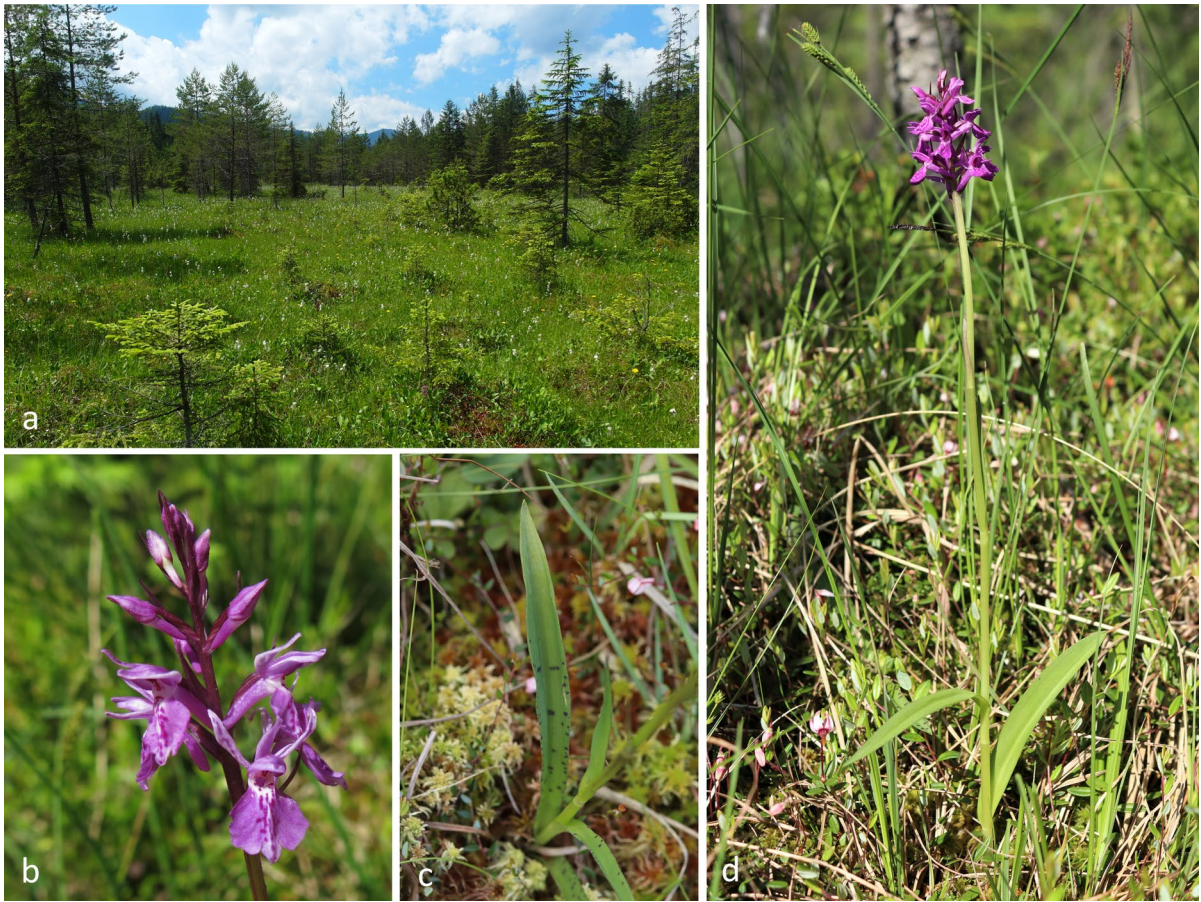


Fig. 11 *Dactylorhiza maculata* subsp. *arcana*: **a** – habitat; **b** – inflorescence; **c** – leaves; **d** – whole plant; all photographs are from loc. 3, Biały potok

it to occur only in North Europe and Siberia, while several others consider it as an arctic–alpine taxon distributed also in Central European mountains (e.g. Soó 1980). In that area, taxonomic ambiguities stem from unresolved relations between *D. *psychrophila* and *D. *sudetica*. The latter was described as ‘*Orchis maculata* var. *sudetica*’ by Reichenbach (1850) based on plant material collected by Poech at an unspecified locality in the Sudeten Mts (Eccarius 2016), almost certainly in the Krkonoše Mts (cf. Klášterský et al. 1982). Both taxa are characterized by a subtle habitus and their affinity to similar habitats. Anyway, several distinctions have been identified between plants from Northern Europe and those from the Sudeten Mts. Nordic *D. *psychrophila* is usually deemed to be diploid (e.g. Averyanov 1990; Eccarius 2016), but plants from the Krkonoše Mts were found to

be tetraploid (Jagiełło and Lankosz-Mróz 1988; Krahulcová 2003), which was also confirmed by our FCM screening. Furthermore, *D. *psychrophila* is considered morphologically close to *D. *fuchsii* (e.g. Averyanov 1983; Eccarius 2016), but Jagiełło (1988) pointed out the similarity of Central European populations to *D. *maculata* rather than *D. *fuchsii*. Also, populations in the Krkonoše Mts either clustered with the *maculata* group in our morphometric analysis or occupied an intermediate position between the groups of *maculata* and *fuchsii*. These circumstances justify the separation of plants from the Krkonoše Mts as distinct from Nordic *D. *psychrophila* as well as from all other Central European members of the *D. maculata* agg. Consequently, they should be recognized as ***D. maculata* subsp. *sudetica*** (Poech ex Rchb.f.) Vöth (cf. Jagiełło 1988; Delforge 2006;



Fig. 12 *Dactylorhiza maculata* subsp. *averyanovii*: **a** – habitat, loc. 42, Rejvíz MMJ; **b** – inflorescence, loc. 60, Zieleniec; **c** – leaves, loc. 60, Zieleniec; **d** – whole plant, loc. 60, Zieleniec

Eccarius 2016). Several populations in the Hrubý Jeseník Mts (55, Velká kotlina) and Krušné hory Mts (e.g. 17, Horská louka u Háje) are sometimes considered taxonomically identical to those from the Krkonoše Mts (e.g. Vlačíha and Dunder 2002; Kubát 2010; Bureš 2013; Kaplan et al. 2017), but this was not unequivocally confirmed in our analysis, and these populations thus remained unclassified. Müller et al. (2021) mentioned *D. fuchsii* var. *sudetica* from the Erzgebirge/Krušné hory Mts, but the same plants had been previously called *D. *transsilvanica* (Jäger and Werner 2006), and their taxonomic identity is unclear. The occurrence of plants morphologically similar to *D. *sudetica* in the Alps (e.g. Hassler and Muer 2022) is likely to be a result of parallelism in alpine habitats (Knotek et al. 2020; Španiel et al. 2023). According

to the current state of knowledge, *D. maculata* subsp. *sudetica* (Fig. 14) should be regarded as an endemic of the Krkonoše Mts.

The *maculata* group did not possess any clearly distinctive characters, so it was the least structured group. *Dactylorhiza maculata* L. was described by Linné (1753:942) as *Orchis maculata* L. in merely a general manner covering virtually all taxa of the *D. maculata* agg. A lectotype was therefore selected by Vermeulen (1947). In the narrow sense, this name applies to the tetraploid taxon, which is quite common in Atlantic and Boreal parts of Europe (e.g. Hansson 1985; Dusak and Prat 2010) but rare in the rest of its distribution area spanning from Europe to Central Siberia (Eccarius 2016). It is reported from all Central European countries, but literature

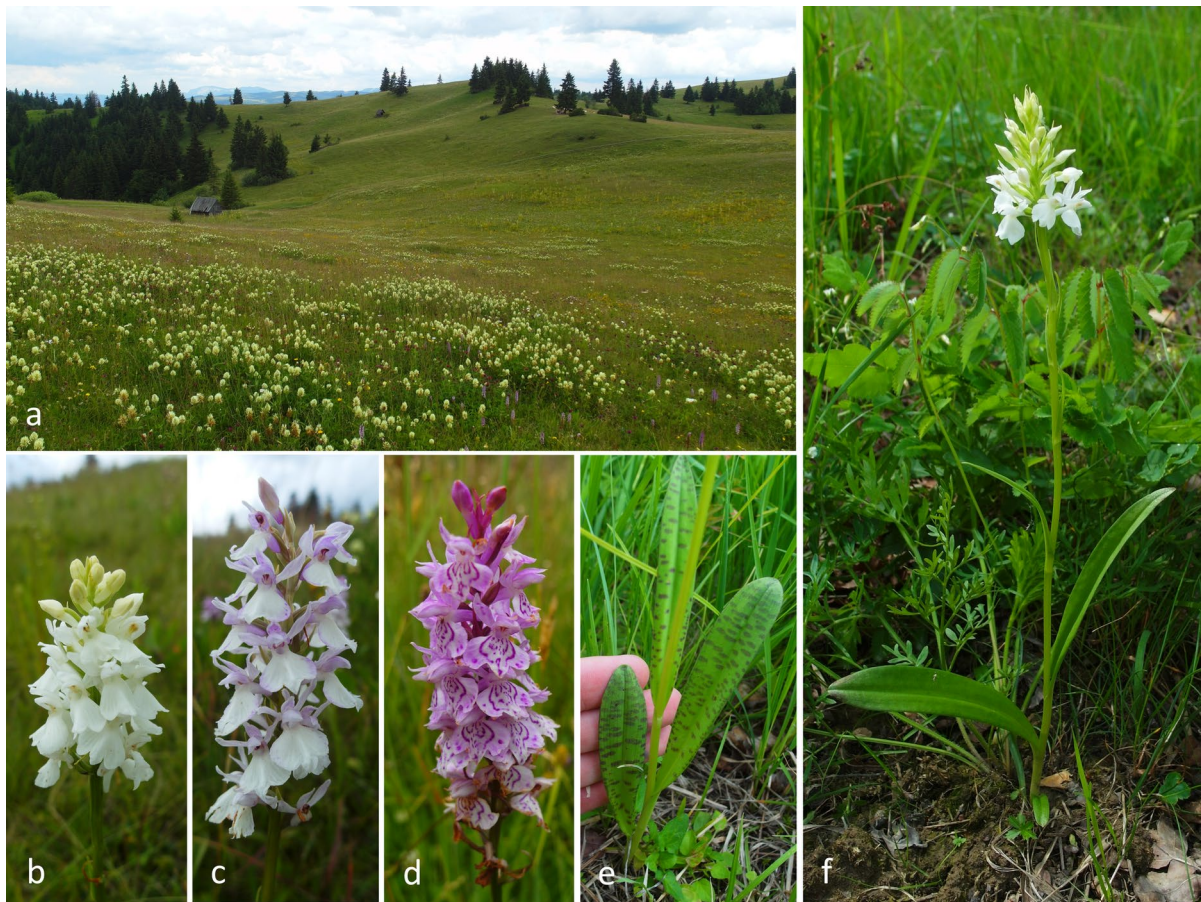


Fig. 13 *Dactylorhiza maculata* subsp. *transsilvanica*: **a** – habitat, loc. 10, Frumoasa; **b, c** – inflorescence, loc. 10, Frumoasa; **d** – inflorescence, loc. 28, Mănăstirea Suzana; **e** – leaves, loc. 10, Frumoasa; **f** – whole plant, loc. 21, Jazeví

records are strongly biased by varying species circumscriptions and taxonomic concepts used by different authors (Kaplan et al. 2017). Only populations strictly corresponding to *D. maculata* s. str. were assigned by us to the *maculata* group (Fig. 15). Yet, some populations with less matching morphological characteristics should be probably included as well, particularly those in the Krušné hory Mts, where the occurrence of the south-west lineage of *D. *maculata* was also confirmed by molecular genetics (Ståhlberg and Hedrén 2010). Some of the local populations were treated as unclassified (the aggregate group) in our analysis, and their addition to the *maculata* group led to an even worse ability to discriminate between the *maculata* and other groups, mainly the *fuchsii* and *psychrophila* groups. On the other hand,

the admittedly low number of *maculata* populations included in the analysis due to strict classification criteria may have contributed to the limited success of the statistical methods at distinguishing this group from all others. Still, *D. maculata* subsp. *maculata* must be regarded as the most average morphotype of the *D. maculata* agg., further challenging the traditional taxonomic concepts with two or more recognized species.

Checklist of recognized subspecies of *D. maculata*

Dactylorhiza maculata subsp. *arcana* Trávn., Taraška, Batoušek et Lamla, subsp. nov. – *D.*

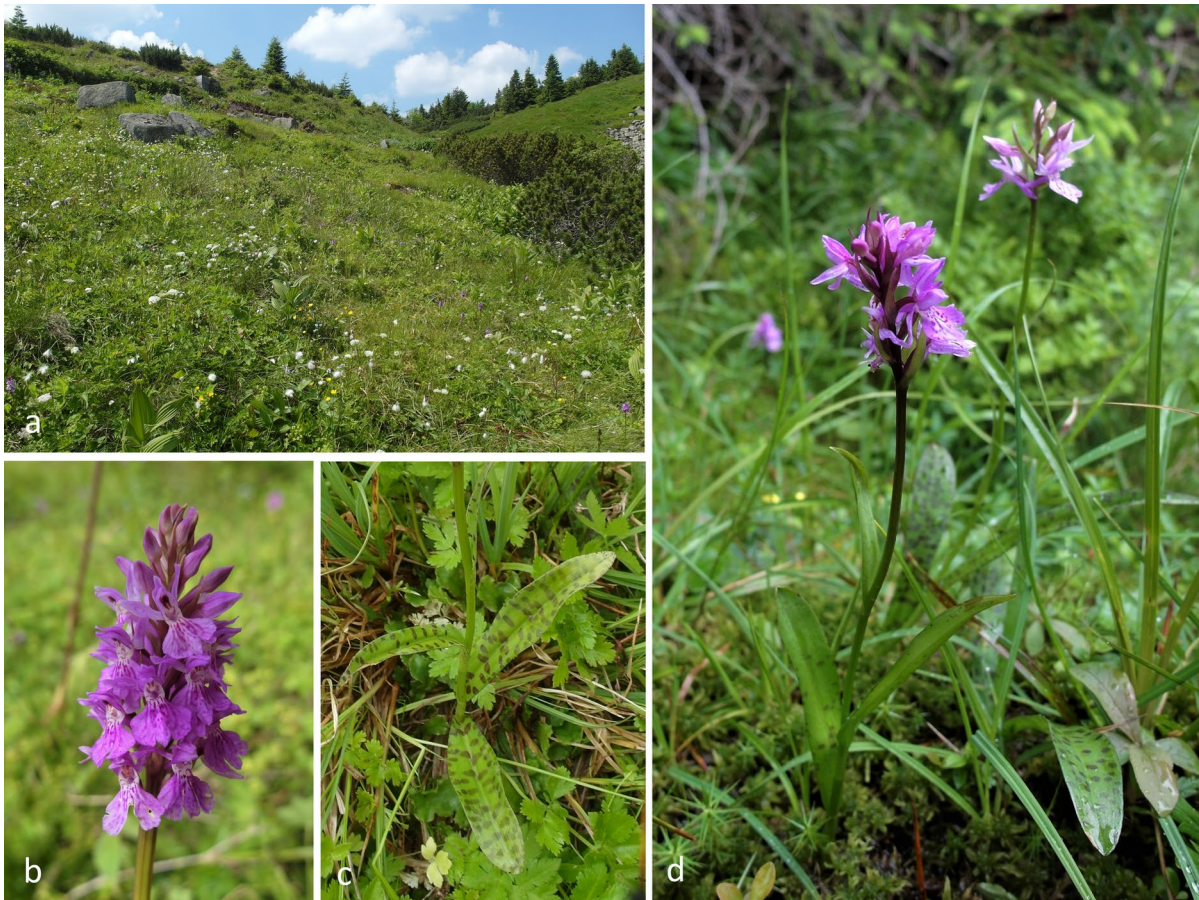


Fig. 14 *Dactylorhiza maculata* subsp. *sudetica*: **a** – habitat, loc. 25, Labský vodopád; **b** – inflorescence, loc. 25, Labský vodopád; **c** – leaves, loc. 25, Labský vodopád; **d** – whole plant, loc. 33, Pančava

maculata subsp. *elodes* auct. non (Griseb.) Soó 1962: Vlčko et al., Orchids of Slovakia 31 (2003)

Holotype: Polsko [Poland]: Tatry Zachodnie Mts, Kościelisko village (near Zakopane town), peat bog west of Biały Potok settlement, west of the village – 905 m.a.s.l.; 49° 16' 59" N, 19° 50' 45" E (WGS-84); 25 June 2016, leg. excursion group; OL 44443! (Table S6 in the electronic supplementary material).

Isotypes: OL 44441!, BRNM 840763!

Description: Perennial herbs with palmate tubers. Plants (26–)27–49(–67) cm high, with (4–)5–7(–8) sheathing leaves and 1–4(–5) bract-like leaves. Sheathing leaves narrowly oblanceolate, usually with bold or pale spots, sometimes unspotted, making an angle of ~30° with the stem; bract-like leaves smaller, lanceolate. The lowermost well-developed leaf (50–)70–141(–174) mm long and

(10–)11–21(–29) mm wide, (1.6–)3.4–8.6(–10.5)× longer than wide, usually subacute at the apex. The 2nd lowermost leaf (80–)97–174(–219) mm long and (9–)11–21(–31) mm wide, (0.7–)5.9–11.5(–13.3)× longer than wide, with the widest dimension in its upper half, usually acute at the apex. Inflorescence a sparse to dense-flowered spike, often with dark reddish-purple anthocyanin pigmentation of the stem, bracts and/or ovaries. Tepals purple, often with bold markings. Lip three-lobed with rather small median lobe, pink to reddish-purple, nearly always with bold markings, the Heslop-Harrison index (1.0–)1.1–1.4(–1.5); spur robust, (7.4–)8.0–10.9(–12.3) mm long and (1.5–)1.9–2.9(–3.3) mm wide in the middle of its length, down-curved, darkly purple; flower colouration and spur shape somewhat resembling that of *D. traunsteineri*. Fruit a capsule with dust-like seeds.

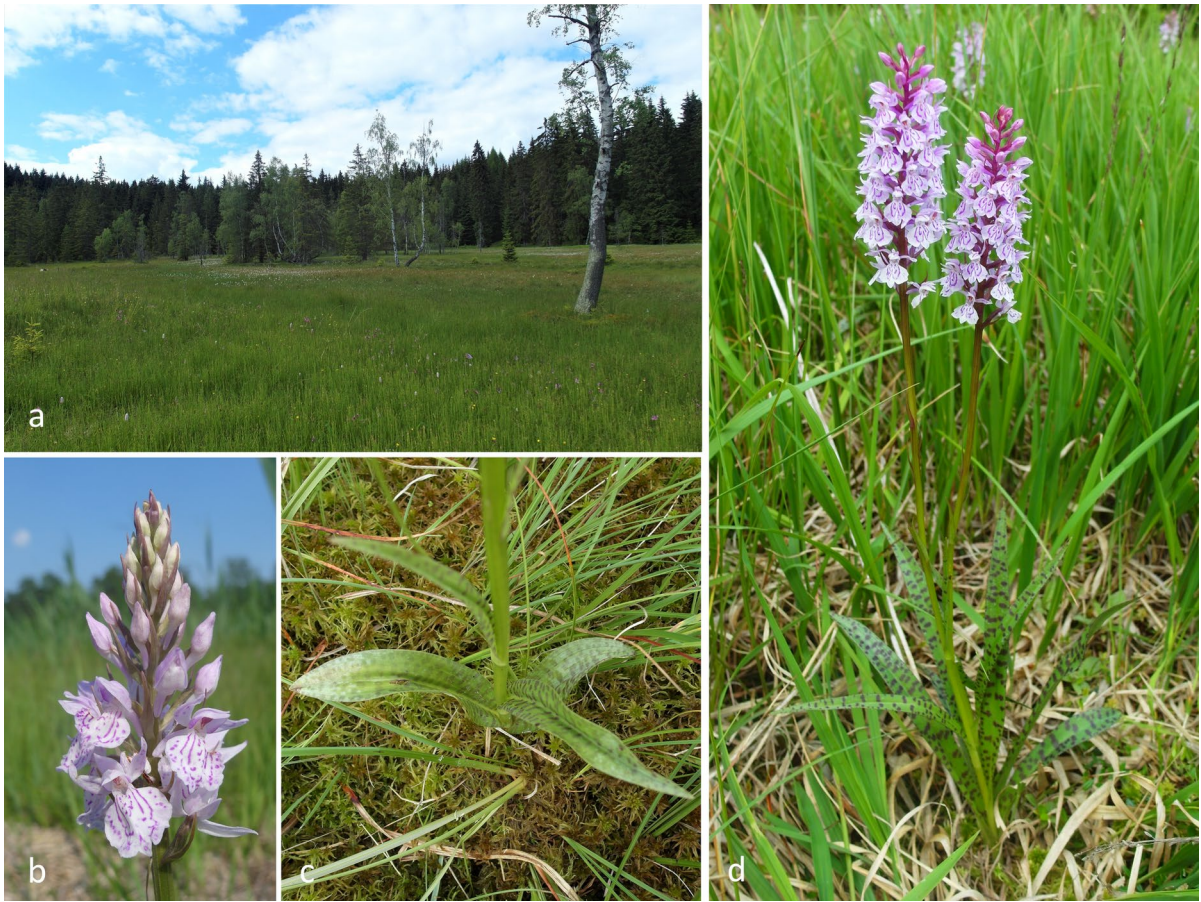


Fig. 15 *Dactylorhiza maculata* subsp. *maculata*: **a** – habitat, loc. 45, Rudné; **b** – inflorescence, loc. 22, Jestřebí; **c** – leaves, loc. 45, Rudné; **d** – whole plant, loc. 40, Přebuz

Similar taxa: *Dactylorhiza maculata* subsp. *arcana* is similar to the type subspecies but differs in having dark (reddish-)purple flowers with robust spurs and narrower leaves, which are subacute at the apex and widest in their upper half. The two taxa also differ in several habitus-related traits, as individuals of *D. maculata* subsp. *arcana* more often have a densely foliated stem, more erect leaves and sparser inflorescences. It may be also confused with plants of the *D. majalis* / *traunsteinerii* complex, from which it differs in having a ‘maculata-like’ lip shape and genome size.

Chromosome counts and ploidy level: $2n = 4x = 80$; exceptionally $2n \sim 6x$.

Habitat and ecology: Moderately calcium-rich sedge-moss fens.

Phytosociological relevé: Poland, Kościelisko-Biały Potok, peat bog 920 m SSW from the confluence of the Kirowa Woda river and Lejowy Potok stream, GPS (WGS-84): 49° 16' 59.8" N, 19° 50' 45.7" E, ca 900 m.a.s.l., decl. 2°, exp. NW, area: 5 × 5 m; 28 June 2021, recorded by V. Taraška, P. Batoušek, F. Lamla and B. Trávníček; taxonomic nomenclature after Kaplan et al. (2019).

Cover – total: 99%; E_3 : 0%; E_2 : 1%, E_1 : 80%, E_0 : 99%. – E_2 : *Salix aurita* +, *Salix caprea* r, *Salix pentandra* r. – E_1 : *Vaccinium oxycoccos* 3, *Carex panicea* 2b, *Eriophorum angustifolium* 2b, *Menyanthes trifoliata* 2m, *Potentilla erecta* 2m, *Carex dioica* 1, *Carex flava* 1, *Dactylorhiza maculata* subsp. *arcana* 1, *Drosera rotundifolia* 1, *Equisetum palustre* 1, *Pedicularis palustris* 1, *Trientalis europaea* 1, *Angelica sylvestris* +, *Calluna vulgaris* +, *Carex echinata*

+, *Carex nigra* +, *Crepis paludosa* +, *Polygala vulgaris* +, *Briza media* r, *Carex rostrata* r, *Equisetum fluviatile* r, *Eriophorum vaginatum* r, *Festuca rubra* r, *Galium palustre* r, *Picea abies* juv. r. – E₀: *Sphagnum* spp., indet. – Species outside the relevé: *Calla palustris*, *Eriophorum latifolium*, *Juncus squarrosus*, *Tofieldia calyculata*.

Threat status: The subspecies should be considered critically endangered [CR B2ab(iii)] because of its rarity in both countries, Slovakia and Poland, at least until comprehensive data on its total distribution and population dynamics is gained.

Etymology: From the Latin word *arcanus* = mysterious, enigmatic. We suggest the epithet ‘tajomný’ for the Slovak and ‘tajemnicza’ for the Polish vernacular subspecies name.

Distribution: Endemic to Poland and Slovakia, with localities known in the foothills of the Oravské Beskydy Mts and Tatry Mts.

***Dactylorhiza maculata* subsp. *averyanovii* Jagiełło**, Acta Univ. Wratislav. 1055: 50 (1990)

≡ *D. maculata* subsp. *elodes* var. *averyanovii* Jagiełło, Fragm. Florist. Geobot. 31–32(3–4): 369 (1988)

– *D. ericetorum* auct. non (Linton) Aver. 1982: Vlčko et al., Orchids of Slovakia 25 (2003)

– *D. maculata* subsp. *elodes* auct. non (Griseb.) Soó 1962: Ponert in Kaplan et al., Key to the Flora of the Czech Republic 185 (2019)

Type (holotype): ‘Zieleniec (Sudeti Orientales, regio urbis Kłodzko), in margine sphagneti’, June 1982, M. Jagiełło, KRAM 297001 (digital image!).

Morphology: Relatively narrow linear leaves with parallel margins and acute apices, up to 19–(23)× longer than wide, avg. Heslop-Harrison index: 1.2.

Chromosome counts and ploidy level: $2n = 4x = 80$.

Habitat and ecology: Open pine and spruce woods in oligotrophic mires, peat bogs and sedge-moss vegetation.

Distribution: Czechia, Poland, Slovakia. The Central and East Sudeten Mts, Beskydy Mts.

Threat status: Czechia: CR B1ab(iii)+2ab(iii). Slovakia: CR; evaluated as *D. ericetorum* (Eliáš et al. 2015). Poland: not evaluated (cf. Zarzycki and Szelağ 2006).

Taxonomic note: This taxon was initially treated at the subspecies level by Jagiełło, who later changed

her opinion and lowered it to the rank of variety (cf. Jagiełło 1988, 1990). Because of a long delay in the publication of the first manuscript written, the subspecies name was unintentionally published later (Jagiełło 1990) than the varietal one (Jagiełło 1988). Nonetheless, both publications include literally the same description and refer to the same type specimen. Both names are therefore validly published, they are legitimate, and neither of them should be regarded as a basionym for the other; instead, they must be considered homotypic synonyms.

***Dactylorhiza maculata* subsp. *elodes* (Griseb.) Soó**, Nom. Nov. Generis Dactylorhiza 7 (1962)

≡ *Orchis elodes* Griseb., Goett. Studien: 276–277 (1845)

≡ *Dactylorhiza elodes* (Griseb.) Aver., Bot. Zhurn. 67(3): 309 (1982)

Type (holotype): ‘[Germany/Netherlands] Bourtangermoor’, sine dato, A. H. R. Grisebach (not signed), GOET 7217 (digital image!).

Morphology: Leaves erect, lanceolate, broadest in their basal part, acute at the apex, avg. Heslop-Harrison index: 1.1, spur usually short and thin.

Chromosome counts and ploidy level: $2n = 4x = 80$.

Habitat and ecology: Sedge and peat-moss vegetation of the raised bogs and wet heath.

Distribution: Northern Lowlands. Germany, Netherlands.

Threat status: Unknown.

***Dactylorhiza maculata* subsp. *fuchsii* (Druce) Hyl.**, Nord. Kärlväxtfl. 2: 238 (1966)

≡ *Orchis fuchsii* Druce, Rep. Bot. Soc. Exch. Club Brit. Isles 4(1): 105 (1915)

≡ *Dactylorhiza fuchsii* (Druce) Soó, Nom. Nov. Gen. Dactylorhiza 8 (1962)

= *Dactylorhiza maculata* subsp. *austriaca* Vöth, Linzer Biol. Beitr. 10(1): 190 (1978)

Type: ‘[Great Britain] Challow Berks’, June 1895, G. C. Druce, OXF 6463 (digital image!; lectotype Vermeulen 1947: 147).

Morphology: Leaves obovate to oblanceolate, relatively broad, obtuse at the apex, lip purple to white, anther caps purple, avg. Heslop-Harrison index: 1.4; populations consist of various proportions of purple-flowered plants with spotted leaves and white-flowered plants with unspotted leaves.

Chromosome counts and ploidy level:
 $2n = 2x = 40$, $2n \sim 3x$, $2n = 4x = 80$.

Habitat and ecology: Broad-leaved and coniferous forests, soft-water springs, forest roadside ditches, wet to mesic mown meadows, moss-sedge vegetation.

Distribution: Throughout temperate Europe and Asia (Eccarius 2016), but regionally rare or absent (e.g. Pannonian Basin, Balkan Peninsula).

Threat status: Czechia: NT. Germany: 'V-Vornwarnliste' (Metzing et al. 2018). Hungary: VU (Király 2007). Poland: VU (Zarzycki and Szela 2006). Slovakia: NT (Eliáš et al. 2015).

Dactylorhiza maculata* (L.) Soó subsp. *maculata = *Orchis maculata* subsp. *ericetorum* E. F. Linton, Fl. Bournemouth 208 (1900) $\equiv$ *Dactylorhiza ericetorum* (Linton) Aver., Bot. Zhurn. 67(3): 309 (1982)

Type: Sweden, unknown locality in the surroundings of Uppsala, sine dato, C. Linnaeus, LINN 1054 (digital image!; lectotype Vermeulen 1947: 130).

Morphology: Leaves narrowly oblanceolate, widest in their middle part, acute or subacute at the apex, avg. Heslop-Harrison index : 1.3.

Chromosome counts and ploidy level:
 $2n = 4x = 80$ (chromosome counts: e.g. Heslop-Harrison 1951; Jagiełło and Lankosz-Mróz, 1988; Aagaard et al. 2005).

Habitat and ecology: Sedge-moss vegetation of calcareous or acidic, usually mineral-rich fens.

Distribution: Atlantic and subatlantic Europe and Fennoscandia, less frequently in Central and East Europe to West Siberia (Eccarius 2016).

Threat status: Czechia: EN B1ab(ii,iii,iv)+2ab(ii,iii,iv). Hungary: VU (Király 2007). Poland: VU (Zarzycki and Szela 2006). Slovakia: EN (Eliáš et al. 2015). In Hungary and Poland, the evaluation relates to the species *D. maculata*, which may include some taxa here recognized as separate subspecies.

***Dactylorhiza maculata* subsp. *sooana* Borsos ex Batoušek, Taraška et Trávn., Plant Syst. Evol. 307: 51(16) (2021)**

– *Dactylorhiza fuchsii* subsp. *sooana* Borsos, Acta Bot. Acad. Hung. 5: 324 (1959), nom. inval. (ICN Art. 40.1)

Type (holotype): 'Slovakia, Štiavnické vrchy Hills, Banský Studenec Village, meadow in the valley of the Bystrý potok brook east of the village, 655 m.a.

s. l., 48° 26' 31" N, 19° 00' 49" E', 13 June 2017, leg. excursion group, OL 37871!

Morphology: Leaves obovate to oblanceolate, relatively broad, obtuse at the apex, always spotted, lip white with or without markings, anther caps white, avg. Heslop-Harrison index: 1.3.

Chromosome counts and ploidy level:
 $2n = 2x = 40$.

Habitat and ecology: Wet to meso-xeric mown meadows, secondary mat-grass swards, basiphilous beech forests and oak forests in warm cool-temperate regions.

Distribution: Endemic to the West Carpathians. Czechia, Hungary, Slovakia. Reports from other parts of the Carpathians (e.g. Loya 2015) must be examined.

Threat status: Czechia: EN B1ab(ii,iii,iv)+2ab(ii,iii,iv); C2a(i). Slovakia: NT (Eliáš et al. 2015). Hungary: VU; evaluated within *D. fuchsii* (Király 2007).

***Dactylorhiza maculata* subsp. *sudetica* (Poech ex Rchb.f.) Vöth, Linzer. Biol. Beitr. 12(2): 430 (1980)**

$\equiv$ *Orchis maculata* var. *sudetica* Poech ex Rchb.f., Icon. Fl. Germ. Helv. 13/14: 66, tab. 56 (1850)

$\equiv$ *Dactylorhiza fuchsii* subsp. *sudetica* (Poech ex Rchb.f.) Verm., Orchideen 37(3): 78 (1975)

$\equiv$ *Dactylorhiza sudetica* (Poech ex Rchb.f.) Aver., Bot. Zhurn. 67(3): 310 (1982)

– *Dactylorhiza fuchsii* subsp. *psychrophila* auct. non (Schltr.) Holub 1964: Procházka, Zpr. Čes. Bot. Společ. 14: 11 (1979)

– *Dactylorhiza fuchsii* var. *psychrophila* auct. non (Schltr.) Soó 1962: Kubát, Flora of the Czech Republic 8: 520 (2010)

Type: Rchb. f., Icon. Fl. Germ. Helv. 13/14: tab. 56. 1850 (lectotype Baumann et al. 2002: 144).

Epitype (designated here): sine loco [Sudeten Mts], sine dato, leg. J. A. Poech, W 0028325!

Note: The protologue contains both an illustration and a reference to the herbarium specimen. The first was selected as a lectotype by Baumann et al. (2002). This typification was later questioned by Eccarius (2011), but it conforms to the ICN (Turland et al. 2018). The illustration must be thus regarded as lectotype, while the herbarium specimen is here designated as an epitype.

Morphology: Dwarf plants with the stem height never exceeding 40 cm, usually with 2–3 elliptic, oblanceolate to obovate sheathing leaves with subacute to obtuse apices, avg. Heslop-Harrison index: 1.2, flowers often darkly reddish-purple, frequent anthocyanin pigmentation of bracts, ovaries and inflorescence axis.

Chromosome counts and ploidy level: $2n = 4x = 80$ (chromosome counts: Krahulcová 2003)

Habitat and ecology: Subalpine oligotrophic water-springs.

Distribution: Endemic to the Krkonoše Mts. Czechia, Poland.

Threat status: Czechia: EN B1ab(iii)+2ab(iii). Poland: not evaluated (cf. Zarzycki and Szelać 2006).

Note: Unlike other taxa of the *D. maculata* agg. classified within the category of EN in Czechia, *D. maculata* subsp. *sudetica* probably did not undergo a significant decrease of its population size, and it also does not exhibit extreme fluctuations (i.e. greater than one order of magnitude; IUCN 2012a) in the number of individuals, as it was assumed in the national Red List (Grulich 2017). Yet, it occurs in the subalpine belt where it faces both climate change and over-tourism (Flousek 2019; Erlebach and Romportl 2021), prospectively leading to changes in habitat extent and quality.

***Dactylorhiza maculata* subsp. *transsilvanica* (Schur) Soó,** Nom. Nov. Gen. *Dactylorhiza* 7 (1962)

≡ *Orchis transsilvanica* Schur, Verh. Mitth. Siebenbürg. Vereins Naturwiss. Hermannstadt 4: 72 (1853)

≡ *Dactylorhiza transsilvanica* (Schur) Aver., Bot. Zhurn. 67(3): 309 (1982)

≡ *Dactylorhiza maculata* var. *transsilvanica* (Schur) P. Delforge, Naturalistes Belges 81(4): 397 (2000)

Type: ‘Auf Moorboden am Schewechbach’, 9 June 1853, leg. P. J. F. Schur, LW (digital image!; lectotype Klein and Deutsch 2005: 231).

Morphology: Leaves oblanceolate to narrowly oblanceolate, usually subacute or obtuse at the apex, avg. Heslop-Harrison index: 1.2; populations formed by a significant proportion of white-flowered plants with unspotted leaves, but often including also purple-flowered plants with spotted leaves, as well as continuous transitions between these two forms.

Chromosome counts and ploidy level: $2n = 4x = 80$ (chromosome counts: Klein and Deutsch 2005; Petrova et al. 2009); rarely $2n \sim 6x$.

Habitat and ecology: Sedge-moss fens, wet to mesic mown meadows and pastures, secondary mat-grass swards and meso-xerophytic grasslands, usually calcareous, mineral-rich and nutrient-poor soils.

Distribution: Bulgaria, Czechia, Romania, Slovakia, Slovenia; mentioned from Hungary (Molnár and Csábi 2021), herbarium specimens of uncertain identity collected in Bosnia and Herzegovina (Loschnigg 1929, OLM !) and Montenegro (Rohlena 1903, PRC !). Carpathians, Dinarides, Stara Planina Mts and Pannonian Basin.

Threat status: Czechia: EN B1ab(ii,iii,iv)+2ab(ii,iii,iv); C2a(i). Slovakia: CR (Eliš et al. 2015). Hungary: EX; evaluated within *D. maculata* (Király 2007).

Determination key to subspecies of *D. maculata* in Central Europe

The key provided here serves to determine populations of *D. maculata* in Central Europe. It gives the most frequent, average and extreme (10–90 percentile, minimum and/or maximum in brackets) values of particular traits, not necessarily individual attributes of each plant. It should therefore not be applied to single plants because of extensive individual variability within the group. Instead, each population must be considered as a whole, and single plants with aberrant phenotypes should be regarded as part of its variation. Populations which do not merit criteria to be assigned to any subspecies should be referred to as *D. maculata* s. lat. or, possibly, as transitional populations among specific subspecies.

(1a) Lowermost well-developed leaf oblong, oblanceolate to obovate, max. $5.2(-7.5) \times$ longer than wide, usually with obtuse apex; avg. Heslop-Harrison index ≥ 1.3 ; $2n = 2x, 3x, 4x$ **2**

(1b) Lowermost well-developed leaf linear, oblanceolate to lanceolate, up to $9.8(-21.7) \times$ longer than wide, with acute, subacute or obtuse apex; avg. Heslop-Harrison index ≤ 1.3 ; $2n = 4x$ (rarely $6x$) **3**

(2a) Leaves always spotted (intensity of leaves spotting does not correlate with intensity of flower colouration and tepal markings); tepals white or,

rarely, pink, lip and anther caps nearly always white (regardless intensity of lip markings); $2n = 2x$. – Mesic meadows, broad-leaved woodlands and forests; Carpathians subsp. **sooana**

(2b) Leaves spotted or unspotted (intensity of leaves spotting positively correlates with intensity of flower colouration and markings); tepals and lip pink or, less often, white, anther caps always purple (excl. achromatic individuals); $2n = 2x, 3x, 4x$. – Forests, meadows, roadside ditches; widespread subsp. **fuchsii**

(3a) Lowermost well-developed leaf (2.1–)3.3–6.9(–12.5)× longer than wide, predominantly obtuse or subacute at the apex **4**

(3b) Lowermost well-developed leaf (3.0–)4.7–12.5(–21.7)× longer than wide, predominantly acute to subacute at the apex **5**

(4a) Plants up to 36(–40) cm high, most often with 5 cauline (incl. bract-like) leaves; lowermost well-developed leaf up to 10(–13) cm long, usually spotted; inflorescence axes, bracts and ovaries usually with purple anthocyanin pigmentation; lip pink to darkly (reddish-)purple with markings (flower colouration often resembling that of *D. majalis*), only rarely white without markings (achromatic plants). – Subalpine springs and grasslands; endemic to the Krkonoše Mts subsp. **sudetica**

(4b) Plants up to 56(–67) cm high, most often with 7 cauline (incl. bract-like) leaves; lowermost well-developed leaf up to 14(–20) cm long, spotted or unspotted; inflorescence axes, bracts and ovaries usually green without anthocyanin pigmentation; lip white or pink, with or without markings. – Populations consisting predominantly, or at least partly of white-flowered plants with unspotted leaves. Mesic to wet meadows and fens; Carpathians, Dinarides, Stara Planina Mts, Pannonia subsp. **transsilvanica**

(5a) Leaves lanceolate, erect, usually widest in their basal half; Heslop-Harrison index ≤ 1.1 (–1.2), spur thin and short, 0.5–0.8(–0.9)× as long as the lip. Leaves with pale spots or unspotted, rarely with bold spots. – Wet heaths; subatlantic West and Central Europe subsp. **elodes**

(5b) Leaves linear to oblanceolate, erect or spread out, usually widest in their upper half; Heslop-Harrison index ≤ 1.4 (–2.1), spur relatively thick and long, (0.6–)0.9–1.3(–1.7)× as long as the lip. – Leaves with pale to bold spots or unspotted **6**

(6a) 2nd well-developed leaf from the base of the stem up to 21(–28) cm long, (6–)8–19(–23)× longer than wide, narrowly linear with $\pm$ parallel margins in the widest part of the leaf, nearly always acute at the apex. – Open pine and spruce woods on mires, rarely open oligotrophic mires subsp. **averyanovii**

(6b) 2nd well-developed leaf from the base of the stem up to 17(–22) cm long, (1–)5–11(–14)× longer than wide, oblanceolate with convex margins in the widest part of the leaf, acute to subacute, rarely obtuse at the apex. – Usually non-woodland habitats **7**

(7a) Stem less densely foliated (avg. 1.7 leaves per 10 cm of the stem length); leaves rather spread out, oblanceolate or lanceolate with the widest place around their middle part; lowermost well-developed leaf typically acute or subacute, rarely obtuse at the apex; inflorescence sparse to dense (compact), lip white to pink, rarely purple, with or without markings, spur usually not conspicuously robust, ca 8.7 mm long and 2.1 mm wide, pink to purple, less often white. – Fens, sedge-moss vegetation; rare but widespread. subsp. **maculata**

(7b) Stem more densely foliated (avg. 2.4 leaves per 10 cm of stem length); leaves rather erect, narrowly oblanceolate with the widest place in their upper half; lowermost well-developed leaf typically subacute, rarely obtuse or acute at the apex; inflorescence usually sparse (not compact), lip purple to darkly (reddish-)purple, with bold or, rarely, pale markings, spur conspicuously robust, ca 9.3 mm long and 2.4 mm wide, purple (flower colouration and spur shape somewhat resembling that of *D. traunsteineri*). – Endemic to the Oravské Beskydy and Tatry Mts subsp. **arcana**

Acknowledgements We are indebted to a number of botanists that accompanied us in the field, helped us with searching localities or collected samples for us. We are also grateful for technical assistance in the FCM labs operated by M. Jandová and J. Vrána. Valuable consultations were kindly provided by P. Trávníček (FCM of orchids), A. Sennikov and J. Danihelka (taxonomic nomenclature). Our thanks also go to M. Hedrén and one anonymous reviewer, as well as to the handling editor P. Koutecký, who significantly contributed to the improvement of this paper. The holotype voucher was prepared and scanned by M. Oulehlová. The respective authorities in nature conservation are acknowledged for granting us with the necessary permits.

Authors' contributions VT, PB, FL and BT conducted the field work and collected morphometric data. VT, MH, FL, EMT and HWS participated in the estimation of genome sizes and ploidy levels. MD and MH designed and performed the statistical analyses. MD analysed the ecological and environmental data. VT and BT carried out the red list categorization. VT drafted the manuscript with significant contributions of MD, MH and BT. All authors commented on and approved the manuscript. BT supervised the project.

Funding Open access publishing supported by the National Technical Library in Prague. The research reported here was supported by an internal grant of Palacký University Olomouc (IGA_PrF_2023_001) and by the Austrian Federal Ministry of Education, Science and Research (BMBWF) within the programme Aktion CZ-AT, administrated by OeAD-GmbH. The article appears through the institutional support of long-term conceptual development of research institutions provided by the Ministry of Culture of the Czech Republic (ref. MK000094862).

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