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Biosystematic and chorological study of *Dactylorhiza maculata* agg. in Central Europe

PhD thesis

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Abstract: Protection of endangered species of the temperate orchids is often complicated due to unsatisfactory knowledge of their variability and taxonomic diversity. This is also the case of the *Dactylorhiza maculata* agg., an evolutionary young group of diploid and autopolyploid taxa with intricate phylogenetic relations and large morphological variability. In this thesis, multivariate morphometrics, flow cytometry and environmental data were employed to reveal the variability of the group in Central Europe.

A special attention was payed to populations traditionally recognized as *D. fuchsii* (s. str.), for which two ploidy levels, namely diploid and tetraploid, had previously been reported, but little has been known about frequency, distribution, and taxonomic value of its cytotypes. This study demonstrates that both diploids and tetraploids occur in Central Europe, where they form either pure ploidy or mixed ploidy populations. Moreover, DNA-triploids sometimes co-occur with the other cytotypes. Plants with different ploidy levels are indistinguishable in morphology and occupy similar habitats, but differences in their distribution patterns were revealed. Polyploidy must be regarded as a hidden source of variation in *D. fuchsii*, which should be taken into consideration in further research and biodiversity protection activities.

The ploidy level was traditionally deemed the most important distinguishing trait between two putative species in Central Europe, namely diploid *D. fuchsii* and tetraploid *D. maculata*. This taxonomic concept was challenged by the discovery of tetraploid *D. fuchsii* as well as rather continuous morphological variability of the *D. maculata* agg. Homoploid hybridization probably allows for genetic admixture between these taxa, or even merging of some distinct evolutionary lineages, which may seem to be well separated in other parts of their distribution areas. Moreover, DNA-triploids may facilitate the gene flow across ploidy levels. Amalgamation of all Central European taxa into a single species, *D. maculata,* is thus advocated here. Eight subspecies with distinct morphology, cytotype diversity and/or ecology may be circumscribed within this species in the study area.

An updated checklist and determination key to Central European *D. maculata* subspecies are provided here, of which two were described as new to science, and one was resurrected in this work. For the Czech Republic, grid-based distribution maps were created, and national Red List categories were assigned for subspecies occurring in this country.

Keywords: cytogeography; distribution; ecology; endangered species; flow cytometry; habitat; morphometrics; Orchidaceae; polyploidy; Red List; taxonomy

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Abstrakt: Ochrana ohrožených druhů temperátních orchidejí je často komplikovaná kvůli neuspokojivým znalostem o jejich variabilitě a taxonomické diverzitě. To je také případ *Dactylorhiza maculata* agg. (okruhu prstnatce plamatého), evolučně mladé skupiny diploidních a autopolyploidních taxonů se složitými fylogenetickými vztahy a značnou morfologickou variabilitou. Předložená práce využívá mnohorozměrné statistiky, průtokové cytometrie a environmentálních dat ke studiu variability této skupiny ve střední Evropě.

Zvláštní pozornost byla věnována populacím tradičně rozlišovaným jako *D. fuchsii* (s. str.), pro něž byly již dříve udávány dva ploidní stupně, diploidní a tetraploidní, avšak existovalo jen málo poznatků o četnosti, rozšíření a taxonomické hodnotě obou cytotypů. Tato práce ukazuje, že ve Střední Evropě se vyskytují diploidi i tetraploidi, již zde tvoří buď čisté, nebo ploidně smíšené populace. Na společných lokalitách s těmito dvěma cytotypy se navíc někdy objevují také DNA-triploidi. Rostliny s rozdílnou ploidní úrovní jsou morfologicky nerozlišitelné a rostou ve stejných biotopech, avšak byly zjištěny rozdíly v charakteru jejich rozšíření. Polyploidie u *D. fuchsii* by měla být nahlížena jako zdroj skryté variability, což je třeba zohlednit v dalším výzkumu i aktivitách na ochranu biodiverzity.

Stupeň ploidie byl ve střední Evropě tradičně považován za nejdůležitější rozlišovací znak mezi dvěma domnělými druhy, diploidní *D. fuchsii* a tetraploidní *D. maculata*. Tento taxonomický koncept byl však zpochybněn kvůli objevu tetraploidní *D. fuchsii* a více méně kontinuální morfologické variabilitě *D. maculata* agg. Homoploidní hybridizace pravděpodobně umožňuje mísení genetické informace obou taxonů, nebo dokonce splývání některých evolučních linií, jež se v jiných částech areálu mohou zdát jako dobře oddělené. DNA-triploidi navíc mohou usnadňovat genový tok napříč ploidními stupni. Výsledky této práce proto podporují taxonomický koncept, který spojuje všechny středoevropské taxony do jediného druhu *D. maculata*. V rámci tohoto druhu může být ve studovaném území rozlišeno osm poddruhů, a to na základě morfologie, cytotypové diverzity a/nebo ekologie.

Práce přináší aktualizovaný seznam středoevropských poddruhů a klíč k jejich určení. Dva poddruhy jsou zde popsány jako nové pro vědu, jeden dříve popsaný druh byl nově rozpoznán. Pro Českou republiku byly dále vytvořeny síťové mapy rozšíření jednotlivých poddruhů, pro něž byly rovněž stanoveny kategorie ohrožení podle národního červeného seznamu.

Klíčová slova: cytogeografie; rozšíření; ekologie; ohrožené druhy; průtoková cytometrie; biotop; morfometrika; Orchidaceae; polyploidie; červený seznam; taxonomie
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DECLARATION

I hereby declare that this thesis has been worked out by myself together with listed co-authors. All literary sources cited in this thesis are listed in the References section.

In Olomouc

Vojtěch Taraška

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AUTHOR CONTRIBUTIONS

CHAPTER 1 **Introduction and aims of the thesis**

VT wrote this text.

CHAPTER 2 **Morphological variability, cytotype diversity, and cytogeography of populations traditionally called *Dactylorhiza fuchsii* in Central Europe**

VT, PB, and BT conceived the project and conducted the field work. VT, EMT and HWS performed the analysis of genome size and chromosome numbers. MD designed and conducted the statistical analysis. VT drafted the manuscript with significant contributions of MD and BT. All authors commented on and approved the manuscript.

CHAPTER 3 ***Dactylorhiza maculata* agg. (Orchidaceae) in Central Europe: Intricate patterns in morphological variability, cytotype diversity and ecology support the single species concept**

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.

CHAPTER 4 **Distribution of the *Dactylorhiza maculata* agg. in the Czech Republic**

VT wrote the Introduction and Addendum. Other parts are adopted from the paper Kaplan et al. (2017) of which the main editor was ZK. VT and BT prepared the maps and comments on *D. maculata* agg.

CHAPTER 5 **Summary and conclusions**

VT wrote this text.

Authors' abbreviations: BT – Bohumil Trávníček, EMT – Eva M. Temsch, FL – František Lamla, HWS – Hanna Wiess-Schneeweiss, MD – Martin Duchoslav, MH – Michal Hroneš, PB – Petr Batoušek, VT – Vojtěch Taraška, ZK – Zdeněk Kaplan.

TABLE OF CONTENT

CHAPTER 1:
Introduction and aims of the thesis

Vojtěch Taraška

FAMILY ORCHIDACEAE

The orchid family (Orchidaceae) constitutes a basal clade of the ordo Asparagales (Stevens 2001), and with approximately 28,000 species it is the most species-rich family of Monocots (Christenhusz and Byng 2016). The centres of its taxonomic diversity were identified in tropical South America and south-east Asia, but its representatives are distributed almost worldwide. The family is divided into five subfamilies, namely (a) Apostasioideae, (b) Vanilloidae, (c) Cypripedioidae, (d) Orchidoidae, and (e) Epidendroidae. However, only the latter two contain about 99% of the total species richness of the family (cf. Stevens 2001; Jersáková et al. 2013; Chase et al. 2015).

Regardless of a few exceptions, members of the family share several common attributes. They are perennial herbs, either epiphytic or terrestrial. One out of six petals in their flowers is different from the others in shape, size and/or colour, and it is called lip or labellum. The number of stamens is reduced to 1(-3) and together with gynoecium they form an accretion called gynostemium. The flowers are often resupinate. The seeds are very small, dust-like, with a small poorly differentiated embryo and a minimum of nutrients, and their germination therefore fully depends on symbiosis with fungi (Buttler 2000; Delforge 2006). An important karyological attribute of orchids is the strict partial endoreplication, i.e. replication of a fixed fraction of the genome in nuclei of differentiated cells (Bory et al. 2009; Trávníček et al. 2015; Hřibová et al. 2016; Brown et al. 2017). The fraction is species-specific, and it may be thus used as a marker in plant taxonomy (Trávníček et al. 2021). The strict partial endoreplication has been detected in many orchid species, but never outside this plant family (Trávníček et al. 2015).

Orchids are known to enter complex relations with other organisms. All species are fully dependent on the mycorrhizal symbiotic fungi at least during the seed germination and in early ontogenetic stages (Li et al. 2021). Many of them, including both mycoheterotrophic and autotrophic species, however, rely on the mycorrhiza their whole life. Little is known about the taxonomic identity of the symbiotic fungi, but investigations from Europe suggest that they belong to common groups of Basidiomycetes in that area (e.g. Jacquemyn et al. 2012, 2016). Orchids usually belong to plants pollinated by insects. Various strategies ensure the pollinator attraction, including food rewarding, food deception and sexual deception. The efficiency of food deception is conditioned by a sufficient number of naive pollinators as well as co-occurrence of imitated species, while sexual deception requires the presence of a specific pollinator (Delforge 2006). The lack of suitable pollinators may be circumvented by autogamy or agamospermy in some species (Neiland and Wilcock 1998).

Relative to their high taxonomic diversity, orchids have little economic importance. Tropical species are popular in horticulture. Members of the genus *Vanilla* are grown in tropical areas for their capsules used as a spice. Tubers of some temperate species, particularly *Orchis* spp., used to be picked up in the Middle East for preparing *sahlep*. Temperate orchids are also popular with the general public and frequently sought after by the amateur botanists as well as photographers because of their attractive appearance. Therefore, they meet all requirements to flagship species in nature protection. The complex interactions with other organisms, including mycorrhizal symbionts, pollinators, grazers and other plants, make them sensitive to any ecosystem changes (Fay and Chase 2009; Štípková and Kindlmann 2021). Especially in European countries, protection of orchids simultaneously aims to protect habitats, biodiversity and landscape in general.

GENUS *DACTYLORHIZA*

Taxonomy and infrageneric treatments

Dactylorhiza Necker ex Nevski is a Holarctic genus of the orchid family (Orchidaceae Juss.), distributed in whole Europe, north Africa, temperate to boreal zone of Asia, with one species exceeding to North America, namely *D. viridis* (L.) R. M. Bateman, Pridgeon & M. W. Chase (Eccarius 2016). Depending on selected taxonomic concept, various numbers of *Dactylorhiza* species are mentioned to exist worldwide, ranging from 37 (Eccarius 2016) up to 61 (Delforge 2006), or even 75 (Averyanov 1990). The centre of the genus' genetic diversity was identified in the Mediterranean area, but the highest number of taxa have been recognized in Central and West Europe (Pillon et al. 2006), which is usually attributed to uneven taxonomic effort and generally high interest in orchids among European botanists (Pillon and Chase 2007).

Formerly, genus *Dactylorhiza* was not distinguished from genus *Orchis* L. A total of five *Orchis* species originally described by Linné (1753) are currently recognized within the genus *Dactylorhiza*, being based on the Linnean epithets *incarnata, latifolia, maculata, sambucina* and *viridis*. Linné (1753) already distinguished between species with simple ("*bulbis indivisis*") and digitate ("*bulbis palmatis*") tubers. These differences were first taxonomically reflected by Necker (1790), who introduced the name *Dactylorhiza* Neck. for orchids with digitate tubers (including species currently assigned to other genera). Later on, Nevski (1935) adopted this generic name in a narrower sense, more or less corresponding to contemporary circumscription of the genus, but it fell into oblivion for a long time. Much more reflected was the treatment by Klinge (1899) who divided genus *Orchis* into two subgenera, namely *Euorchis* Klinge and *Dactylorchis* Klinge. The latter was promoted to the genus level by Vermeulen (1947). However, the name *Dactylorhiza* Nevski has a priority over *Dactylorchis* (Klinge) Verm. in that rank.

Separation of *Orchis* and *Dactylorhiza* is nowadays broadly accepted. The most significant distinguishing morphological traits are the shape of tubers (ovoid vs digitate), size of bracts (minute vs conspicuous), and shape of lips (four- vs three-lobbed). This treatment was also supported by molecular phylogeny. Although genus *Orchis* in its traditional circumscription split into three genera (*Orchis* s. str., *Anacamptis* s. lat., *Neotinea;* Bateman et al. 1997), none of them is closely related to *Dactylorhiza*. Instead, *Dactylorhiza* (incl. *Coeloglossum*, see below) is considered a monophyletic genus close to other genera with digitate tubers, i.e. *Gymnadenia* s. lat., *Pseudorchis* and *Platanthera* (Bateman et al. 2003, 2018).

First attempt to taxonomic division of (current) genus *Dactylorhiza* may probably be attributed to Camus and Camus (1928), who recognized four subsections within the subgenus *Dactylorchis* (recently accepted names at the species levels are given in the brackets): subsect. *Conniventes* (*D. iberica*), subsect. *Sambucinae* (*D. sambucina, D. romana*), subsect. *Latifoliae* (e.g. *D. cordigera, D. praetermissa, D. foliosa, D. incarnata, D. majalis, D. traunsteineri*), and subsect. *Maculatae* (*D. maculata* s. lat., incl. *D. fuchsii, D. saccifera, D. elodes* etc.). This treatment was revised by (among others) Keller & Schlechter (1928), Nevski (1935), Vermeulen (1947), Soó (1960), Averyanov (1990) and, most recently, Eccarius (2016). In general, there is a consensus on the separation of *D. iberica* as well as *D. sambucina* and its close relatives. The Eccarius' approach to section delimitation was found to be highly controversial as obviously inconsistent with revealed phylogenetic relations (Bateman 2021). Thus, somewhat earlier classification by Averyanov (1990) seems to be the most reliable up to

date, treating the genus *Dactylorhiza* as follows (names of taxa as stated in the reference source):

Sect. *Iberanthus*	*D. iberica*
Sect. *Aristatae*	*D. aristata*
Sect. *Sambucinae*	*D. sambucina* agg., *D. romana*, and others
Sect. *Dactylorhiza*	
Subsect. *Dactylorhiza*	*D. incarnata* agg., *D. euxina*, *D. umbrosa*, *D. hatagirea*, *D. salina*, and others
Subsect. *Maculate*	*D. maculata* agg., *D. foliosa*, *D. fuchsii* agg. *D. saccifera* agg.
Subsect. *Latifoliae*	*D. majalis* agg., *D. cordigera* agg, *D. traunsteineri* agg., and others
Subsect. *Sesquipedales*	*D. elata* agg.

Molecular phylogenetics revealed that monotypic genus *Coeloglossum* Lindl. should be also incorporated into genus *Dactylorhiza* (Bateman et al. 1997, 2018), although its taxonomic position within this genus is still unclear (cf. Devos et al. 2006, Bateman and Rudall 2018, Brandrud et al. 2020). Conservation of the name *Dactylorhiza* against by far older name *Coeloglossum* was thus needed in order to stabilise the nomenclature (Brummitt 2004). *Dactylorhiza viridis* (syn. *C. viride*), the only representative of the abolished genus *Coeloglossum*, differs from other congeners in several traits, among other in presence of the nectar in its spur and rewarding pollination strategy (Tyteca and Klein 2008), and it definitely deserves the highest infrageneric rank within genus *Dactylorhiza*. The taxonomic categories of sections and subsections are, however, only rarely used in recent literature concerning *Dactylorhiza*. Instead, particular taxa are assembled into informal groups or aggregates (Fig. 1), representing distinctive evolutionary units with a peculiar role in the polyploid evolution of the genus (cf. Hedrén 2001; Delforge 2006; Bateman 2021). These are particularly *D. sambucina, D. incarnata, D. maculata, D. euxina, D. majalis* and *D. traunsteineri* groups. Along with them, *D. aristata*, *D. iberica* and *D. viridis* are treated as phylogenetically isolated species.

Figure 1. Examples of several Central European taxa of genus *Dactylorhiza*, representing different taxonomic groups. (**a**) *D. sambucina*; Czechia, White Carpathians Mts, 14 May 2015. (**b**) *D. incarnata* subsp. *incarnata*; Czechia, Moravian Karst, 4 June 2022. (**c**) *D. maculata* subsp. *fuchsii*; Czechia, Hrubý Jeseník Mts, 26 June 2023. (**d**) *D. majalis* subsp. *majalis*; Czechia, Vidnava Lowland, 13 May 2022. (**e**) *D. traunsteineri* subsp. *traunsteineri*; Austria, Kitzbühel Alps Mts (*locus classicus*), 15 June 2023. (**f**) *D. viridis*; Czechia, Hrubý Jeseník Mts, 27 June 2014. – Photo: V. Taraška.

Sources of variability and taxonomic diversity in genus *Dactylorhiza*

High variability in morphological traits has been revealed in genus *Dactylorhiza* at both among-population and within-population levels, as well as at various geographic scales. The most striking differences have been observed in plant height, number of leaves, lip shape, flower colouration, leaf shape and, eventually, spotting (e.g. Vermeulen 1947; Bateman and Denholm 1983, 1985, 1988; Naczk et al. 2015). In addition, variability in ploidy levels and chromosome numbers has been found for some groups of the genus, particularly among the members of the so-called *D. incarnata/maculata* polyploid complex (Heslop-Harrison 1951; Jagiełło 1988; Aagaard et al. 2005; Ståhlberg and Hedrén 2010). The genetic variability of some *Dactylorhiza* taxa may also be extensive (e.g. Pillon et al. 2007; Naczk et al. 2015; Brandrud et al. 2020). Correlation between morphology, ploidy level and genetics is often weak or intricate, which is the main challenge for taxonomy of the genus. The main sources of variability and taxonomic

diversity of the genus are related to its migration history, reproduction strategy, and current evolutionary processes including hybridization and polyploidization.

Migration history and geographical determinants

The highest genetic diversity of the genus was found in the Mediterranean area and the Caucasus, which were thus considered as potential glacial refugia for most European *Dactylorhiza* species by Pillon et al. (2006). This hypothesis was later supported by ecological niche modelling (Naczk and Kolanowska 2015). Nevertheless, some temperate species, including the woody plants, probably survived in northern refugia during the LGM (Schönswetter et al. 2005; Douda et al. 2015; Mitka et al. 2023; Molnár et al. 2023). This may be also the case of some representatives of genus *Dactylorhiza* whose refugia may have been situated in areas with sheltered topography in Central Europe (e.g. Alps, Carpathians) and in the Russian Plain east of the continental ice sheet (Nordström and Hedrén 2008, 2009; Ståhlberg and Hedrén 2010; Balao et al. 2016). In the Holocene, after the recession of the continental ice sheet, various *Dactylorhiza* species expanded from their refugia northwards to previously glaciated areas in Fennoscandia (Nordström and Hedrén 2008; Ståhlberg and Hedrén 2010), British Isles (Hedrén et al. 2011), or Siberia (Averyanov 1990). Thus, strong geographic pattern in genetic diversity is apparent in genus *Dactylorhiza* as a result of postglacial migration. Interestingly, unlike in many other plant groups, this pattern does not simply consist in decrease of variability from south to north (Pillon et al. 2007), being affected by various evolutionary processes.

Variability of populations in newly colonised territories may be increased due to multiple colonisations by the same species from different source areas. Seeds of the orchids are very small, dust-like, which is an adaptation for their transfer by wind (Arditti and Ghani 2000). Long-distance seed dispersal is considered to be an important mechanism of genetic homogenization of orchid species across their distribution range (Brzosko et al. 2017), and it was proved to shape the genetic structure in some allotetraploid *Dactylorhiza* taxa in North Europe, namely in Gotland (Hedrén et al. 2018) and Scandinavian Peninsula (Hedrén and Nordström Olofsson 2018). On the other hand, the role of seed transportation in orchids must not be overestimated, as most seeds are only spread in the vicinity of the mother plant (Machon et al. 2003; Jacquemyn 2007). In fact, the seed dispersal is likely important during the periods of expansion and colonisation, but its contribution to gene flow among established populations is rather low (cf. Balao et al. 2015, Hedrén et al. 2018, Hedrén & Nordström Olofsson 2018).

Another case of processes leading to regionally increased variability is the fusion of genetically distinct lineages. Gradual but slow melting of continental ice sheet, followed by fast colonisation of uncovered territories by plants, led to the establishment of secondary contact zones of particular genotypes of *D. traunsteineri* agg. in central Scandinavian Peninsula and Baltic states (Nordström and Hedrén 2008). Similarly, two lineages of *D. maculata* subsp. *maculata* expanded via two migration routes from geographically distinct glacial refugia to Scandinavia, where they intermingled (Ståhlberg and Hedrén 2008, 2010). Even Central Europe has proved to be an area of secondary contact between two autotetraploid *D. maculata* agg. taxa, namely autochthonous *D. maculata* subsp. *fuchsii* and South-West European lineage of *D. maculata* subsp. *maculata* (cf. Ståhlberg and Hedrén 2010; Naczk et al. 2015), but their variations in that region were poorly explored.

Despite the presumed ability of the long-distance dispersal in orchids, the geographic isolation may also be important for shaping the variability and diversity of genus *Dactylorhiza*. A very good example is the Madeiran island endemic *D. foliosa*, which is phylogenetically close to European continental *D. maculata* (Ståhlberg & Hedrén 2010; Brandrud et al. 2020), but gradually accumulated morphological, karyological and genetic changes between these two taxa justify their separation at the species level (Bateman 2021; Hedrén 2022). On the other hand, populations form Iceland used to be also treated as endemic species *D. islandica* (e.g. Delforge 2006), but in fact they are indistinguishable from continental *D. maculata* (Bateman 2021). Much depends on the colonisation history, with differences between relic populations and recent colonisers. Terrestrial island-like systems, including mountaintops, outcrops, and edaphic islands, may affect the ecological-evolutionary processes and regional biodiversity in similar ways as the 'true' islands (Dawson et al. 2016; Itescu 2019; Mendez-Castro 2021). This may at least partly explain a relatively high variability and taxonomic diversity of genus *Dactylorhiza* in areas with complex topography and mosaic landscape structure such as Central and West Europe.

In contrast to relic populations, there are the newly established taxa in the postglacial period, which occupy just a small area close to the place of their origin. Particularly this is the case of some allopolyploids arisen from hybridization between various species (e.g. Hedrén 2001; Hedrén et al. 2011). These taxa contribute very little to overall genetic variability of genus *Dactylorhiza*, because they share most of their genomes with their progenitors. However, they seriously increase the taxonomic diversity of the genus if they are formally described (Pillon and Chase 2007). Moreover, the origin of many locally distributed taxa is unknown or just speculative, which also complicates their taxonomic positioning and ranking, as is the case with *D. bohemica* (Businský 1989), *D. carpatica* (Batoušek and Kreutz 1999), *D. isculana* (Seiser 2002), or *D. majalis* subsp. *turfosa* (Procházka 1982). It is thus a matter of disputation whether such taxa should or not be recognized taxonomically (cf. Pedersen 1998; Pillon and Chase 2007; Bateman 2021).

Pollination system and sexual reproduction

Except for the rewarding species *D. viridis*, all members of the genus belong to food deceptive orchids with sexual mode of reproduction. Representatives of insect genera *Apis*, *Bombus*, *Volucela* (Hymenoptera), *Alosterna* and *Strangalia* (Coleoptera) were identified as pollinators of various *Dactylorhiza* species (Dafni and Woodell 1986; Gutowski 1990; Ostrowiecka et al. 2019; Wróblewska et al. 2019). High variability in floral sizes, morphology, colouration as well as floral scents increases the probability of repeated visits by deceived pollinators, which in turn increases the individual reproductive success (Jersáková et al. 2006; Vallius et al. 2007; Pellegrino et al. 2008; Wróblewska et al. 2019). This strategy thus strongly supports disruptive evolution in floral traits, resulting in distinct morphotypes / phenotypes present in the population (e.g. Ackerman et al. 2011). This effect was most convincingly demonstrated in *D. sambucina* (Gigord et al. 2001) and *D. incarnata* (Vallius et al. 2008) with dimorphism in flower colouration. On the other hand, the food deception also promotes the interspecific competition for pollinators (Lammi and Kuitunen 1995), which may enhance hybridization in sympatric populations of two or more *Dactylorhiza* taxa (Neiland and Wilcock 1998). Occasional gene flow between distinct taxa may increase their genetic variability (e.g. Balao et al. 2016; Brandrud et al. 2020), but it may also end up with the genetic corrosion and elimination of one species (e.g. Krahulcová et al. 1996; Musilová 2013), which must be definitely regarded as decrease in local species diversity.

The variability depletion at the population level may also be driven by self-pollination, which was experimentally proved in several *Dactylorhiza* species (e.g. Juillet et al. 2007; Vallius et al. 2008). Autonomous self-pollination through the mechanism of caudicle reconfiguration was observed in *D. fuchsii* (Tałałaj et al. 2019). This kind of selfing was prevented by caudicle removal by insects, and it may be thus regarded as an adaptation to the lack of pollinators. In contrast, a relatively high level of pollinator-mediated geitonogamy was observed in natural populations of *D. sambucina* (Kropf and Renner 2008), which related to the low density of conspecific plant individuals but abundant pollinators. Self-pollination may be beneficial for persistence of the populations under suboptimal conditions, but it inevitably leads to lower fitness, inbreeding depression, and reduction of population genetic variability (Jersáková et al. 2006).

Interspecific hybridization and polyploidization

The most important sources of variability and taxonomic diversity in genus *Dactylorhiza* are hybridization and polyploidization. Homoploid hybridization is probably more frequent, but even hybrids between diploids and tetraploids have been reported (e.g. Ståhlberg 2009; Balao et al. 2016; Kantor 2019). Primary hybrids of *Dactylorhiza* species may be at least partly fertile and backcross with their parents, which sometimes results in formation of hybrid swarms (e.g. Lord and Richards 1977; Bertolini et al. 2000; Aagaard et al. 2005; De hert et al. 2012; Balao et al. 2016). Past introgressive gene flow is sometimes detected also in plants whose phenotype does not exhibit any traces of hybridization (e.g. Ståhlberg & Hedrén 2008; Naczk et al. 2015). Primary hybrids of various *Dactylorhiza* species have been formally described (e.g. Businský 1989; Batoušek 1997), but more extensive hybridization may even lead to establishment of new hybridogenous taxa (e.g. Pedersen 2006).

Polyploidization occurs in several *Dactylorhiza* sections / groups. The basic chromosome number for the genus is $n = 20$ (Averyanov 1990). The polyploid series includes diploids ($2n = 40$), triploids ($2n = 60$), tetraploids ($2n = 80$), pentaploids ($2n = 100$), and hexaploids ($2n = 120$; Kliphuis 1963; Heslop-Harrison 1968; Averyanov 1979; Vöth and Greilhuber 1980; Averyanov et al. 1982; Cauwet-Marc and Balayer 1984; Jagiełło and Lankosz-Mróz 1988; Jagiełło 1989; Bertolini et al. 2000; Efimov 2023), which may be arisen from both auto- and allopolyploidization (Hedrén 1996, 2001; Hedrén et al. 2001; Pillon et al. 2007). Aneuploid chromosome numbers have also been reported in genus *Dactylorhiza*, but they were connected either to infraspecific variability (Averyanov et al. 1982), or hybridization (Lord & Richards 1977), or they eventually resulted from erroneous counting, and it is thus lacking any taxonomic significance. No polyploids have been reported in *D. iberica, D. aristata* nor *D. viridis*. One allotriploid and one allotetraploid species have been revealed in the *D. sambucina* group (Pedersen 2006). Members of the *D. incarnata* group are considered strictly diploid in Europe (Kantor 2019), but a tetraploid species *D. armeniaca* is known from the Caucasus (Hedrén 2001). Besides diploids, *D. maculata* group involves several independently established autotetraploid lineages (Ståhlberg and Hedrén 2010). Exclusively allopolyploid taxa are assembled in the *D. majalis / traunsteineri* group (e.g. Hedrén 1996; Pillon et al 2007).

Interspecific hybridization and polyploidization are often joint phenomena in *Dactylorhiza*. Allopolyploid hybridization occurred repeatedly between various taxa of the *D. incarnata* and *D. maculata* groups, which resulted in formation of numerous allotetraploid taxa, currently distributed across temperate Eurasia (e.g. Hedrén 1996, 2001; Devos et al. 2003; Pillon et al. 2007; Naczk et al. 2015). Their taxonomic

classification is a tough nut to crack because of similar genome composition, morphological convergence, and virtually unlimited gene flow between distinctive allopolyploid lineages, as well as frequent hybridization with their progenitors. Some of the allotetraploid taxa are considered to be polyphyletic, and obviously reticulate evolution (cf. Devos et al. 2003; Pillon et al. 2007; Balao et al. 2016) prevents application of strictly cladistic approach. Two major groups of allotetraploids are usually recognized at the species level, namely *D. majalis* s. lat. established in pre-Holocene and spread from its refugium after deglaciation ('old' allotetraploids), and genetically heterogeneous *D. traunsteineri* s. lat. arisen polytopically during Holocene ('young' allotetraploids; Pillon et al. 2007; Balao et al. 2016). However, both old and young allopolyploids are sometimes amalgamated into a single species, *D. majalis sensu latissimo* (Bateman and Denholm 1983; Pedersen et al. 2003; Nordström and Hedrén 2008, 2009).

Variability vs plasticity

The main challenge for morphological studies in *Dactylorhiza* is to distinguish between variability on the genetic background and plasticity induced by the environment (e.g. Heslop-Harrison 1948; Bateman & Denholm 1989; Faltyn and Jakubska-Busse 2008; Naczk et al. 2015; Efimov et al. 2023). The individual morphology may be influenced by both abiotic and biotic factors. The abiotic factors relate mainly to the soil reaction, water supply, annual temperature regime and insolation (e.g. Heslop-Harrison 1948; Jagiełło 1988; Blinova 2004). The biotic factor with strong impact on flower morphology is the behaviour of pollinators (Heslop-Harrison 1968; Dufrêne et al. 1991), but also mycorrhizal symbiosis, intensity of grazing, or interspecific interactions among plants may be of significant importance (cf. Callaway et al. 2003; Wang et al. 2017; Puy et al. 2021; Whyle et al. 2022). Over a long period, joint effects of these factors may shape the phenotypes in local populations of *Dactylorhiza* via cumulative genetic changes under the selection pressure.

Moreover, adaptive epigenetic changes have been observed in *Dactylorhiza* populations with similar genome compositions growing under different ecological conditions (Balao et al. 2017). Their epigenetic variation correlates with eco-environmental conditions, such as the water availability and temperature, and it is thus considered as an important adaptive mechanism after colonisation of new sites (Paun et al. 2010). Some of these epigenetic changes are stable and heritable, and they may be responsible for persistent ecological divergence between sibling taxa arisen from hybridization between the same parental taxa (Paun et al. 2010, 2011; Wolfe 2023). In such cases, observed morphological variability may be of a taxonomic importance although it is not accompanied with genetic differences.

DACTYLORHIZA MACULATA GROUP

The *D. maculata* group, often reffered to as *D. maculata* agg., represents a diverse group of closely related diploid and autopolyploid taxa. Although it is considered monophyletic, its delimitation from other *Dactylorhiza* groups is difficult. In general, members of the *D. maculata* group may be recognized from other groups by a combination of several morphological characters: tubers deeply two to five-fid; stolones absent; stems rather thin and solid; leaves often spotted; flowers pink, purple or white (but not yellow); lip relatively wide, shallowly to deeply three-lobed (cf. Vermeulen 1947; Soó 1980;

Averyanov 1990; Delforge 2006; Eccarius 2016). Furthermore, monoploid genome size (1Cx value) of *D. maculata* group members is consistently lower than that of all examined representatives of other groups (cf. Aagaard et al. 2005; Siljak-Yakovlev 2010; Šmarda et al. 2019; but note that the latter incorrectly identified the ploidy level of *D. fuchsii*, which was probably tetraploid). Therefore, genome size also allows for identification of hybrids with members of the other groups (but not within the group). In the field, the flowering time may also be a useful trait, as the *D. maculata* group members usually reach their phenological optimum later than those of *D. sambucina, D. incarnata* and *D. majalis* (but not *D. traunsteineri*) groups.

Taxonomic classification of the *D. maculata* group underwent many changes in the past, and it is not even consensual until today. *Orchis maculata* was described by Linné (1753) in his *Species plantarum*. Several further taxa of the group have been described since the 19th century, either as varieties of *O. maculata* (e.g. *O. maculata* var. *sudetica* Poech ex Rchb.), or as separate species (e.g. *O. fuchsii* Druce, *O. transsilvanica* Schur), of which many are taxonomically recognized until today. In the middle of the 20th century, a taxonomic concept was introduced according to which the whole aggregate should be divided into two species, namely *O. maculata* and *O. fuchsii* (Heslop-Harrison 1951). They were supposed to differed in morphology, ecology as well as ploidy levels, as the first was considered to be tetraploid ($2n = 80$), while the latter diploid ($2n = 40$; e.g. Heslop-Harrison 1951, 1968). Since then, there was an obvious effort to subordinate any other member of the group to either of these species, mainly with emphasis on the ploidy level (cf. Vöth & Greilhuber 1978).

Nomenclatoric changes followed after the split of genus *Orchis* in 1960th, which resulted in many new combinations within genus *Dactylorhiza* (e.g. Soó 1962; Hunt and Summerhayes 1965), but the taxonomic concept of two species persisted in the *D. maculata* group. Morphological variability, ploidy level diversity, and their correlations were further inquired in the group. In West and North Europe, distinctions of *D. maculata* and *D. fuchsii* were usually confirmed (e.g. Tyteca and Gathoye 2003; Bateman and Denholm 2003; Ståhlberg and Hedrén 2008), justifying their separation at the species level. By contrast, rather ambiguous results were obtained from investigations in Central and East Europe. In this region, tetraploid populations morphologically corresponding to *D. fuchsii* were identified (e.g. Vermeulen 1968; Vöth 1978; Jagiełło 1988; Bertolini et al. 2000; Měsíček and Javůrková-Jarolímová 1992). Moreover, the circumscription of both species, *D. maculata* and *D. fuchsii*, appeared to be problematic in this area, and the taxonomic positioning of some local taxa within the abovementioned species seemed to be rather artificial or disputable (cf. Potůček 1969; Procházka 1979; Vöth & Greilhuber 1980; Jagiełło 1988; Averyanov 1990; Naczk et al. 2015; Efimov et al. 2023).

Investigations based on molecular markers shed new light to the phylogeny and phylogeography of the group. First insight revealed that *D. maculata* comprised two distinct lineages, of which one is genetically closer to *D. fuchsii* than to the other *D. maculata* populations (Shipunov et al. 2004). Later on, a large-scale analysis of ITS and plastid haplotypes (Ståhlberg and Hedrén 2010) identified much more intricate evolutionary history of the group, which supports amalgamation of *D. maculata* and *D. fuchsii* into a single species. The diploid Madeiran endemic *D. foliosa* represented a sister group of *D. maculata* s. lat., which comprised a total of five evolutionary lineages, namely (i) south-west European and (ii) north-east European subsp. *maculata* (both autotetraploids of independent origins), (iii) south-east European diploid subsp. *saccifera*, (iv) a widespread Eurasian diploid subsp. *fuchsii*, and (v) an autotetraploid segregate of

subsp. *fuchsii* distributed in Central Europe. A geographically restricted contact zone with reciprocal gene flow between both *maculata*-lineages was revealed in Scandinavia (Ståhlberg and Hedrén 2008). Distinct tetraploid lineages, namely south-west *maculata* and *fuchsii*, however come into contact also in Central Europe.

The latest attempt to reveal the phylogeny of the group employed the RAD-seq data from populations across Europe and the Caucasus (Brandrud et al. 2020). The *D. maculata* group clearly split into four clades, namely *gervasiana*-clade, *saccifera*-clade, *fuchsii*-clade and *maculata*-clade. Consistently with previous findings (Ståhlberg & Hedrén 2010; Naczk et al. 2015), the *fuchsii*-clade exhibited a large level of genetic diversity, but strong cohesion and no geographic structure across its distribution range. In contrast, the *maculata*-clade comprised genetically heterogeneous populations, including diploid *D. foliosa* and several autotetraploids recognized as *D. *kolaënsis* (the asterisk here and further on is used when dealing with taxa regardless of their taxonomic rank), *D. *transsilvanica*, *D. *islandica*, *D. *savogiensis*, *D. *ericetorum*, and *D. *caramulensis* (Brandrud et al. 2020; summarised by Bateman 2021). According to Bateman (2021), the four clades recognized by Brandrud et al. (2020) should be taxonomically recognized as separate species, with an additional species to be the ancestral diploid Madeiran endemic *D. foliosa*. However, this approach possibly underrates the role of hybridization and gene introgression between *D. *maculata* and tetraploid *D. *fuchsii* in Central Europe (e.g. Ståhlberg & Hedrén 2010; Naczk et al. 2015; Brandrud et al. 2020). Some regionally distributed taxa were missing or undersampled by Brandrud et al. (2020), which is most striking for completely lacking representatives of Central European tetraploid *D. *fuchsii*. On the other hand, several accessions of *D. *sooana* from Czechia and Hungary were included in *D. *fuchsii* without any remark. Moreover, the topology of major clades was unstable and strongly dependent on inclusion/exclusion of several *D. *maculata* accessions from North Europe (Brandrud et al. 2020; Bateman 2021). In this light, the phylogeny of the group remains unresolved, and any attempt to its taxonomic classification may be disputable.

PROTECTION AND CONSERVATION OF GENUS *DACTYLORHIZA*

Members of the genus *Dactylorhiza* are included in national Red Lists of all Central European countries, including Austria (Niklfeld and Schratt-Ehrendorfer 1999), Germany (Metzing et al. 2018), Hungary (Király 2007), the Czech Republic (Grulich 2017), Poland (Mirek et al. 2021), and Slovakia (Eliáš et al. 2015). As the other temperate orchids, they often act as the flagship and umbrella species in nature protection. Much attention has been paid to their ecological requirements, finding suitable management, and describing the reasons of their decline (e.g. Jersáková and Kindlmann 2004; Štípková & Kindlmann 2021). Yet, the knowledge of variability, taxonomic diversity and chorology of rare and protected plants has an impact on the nature conservation issues, as well (Pillon & Chase 2007; Joffard et al. 2022). National or international policy on biodiversity conservation is usually implemented as the legal protection of species (or other taxa), and unresolved taxonomy thus seriously complicates the legislation measures. Furthermore, the threat status of rare species is usually stated following the methodology of IUCN (2012) which takes into consideration several criteria, including population size, geographic range, and the temporal changes of both these attributes. Clear delimitation of particular taxa and the knowledge of their distribution areas are thus prerequisites for a successful evaluation against the red list criteria.

Recent studies focused on variability and taxonomy of genus *Dactylorhiza* often emphasise their implications for biodiversity conservation. Above all, the understanding of how to handle the diversity dramatically changed since the advances in molecular biology. The number of taxa recognized in some territories appeared to be exaggerated, not corresponding to real variation of the genus *Dactylorhiza* (Pedersen 1998; Pillon et al. 2006). Thus, it was suggested that conservation activities should focus on areas with high genetic variability, particularly former glacial refugia and secondary contact zones of distinct lineages (e.g. Pillon et al. 2006). Conservation importance of the refugial populations compared to those on the newly colonised margins of the species' distribution range have been demonstrated by Hedrén and Nordsrtröm Olofsson (2018). Nevertheless, information on genetic variation and phylogenetic position is still lacking for many taxa delimited solely on the ground of morphology. For example, several (steno)endemics have been reported from Central Europe, namely *D. bohemica*, *D. carpatica*, *D. fuchsii* subsp. *sooana* (Kliment 1999; Kubát 2010). In this area, endemism may be related to hypothetical presence of glacial refugia, but also excessive enthusiasm in finding new taxa among local botanists (Pillon and Chase 2007).

A great progress has been made in the perception of allopolyploids. They had previously been regarded as of lower conservation importance than their diploid progenitors (Hedrén 2001), but better insight into their genetic variation dramatically changed this view (Pillon et al. 2006; Nordström and Hedrén 2009). Despite an increasing number of studies on genetic variation of genus *Dactylorhiza*, its morphological and karyological variability has been underestimated in the last years. This is particularly true for the cytotype diversity in diploid-autopolyploid complexes, which is standing aside even in some recent phylogenetic studies (cf. Brandrud et al. 2020). Frequent occurrences of minority cytotypes have been revealed in a sister genus *Gymnadenia*, namely *G. conopsea*, whose cytotype diversity has been recommended as an important attribute to be taken into consideration while setting conservation priorities (Trávníček et al. 2011, 2012). Detailed information on cytotype diversity, frequency and spatial patterns in *D. maculata* agg. might by of similar importance, but they are missing for most of its distribution area.

Unresolved taxonomy of closely related species aggregates has also negative impact on conservation issues, as it prevents the determination of distribution ranges for particular taxa. Previously this was the case of allopolyploids *D. *traunsteineri*, *D. *lapponica* and *D. *russowii* traditionally recognized as separate species, but recently amalgamated into a single subspecies of widely distributed *D. majalis* s. lat. (Nordström and Hedrén 2008). More intricate relations were revealed among *D. incarnata* s. lat. with two colour morphs and its segregate *D. incarnata* var. *ochroleuca* (Hedrén & Nordström 2009; Pedersen 2009), which deserves a higher taxonomic status and, thus, conservation value. In contrast, the taxonomy remains unresolved for the Central European alpine populations of the *D. maculata* agg., namely *D. *savogiensis* and *D. *sudetica*, which are sometimes considered as endemics to particular mountain ranges (e,g. Delforge 2006; Eccarius 2016), while they are alternatively merged with Nordic *D. *psychrophila* (e.g. Soó 1980; Averyanov 1990). Depending on the taxonomic concept elected for these plants, very contrasting approach may be required from local authorities in nature protection. This is a very good example of how taxonomy affects the practices in nature and biodiversity conservation.

AIMS OF THE THESIS

Most species of the temperate orchids underwent a serious decline during the 20th century, and they are thus in the spotlight of nature conservationists. The reasons for their threat are quite well-understood (cf. Štípková and Kindlmann 2021). Yet, all activities related to the species protection are problematic unless the taxonomy of the target group/species aggregate is resolved. This is also the case of the *Dactylorhiza maculata* agg., whose members are often bound to vanishing habitats such as fens, peat bogs and wet meadows. The group comprises some widely distributed and locally abundant taxa along with putative endemics of small areas. Genus *Dactylorhiza* is also famous for its extreme variability (morphological, karyological, genetical) as well as morphological convergences, and delimitation of some taxa is thus unclear. Furthermore, about one-hundred-year-lasting research in distinct (often isolated) parts of Europe inevitably resulted in many ambiguities in taxonomic nomenclature, including frequent synonyms, illegitimate names, misinterpretations and misapplications of valid names, etc. For these reasons, it is very difficult to identify the most endangered taxa or areas of special conservation importance. The aim of this thesis was to reveal the variability and taxonomic diversity of the *D. maculata* agg. in Central Europe, and to provide a unified taxonomic concept applicable throughout the area. These issues are solved in following chapters:

Chapter 2: Morphological variability, cytotype diversity, and cytogeography of populations traditionally called *Dactylorhiza fuchsii* in Central Europe

This chapter is focused on Central European populations of *D. *fuchsii*, which previously proved to be variable in terms of ploidy-level. Frequency, distributions and morphological variability of its particular cytotypes are investigated. Taxonomic status of *D. *sooana*, an ambiguous taxon with apparent affinity to *D. *fuchsii*, is resolved here.

Chapter 3: *Dactylorhiza maculata* agg. (Orchidaceae) in Central Europe: Intricate patterns in morphological variability, cytotype diversity and ecology support the single species concept

In this chapter, taxonomic reassessment of the *D. maculata* agg. in Central Europe is done using morphometrics, ploidy level estimations, and analysis of eco-environmental traits. An overview of taxa occurring in this area is provided, including key to their determination.

Chapter 4: Distributions of *D. maculata* agg. taxa in the Czech Republic

Distribution data of *D. maculata* agg. and its individual taxa were integrated for territory of the Czech Republic, with emphasis on the revised herbarium specimens. Annotated grid-based distribution maps are presented as the main outputs in this chapter.

CHAPTER 2:

Morphological variability, cytotype diversity, and cytogeography of populations traditionally called *Dactylorhiza fuchsii* in Central Europe

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ABSTRACT

The morphological variation and cytotype diversity were investigated among Central European populations traditionally recognized as *Dactylorhiza fuchsii*, recently incorporated in *D. maculata* s.l. Flow cytometry was employed to assess the ploidy levels of 738 individuals from 77 localities and multivariate morphometrics for a total of 531 individuals from 27 localities. Three ploidy levels were found: diploid ($2n = 2x = 40$), DNA-triploid and tetraploid ($2n = 4x = 80$). Whereas diploids and tetraploids often occurred as pure-cytotype populations, individuals of DNA-triploids always co-occurred with at least one of the other cytotypes. Qualitative morphological traits were inferred to be the most important drivers of morphological variation among the investigated plants, with the most striking differences in flower colouration and leaf spotting. The combination of morphological and cytological characters enabled to delimit two separate groups of populations. The first corresponded to *D. maculata* subsp. *fuchsii* with morphologically indistinguishable diploid, DNA-triploid and tetraploid individuals, sometimes occurring in mixed-ploidy populations. A complex geographical pattern of cytotype distributions was observed, with diploids scatteredly occurring throughout Central Europe except for Bohemian Massif, which was dominated by tetraploids. The other group of populations represented newly described in this study *D. maculata* subsp. *sooana,* subsp. nova, morphologically well-defined and strictly diploid taxon with a restricted geographical range, occurring in the Western Carpathians. A new combination for a hybrid taxon *D.* × *dinglensis* nothosubsp. *smitakii,* comb. nova (= *D. maculata* subsp. *sooana* × *D. majalis* subsp. *majalis*), was also proposed.

INTRODUCTION

The genus *Dactylorhiza* Nevski belongs to the taxonomically most complicated groups of the orchid family in Europe (Heslop-Harrison 1968; Reinhard et al. 1991; Pedersen 1998; Delforge 2006; Pillon et al. 2006). Frequent polyploidization, hybridization, and gene introgression have resulted in reticulate evolution and multiple origins of some of its taxa (Lord and Richards 1977; Hedrén 1996; Hedrén et al. 2001; Pillon et al. 2007; Nordström and Hedrén 2009; De hert et al. 2012; Balao et al. 2016; Brandrud et al. 2020). High morphological variation, phenotypic plasticity (Meyer 1968) and putative epigenetic changes (Paun et al. 2010) further complicated the reconstruction of the phylogeny and taxonomic inferences within this group. The biosystematics and evolution of the genus has recently been a subject of many investigations, with the main focus on the *D. incarnata/maculata* polyploid complex, which consists of three groups of taxa: the diploid *D. incarnata* group, the diploid and autopolyploid *D. maculata* group, and the allopolyploid taxa of the *D. majalis/traunsteineri* group (Hedrén 2001; Devos et al. 2003; Pillon et al. 2007; Hedrén et al. 2008; Nordström and Hedrén 2009; Naczk et al. 2015; Bateman et al. 2018).

A number of taxa have been recognized within the *D. maculata* group across its distribution range from Europe to East Asia (cf. Vermeulen 1947; Senghas 1968; Soó 1980; Delforge 2006; Eccarius 2016), but no consensus on taxonomic treatment has been introduced to date, and the number of currently recognized species ranges from three to 15 (cf. Buttler 2000; Delforge 2006; Eccarius 2016). Two species are traditionally recognized in Central Europe within the *D. maculata* group: *D. maculata* (L.) Soó (s. str.)

and *D. fuchsii* (Druce) Soó (e.g. Soó 1980; Reinhard et al. 1991; Delforge 2006; Danihelka et al. 2012; Eccarius 2016). Both taxa were lectotypified by Vermeulen (1947); the type specimen of *D. maculata* was selected from Linné's material collected in the surroundings of Uppsala, while the name of *D. fuchsii* is based on Druce's collection from Wantage in Oxfordshire. They were distinguished based on their morphology (Druce 1915; Vermeulen 1947; Heslop-Harrison 1951; Gathoye and Tyteca 1987; Dufrêne et al. 1991; Tyteca and Gathoye 2003; Ståhlberg and Hedrén 2008): plants with narrow, acute leaves and broad labellum with a small and thin middle lobe were assigned to *D. maculata*, while *D. fuchsii* was characterized by broad, obtuse leaves and deeply three-lobed labellum with the wide and long middle lobe. Later, some differences were stated also in ecology (Heslop-Harrison 1951; Jagiełło 1988; Dufrêne et al. 1991; Ståhlberg 2009) and, above all, in chromosome numbers: diploids with $2n = 40$ were considered *D. fuchsii*, while tetraploids with $2n = 80$ were assigned to *D. maculata* (Heslop-Harrison 1951; Vöth and Greilhuber 1980; Averyanov 1982, 1990).

However, subsequent research disproved the correlation between morphology and ploidy levels of *D. maculata* group, particularly in Central Europe. Whereas *D. maculata* has always been found to be tetraploid, plants morphologically corresponding to *D. fuchsii* were reported to be either diploid or tetraploid (cf. Vermeulen 1968; Májovský 1976, 1978; Vöth 1978; Jagiełło and Lankosz-Mróz 1988; Měsíček and Javůrková-Jarolímová 1992; Krahulcová 2003). Moreover, the morphological differences between both taxa in Central Europe seem to be rather weak based on sparsely published data (Jagiełło 1988; Ståhlberg and Hedrén 2010; Kaplan et al. 2017). Therefore, many authors prefer to merge both these taxa into a single species *D. maculata* s.l. and recognize them as subspecies (e.g. Cauwet-Marc and Balayer 1984; Reinhard et al. 1991; Buttler 2000; Baumann et al. 2002; Ströhle 2003; GIROS 2009; Ståhlberg and Hedrén 2008; Naczk et al. 2015; Kurtto et al. 2019). This treatment also better reflects the genetic structure of the *D. maculata* group (Ståhlberg and Hedrén 2010). On the other hand, a recent molecular study of Brandrud et al. (2020) recognized *D. maculata* and *D. fuchsii* as two well-separated evolutionary lineages; their sampling in Central Europe was however scarce and did not include polyploid individuals of the latter taxon. It follows that *D. fuchsii* (*D. *fuchsii* from hereafter) has still an undefined taxonomic position within the *D. maculata* group and requires more detailed studies.

Considering all previous findings, it is obvious that Central European populations of *D. *fuchsii* are considerably variable both concerning morphological traits and ploidy levels (e.g. Delforge 2006; Eccarius 2016). However, little is known about the correlation between morphological variation and ploidy level, as well as the distribution patterns of particular cytotypes. This also applies to the most peculiar morphotype of white-flowering populations clearly derived from *D. *fuchsii* and sometimes recognized as *Dactylorhiza fuchsii* subsp. *sooana* Borsos, which is however an invalid name. This taxon was first mentioned from Northern Hungary (Borsos 1959) and nowadays is considered endemic to the Czech Republic, Slovakia, and Hungary (Kliment 1999; Vlčko et al. 2003). A brief description of this taxon provided by Borsos (1959, 1961) was supplemented by Batoušek (1995), referring to *D. fuchsii* subsp. *sooana* as possessing white flowers (with or without markings), white anther caps, and spotted leaves. Nonetheless, the range of morphological variation of this taxon overlaps with *D. fuchsii* subsp. *fuchsii* according to some authors (Borsos 1961; Soó 1980; Vlčko et al. 2003), and the delimitation of these taxa is thus complicated, which also causes taxonomic ambiguities. Kreutz (2004) recognized these two taxa as varieties of *D. fuchsii* subsp.

fuchsii, while Eccarius (2016) listed *D. fuchsii* subsp. *sooana* just among synonyms of *D. fuchsii*. A population of *D. *fuchsii*,* labelled as '*sooana*', was also included in the analysis by Ståhlberg and Hedrén (2010) as *D. maculata* subsp. *fuchsii*, with a note that it may be classified into a lower taxonomic unit because of possible morphological and/or geographical distinctions; nonetheless, the distinctions have not been scrutinized. Even the ploidy level of this putative taxon is unknown, and though both diploids and tetraploids have been mentioned in literature, reliable data are missing (Kubát 2010). Moreover, *D. fuchsii* subsp. *sooana* has never been validly described, as Borsos (1959) did not state the type specimen along with the protologue, and the epithet '*sooana*' has never been validated.

Several more taxa in various taxonomic ranks were recognized within *D. *fuchsii* in Central Europe (e.g. '*longibracteata*', '*meyeri*'), but they are usually not accepted in recent literature (cf. Kubát 2010, Eccarius 2016). Besides *D. fuchsii* subsp. *sooana*, the only widely accepted taxon is *D. fuchsii* subsp. *sudetica* (Rchb.) Verm., often synonymized with *D. fuchsii* subsp. *psychrophila* (Schlecht.) Holub. (e.g. Procházka 1979; Ponert 2019), resp. *D. fuchsii* subsp. *fuchsii* var. *psychrophilla* (Schlecht.) Soó (e.g. Kubát 2010; Danihelka et al. 2012). These names are applied to plants of subtle habitus and strikingly coloured flowers, occurring in mountain regions of Central Europe (Devillers and Devillers-Terschuren 2000). However, it was shown that the populations from the Sudeten Mts are rather transitional between *D. maculata* s. str. and *D. *fuchsii* in their morphology (Jagiełło 1988), and only tetraploid chromosome numbers have been found in these plants (Jagiełło 1988; Krahulcová 2003). Therefore, they are often incorporated into *D. maculata* s. str., under the name of *D. maculata* subsp. *sudetica* (Rchb.) Vöth (e.g. Vöth & Greilhuber 1980; Jagiełło 1988; Eccarius 2016). The taxonomic riddle of this taxon must be solved in a larger taxonomic and geographical context.

Flow cytometry provides a rapid estimate of the ploidy level of large populational samples and may be considered a useful non-invasive method (Doležel et al. 2007; Loureiro et al. 2010). This method was employed to assess the cytotype diversity of Central European populations of *D. *fuchsii* (including '*sooana*'). Simultaneous analyses of cytogenetic and morphological variation allowed us to address the following questions: (1) What is the extent and structure of morphological and genome size (cytotype) variation within this group in Central Europe? (2) What are the morphological characters diagnostic for the ploidy levels (cytotypes)? Revealed patterns of morphological and cytotype diversity allowed us to make some taxonomical inferences which follow here.

MATERIALS AND METHODS

Plant material

Only populations morphologically corresponding to *D. *fuchsii* according to literature (Soó 1980; Delforge 2006; Eccarius 2016) were studied. Each population was further classified as belonging to informal groups, either 'fuchsii' or 'sooana' (not italicized). Populations consisting of plants predominantly (with at least 95% individuals) with spotted leaves, white flowers (both with or without markings), and white anther caps were classified as 'sooana', while all others were considered 'fuchsii', comprising plants with

spotted or unspotted leaves, white to purple flowers and mostly purple anther caps (Batoušek 1995). Flow cytometric estimation of ploidy levels enabled further assignment of the populations belonging to the fuchsii group as 'fuchsii-2x', 'fuchsii-3x' and 'fuchsii-4x'. In mixed ploidy populations, each ploidy level was analysed as a separate subpopulation. The sooana group was uniform in ploidy level, and any further division of the group was thus not applicable.

Plant material and data were collected in 2011–2018 from 77 localities in Central Europe (Online Resource 1), including Austria (11), Czech Republic (29), Germany (3), Hungary (5), Poland (6), Romania (3), Slovakia (16) and Slovenia (4). In total, 738 individuals were investigated for their DNA-ploidy levels (Suda et al. 2006). Morphological data were collected for 531 individuals from 27 populations (Online Resource 2). Preferably, individuals with estimated DNA-ploidy level were used for morphometric analysis. In some cases also other plants were used, but DNA-ploidy level was estimated from a representative number of other plants in the same population, and the population must have shown to be pure-cytotype. Because of the conservation status of the studied taxa, herbarium vouchers were usually not collected; instead, a series of photographs was taken for most of the individuals used in the morphometric analysis and stored in archive of the first author.

Analyses of chromosome numbers, DNA-ploidy levels and genome sizes

Number of chromosomes was established from chromosomal spreads prepared from microspores (haplophasic chromosome number, *n*). Flower buds were collected in the field ca 10 days before blossoming, fixed in acetic acid: ethanol (1: 3) and stored at –20 °C until processed. Standard protocol of Feulgen staining was used to stain the tissue (Weiss et al. 2003). Briefly, flower buds were hydrolyzed in 5 N HCl for 30 min in 20 °C, washed with water and stained with Schiff's reagent (Sigma, Vienna, Austria) for 1–2 h in darkness. The anthers were dissected and squashed in a drop of 60% acetic acid. Chromosome spreads were analysed under 1000 × magnification using Axioplan light microscope (Carl Zeiss, Vienna, Austria).

DNA-ploidy level was estimated by flow cytometry (FCM) following a standard protocol with internal standards (Doležel et al. 2007) and ploidy level was assessed based on calibration with plants for which chromosome numbers were counted. *Pisum sativum* cv. 'Ctirad' (2C = 9.09 pg; Doležel et al. 1998) was used as the internal standard for diploids and tetraploids, and *Zea mays* cv. 'CE-777' (2C = 5.43 pg; Lysák and Doležel 1998) for DNA-triploids. Fresh ovaries of *Dactylorhiza* were used for the analysis because the vegetative plant tissues (typically leaves) may provide erroneous results due to more prominent occurrence of progressively partial endoreplication (PPE; Trávníček et al. 2015), alternatively mentioned as strict partial endoreplication (Brown et al. 2017). This is a specific process of DNA endopolyploidization characteristic for the orchid family, leading to a disproportional increase in nuclear DNA content of somatic cells, including those of ovaries, which however contain sufficiency of non-replicated nuclei, yielding to 2C peaks in FCM analysis (Trávníček et al. 2015; Hřibová et al. 2016). Ovaries were collected in the field and stored in wet paper tissue in 4 °C until processed, typically up to 5 days, but no more than 10 days. In the laboratory, one or two ovaries and 0.5 × 0.5 cm of internal standard tissue were co-chopped using a razor blade (Galbraith et al. 1983) in

a Petri dish in LB01 buffer with PVP (Doležel et al. 2007). The nuclei solution was filtered through the 40 µm nylon mesh and stained with 30 µl of either 4,6-diamidino-2-phenylindole (DAPI, 4 µg/ml) or propidium iodide (PI, 50 µg/ml). In the analysis with PI, 30 µl of RNase was added to the sample to digest the RNAs.

The analysis was conducted with the following instruments: BD Accuri C6 (BD Biosciences, San Jose, CA, USA); Partec Cy Flow ML (Partec GmbH, Münster, Germany), both at the Department of Botany, Palacký University Olomouc; Partec Cy Flow ML at the Department of Botany and Biodiversity Research, University of Vienna; and Partec Cy Flow ML at the Institute of Experimental Botany, Olomouc. Each individual was analysed separately and the fluorescence of at least 3,000 particles was recorded. Only results with peak CV $\leq$ 5.0 were accepted. Several diploid and tetraploid individuals were analysed with both PI and DAPI to calibrate the position of the peaks for the different dyes used.

BD Accuri software and Partec FloMax software were used to evaluate the histograms with two or more (because of frequent endoreplication) peaks. The G_0/G_1 peak of the standard and G_0/G_1 peak (2C-peak after Trávníček et al. 2015) of the analysed plant were identified. For every plant, an index (relative genome size) was calculated as the ratio of the mean G_0/G_1 peak of the *Dactylorhiza* / mean G_0/G_1 peak of the internal standard. The ratios obtained from the analysis using *Z. mays* as the standard were recalculated to the values expected from the measurement with *P. sativum*.

Absolute genome size was measured for several plants, using a similar protocol as for DNA-ploidy level estimation with the following settings: suspension was stained with PI, each plant was measured three times, and at least 3,333 nuclei were analysed in each measurement with a maximum peak CV = 3.5%. The peak ratios obtained for each plant were averaged and the genome size was calculated as the average peak ratio multiplied with the genome size of the internal standard.

Morphological data recording and analyses

Twenty-four morphological characters were measured (16 characters), numbered (four characters) or scored (three binary characters and one multistate character) (Tables 1 and 2; Online Resource 3). Characters studied included morphological characters traditionally used in the determination keys and special taxonomic literature for delimitation of various *Dactylorhiza* taxa as well as characters found useful in our preliminary screening of Central European populations of *D. maculata* group. Vegetative traits were measured with an adjusted ruler on living plants directly in the field, to minimize the damage of the individuals. Floral traits were measured from a digital picture. For each individual, one flower from the middle-low part of the inflorescence (typically the 4th flower from the bottom) was removed. The lip was separated, put on the scanner glass, and weighted down with a microscope slide; this led to flattening of the lip, which was subsequently digitized by a scanner with high resolution (1200 dpi). ImageJ software (Schneider et al. 2012) was used for the size measurement of the traits. Besides the primary traits, 15 additional ratios and indices were derived from primary traits for further analyses.

Table 1. Descriptive statistics of all quantitative primary characters studied and their ratios (mean, SD = standard deviation, minimum, 10% & 90% quantile, and maximum) for the groups of *Dactylorhiza *fuchsii* (fuchsii-2x, N = 111; fuchsii-4x, N = 284; sooana, N = 136) in Central Europe. Nested ANOVA with populations nested within groups was used for comparison of means among groups. Before statistical tests, some quantitative characters were log-transformed to improve their normality. Descriptive statistics based on the original (untransformed) values are presented in table. Characters with significant ANOVAs ($P \leq 0.05$) are indicated by boldface. Tukey multiple comparison test was used after a significant result of ANOVA; different letters rowwise indicate significant differences between groups at $P \leq 0.05$. Abbreviations of each character are added before the name of the respective character (first column).

Character	**Group**			**F**	**P**
	fuchsii-2x	**fuchsii-4x**	**sooana**		
plH: plant height (cm)	45.2 ± 12.9	42.4 ± 11.7	48.6 ± 8.7	1.50	0.243
	(23.0-) 30.2–64.0 (-82.0)	(17.0-) 27.0–58.8 (-77.0)	(26.0-) 37.0–61.3 (-67.0)		
in1: length of the 1st internode (mm)	36.88 ± 15.07[ab]	27.94 ± 15.35[a]	39.25 ± 16.67[b]	5.90	**0.009**
	(12.0-) 19.0–59.0 (-81.0)	(3.0-) 12.0–47.7 (-121.0)	(9.0-) 21.0–62.0 (-92.0)		
in2: length of the 2nd internode (mm)	61.50 ± 19.24[a]	48.90 ± 17.60[b]	65.10 ± 16.85[a]	9.38	**0.001**
	(9.0-) 38.4–87.8 (-133.0)	(6.0-) 28.3–68.7 (-158.0)	(23.0-) 43.0–88.0 (-112.0)		
nrL: number of leaves	6.21 ± 1.24[a]	6.55 ± 1.54[ab]	7.15 ± 1.54[b]	3.49	**0.047**
	(4.0-) 5.0–7.8 (-11.0)	(4.0-) 5.0–9.0 (-13.0)	(4.0-) 5.0–9.0 (-13.0)		
IL1: length of the 1st leaf (mm)	100.13 ± 25.86	92.21 ± 25.80	106.33 ± 25.81	2.69	0.090
	(34.0-) 68.4–134.0 (-173.0)	(28.0-) 60.5–130.5 (-165.0)	(46.0-) 73.7–141.3 (-180.0)		
wL1: width of the 1st leaf (mm)	27.99 ± 7.75	24.92 ± 7.81	26.30 ± 6.64	1.01	0.380
	(15.0-) 19.0–40.0 (-53.0)	(10.0-) 15.0–35.0 (-51.0)	(14.0-) 18.0–35.3 (-52.0)		
aL1: angle between the stem and the 1st leaf (degrees)	46.4 ± 13.28	53.42 ± 18.38	51.58 ± 15.64	1.27	0.298
	(15.0-) 30.0–70.0 (-80.0)	(10.0-) 30.0–80.0 (-90.0)	(10.0-) 30.0–70.0 (-90.0)		
IL2: length of the 2nd leaf (mm)	130.24 ± 29.61	121.56 ± 31.60	132.56 ± 23.71	1.00	0.381
	(70.0-) 92.0–170.8 (-203.0)	(46.0-) 82.0–164.5 (-214.0)	(82.0-) 102.7–164.5 (-200.0)		
wL2: width of the 2nd leaf (mm)	26.27 ± 8.64	23.66 ± 8.37	25.00 ± 6.89	0.62	0.544
	(9.0-) 17.0–37.8 (-51.0)	(6.0-) 14.0–35.0 (-50.0)	(11.0-) 17.0–34.0 (-52.0)		
dBW: distance between the base of the 2nd leaf and its widest part (mm)	84.28 ± 22.84	76.27 ± 25.31	89.40 ± 18.72	2.28	0.124
	(20.0-) 58.4–117.8 (-149.0)	(16.0-) 44.5–110.0 (-143.0)	(37.0-) 68.4–115.0 (-138.0)		
aL2: angle between the stem and the 2nd leaf (degrees)	35.99 ± 13.45	39.68 ± 16.82	44.93 ± 14.47	1.83	0.281
	(10.0-) 20.0–50.0 (-90.0)	(5.0-) 20.0–60.0 (-90.0)	(20.0-) 30.0–65.0 (-80.0)		

Character	Group			F	P
	fuchsii-2x	**fuchsii-4x**	**sooana**		
A: length of the labellum from its base to the tip of the middle lobe (mm)	7.84 ± 1.12[ab]	8.26 ± 1.03[b]	7.65 ± 0.87[a]	3.52	**0.045**
	(5.34-) 6.53–9.41 (-10.95)	(5.47-) 6.99–9.66 (-11.25)	(5.88-) 6.46–8.92 (-10.33)		
B: length of the labellum from its base to the tip of the lateral lobe (mm)	6.40 ± 1.03[a]	6.98 ± 1.00[b]	6.76 ± 0.90[ab]	3.90	**0.034**
	(3.75-) 5.11–7.88 (-9.27)	(4.77-) 5.84–8.28 (-10.72)	(5.04-) 5.57–7.99 (-9.50)		
C: length of the labellum from its base to the base of the incision (mm)	4.28 ± 0.75[a]	4.83 ± 0.81[b]	4.73 ± 0.68[b]	7.14	**0.004**
	(2.46-) 3.35–5.27 (-6.42)	(2.55-) 3.95–5.94 (-7.79)	(3.50-) 3.88–5.57 (-7.43)		
E: width of the middle lobe (mm)	3.09 ± 0.53[a]	3.42 ± 0.56[b]	3.28 ± 0.46[b]	3.51	**0.004**
	(2.09-) 2.52–3.70 (-4.62)	(1.94-) 2.72–4.17 (5.34)	(2.20-) 2.77–3.92 (-4.41)		
F: width of the labellum (mm)	10,8 ± 1.57	11.51 ± 1.67	11.41 ± 1.32	1.37	0.272
	(7.37-) 8.93–12.56 (-16.15)	(7.40-) 9.30–13.47 (-16.85)	(8.53-) 9.60–13.10 (-15.31)		
HH: Heslop-Harrison index [= (2A)/(B + C)]	1.48 ± 0.16[a]	1.41 ± 0.16[ab]	1.34 ± 0.12b	8.12	**0.002**
	(1.17-) 1.26–1.70 (-1.94)	(1.07-) 1.24–1.61 (-2.18)	(1.09-) 1.20–1.51 (-1.78)		
A/D [= A/(A-C)]	2.27 ± 0.40[a]	2.52 ± 0.58[ab]	2.71 ± 0.55[b]	5.92	**0.008**
	(1.60-) 1.84–2.87 (-3.66)	(1.54-) 1.92–3.29 (-6.74)	(1.75-) 2.16–3.26 (-5.18)		
F/E	3.55 ± 0.51	3.40 ± 0.42	3.51 ± 0.43	1.97	0.161
	(2.46-) 3.00–4.21 (-5.27)	(2.26-) 2.83–3.94 (4.46)	(2.61-) 2.96–4.06 (-4.74)		
BBC [= B/(B-C)]	3.17 ± 0.70	3.44 ± 0.93	3.49 ± 0.79	1.55	0.231
	(2.02-) 2.37–4.04 (-5.98)	(2.02-) 2.52–4.76 (-9.02)	(2.20-) 2.66–4.57 (-6.71)		
plH/lL1	4.68 ± 1.38	4.81 ± 1.45	4.81 ± 1.42	0.66	0.940
	(2.21-) 2.98–6.73 (-9.44)	(2.10-) 3.11–6.82 (-9.66)	(2.65-) 3.30–6.71 (-10.0)		
plH/lL2	3.50 ± 0.74	3.55 ± 0.76	3.73 ± 0.73	0.53	0.594
	(1.90-) 2.51–4.50 (-5.35)	(1.59-) 2.61–4.52 (-6.25)	(2.42-) 2.85–4.69 (-6.50)		
plH/nrL	7.35 ± 1.83	6.64 ± 1.89	7.01 ± 1.62	1.61	0.379
	(3.83-) 5.00–9.74 (-13.6)	(2.83-) 4.40–9.29 (-16.13)	(3.82-) 5.18–9.03 (-13.50)		
lL1/wL1	3.71 ± 1.02	3.90 ± 1.17	4.16 ± 1.00	0.91	0.417
	(1.50-) 2.55–5.08 (-6.67)	(1.91-) 2.47–5.53 (-8.18)	(2.30-) 3.12–5.56 (-7.47)		
lL2/wL2	5.30 ± 1.48	5.52 ± 1.65	5.60 ± 1.46	0.21	0.812
	(2.84-) 3.49–7.69 (-9.67)	(2.41-) 3.66–7.50 (-11.75)	(2.40-) 4.03–7.33 (-11.55)		

Character	Group			F	P
	fuchsii-2x	**fuchsii-4x**	**sooana**		
lL2/dBW	1.58 ± 0.24[ab]	1.66 ± 0.37[a]	1.50 ± 0.16[b]	8.82	**0.001**
	(1.28-) 1.39–1.80 (-3.50)	(1.10-) 1.38–1.98 (-4.13)	(0.93-) 1.35–1.66 (-2.49)		
plH/in1	14.42 ± 6.26[a]	19.10 ± 11.95[b]	14.73 ± 7.34[a]	5.51	**0.012**
	(5.24-) 8.54–21.51 (-36.67)	(5.33-) 9.34–32.47 (-97.50)	(5.29-) 8.05–21.67 (-49.09)		
plH/in2	8.31 ± 4.13[ab]	9.70 ± 5.21[a]	7.99 ± 2.76[b]	3.85	**0.038**
	(4.14-) 4.92–12.02 (-40.00)	(3.51-) 5.52–14.77 (-56.67)	(4.48-) 5.41–11.57 (-22.17)		
in2/in1	1.83 ± 0.66	2.10 ± 1.17	1.86 ± 0.69	1.62	0.222
	(0.33-) 1.18–2.65 (-4.75)	(0.26-) 1.18–3.22 (-12.00)	(0.80-) 1.20–2.59 (-5.36)		
lL1/in1	3.27 ± 1.50[a]	4.20 ± 2.48[b]	3.24 ± 1.66[a]	3.53	**0.048**
	(1.31-) 1.72–5.16 (-8.83)	(0.98-) 1.88–7.41 (-18.00)	(1.10-) 1.61–5.14 (-10.36)		
lL2/in2	2.47 ± 1.44	2.87 ± 1.70	2.21 ± 0.88	3.18	0.622
	(1.00-) 1.57–3.64 (-14.11)	(0.73-) 1.47–4.39 (-17.33)	(0.92-) 1.36–3.05 (-7.74)		
LAS1: leaf apex shape index of the 1st leaf [(nr. of plants with acute apex—nr. of plants with obtuse apex)/total nr. of plants]	evaluated solely at the population level; in analysis based on individuals, this trait was substituted by sL1A, sL1S, sL1O (see Table 2)				
LAS2: leaf apex shape index of the 2nd leaf; [(nr. of plants with acute apex—nr. of plants with obtuse apex)/total nr. of plants]	evaluated solely at the population level; in analysis based on individuals, this trait was substituted by sL2A, sL2S, sL2O (see Table 2)				

Table 2 (on the next page). Descriptive statistics of all qualitative characters studied (percentage of each category for each studied categorical variable within each group) for the groups of *Dactylorhiza *fuchsii* (fuchsii-2x, N = 111; fuchsii-4x, N = 284; sooana, N = 136) in Central Europe. GLMM with the logit link function and binomial distribution was used for the analysis of binary characters. LRT test was used for the estimation of significance level. Multiple comparisons between groups were analysed using Tukey method with p value adjustment. Multistate categorical characters were analysed by log-linear models. Different letters rowwise indicate significant differences between groups at $P \leq 0.05$. Characters with significant differences among groups ($P \leq 0.05$) are indicated by boldface. Abbreviations of each character/category are added before the name of the respective character/category (first column).

Character	Group			$\chi 2$	P
	fuchsii-2x	**fuchsii-4x**	**sooana**		
pAx: presence of dark anthocyanin pigmentation on the inflorescence axis	ab	a	b	8.5	**0.010**
	2.0	14.0	1.0		
pPe: presence of anthocyanin pigmentation on the perianth, excluding labellum	a	a	b	31.0	**< < 0.001**
	78.0	94.0	6.0		
pAc: presence of anthocyanin pigmentation on the anther cap	a	a	b	39.1	**< < 0.001**
	87.0	98.0	5.0		
pLe: spots on leaves	a	b	c	166.8	**< < 0.001**
pLeA: absent	46.9	8.5	0.0		
pLeP: pale	33.3	60.5	29.4		
pLeB: bold	19.8	31.0	70.6		
pLa: labellum markings	a	b	c	96.8	**< < 0.001**
pLaA: absent	12.6	3.9	9.6		
pLaP: pale	8.1	5.6	40.4		
pLaB: bold	79.3	90.5	50.0		
cLa: labellum colour	a	b	c	344.8	**< < 0.001**
cLaW: white	29.7	10.6	97.1		
cLaB: bicolour, white-purple	23.4	27.1	2.9		
cLaP: purple	46.9	62.3	0.0		
sL1: shape of the first leaf apex	a	b	c	29.7	**< < 0.001**
sL1A: acute	6.3	9.9	1.5		
sL1S: subacute	17.1	22.5	8.1		
sL1O: obtuse	76.6	67.6	90.4		
sL2: shape of the second leaf apex	a	b	c	33.2	**< < 0.001**
sL2A: acute	34.2	47.2	21.3		
sL2S: subacute	37.9	27.5	32.4		
sL2O: obtuse	27.9	25.3	46.3		

In total, 531 individuals from 27 populations of *D. *fuchsii* were included in the morphometric analyses. Several datasets were used: (1) matrix 1 – complete dataset including all 531 individuals as OTU and all primary and derived characters; (2) matrix 2 – complete dataset including all 531 individuals as OTU and reduced set of characters. Specifically, two primary characters (in1, in2) and 5 ratios derived from these characters (plH_in1, plH_in2, in2_in1, lL1_in1, lL2_in2) were excluded from the dataset due to the absence of their records for some populations. Problem of multicollinearity was assessed by variance inflation factor (VIF) for quantitative traits using the library usdm (Naimi 2017) in R (R Foundation for Statistical Computing, Vienna, Austria). Only those variables were retained in the analyses whose VIF was lower than 15, which is slightly higher than the recommended $VIF \leq 10$ (O'Brien 2007). Consequently, six primary quantitative characters were excluded (plH, wL1, lL2, A, C, E). The potential problem of multicollinearity in categorical characters was accessed by Cramer's V (Legendre and Legendre 2012). Only one variable (pPe) had Cramer's V higher than 0.9 in two paired analyses (with pAc and cLaW) and therefore it was excluded from the dataset; (3) matrix 3 – complete dataset including all 531 individuals as OTU and reduced set of characters. Only quantitative characters and their ratios identical to those in matrix 2 were considered. All nominal variables, including those considered as diagnostic for the sooana group, were excluded from the matrix; (4) matrix 4 – a dataset with 27 population samples as OTU characterized by the population's median values of quantitative characters and their ratios and proportional representation of each category for each studied categorical variable per each population. After excluding the collinear variables with $VIF \geq 15$, just 13 variables remained as follows: plH/in1, in2/in1, lL1/in1, lL2/in2, pLeP, pLeB, pLaA, pLaP, pAx, cLaB, cLaP, LAS1, LAS2.

To compare groups (as defined above), the matrix 1 was firstly analysed using univariate statistics. Nested ANOVA with populations nested within groups and Tukey multiple comparison test were used for quantitative characters and their ratios using NCSS 9 (NCSS, LLC., Kaysville, Utah, USA, ncss.com/software/ncss). Bonferroni correction of P-values of ANOVAs was applied additionally. Before statistical tests, some quantitative characters were log-transformed to improve their normality. Descriptive statistics based on the original (untransformed) values are presented in tables and visualized in plots.

Generalized linear mixed model (GLMM) with the logit link function and binomial distribution was used for the analyses of binary characters. In the GLMM, a group was considered a fixed factor and a population a random factor nested within groups. For GLMM, the lme4 library (Bates et al. 2019) and afex library (Singmann et al. 2016) in R were used. LRT test was used for the estimation of significance level and emmeans library (Lenth et al. 2020) was used for multiple comparisons between groups using Tukey method with P value adjustment. Due to convergence problems when using GLMM with multinomial distribution of multistate categorical characters in Statistica 10 software (StatSoft, Inc., Tulsa, USA) using population as a nested random factor, log-linear models were calculated instead, using likelihood ratio χ^2 test on pooled data (i.e. ignoring population identity within each group) in NCSS 9. After significant overall χ^2 test, separate χ^2 tests were done for each pair of groups and P-values were adjusted using Bonferroni correction. Small value (0.2; i.e. delta value) was added to each cell count when 0's were present in the table.

Principal coordinate analysis (PCoA), using a Gower's dissimilarity coefficient for mixed data consisting a mixture of quantitative, count and qualitative characters (Legendre and

Legendre 2012), was used to obtain insight into the phenetic relationships among all studied individuals (matrix 2). Principal component analysis (PCA) was done based on the correlation matrix of the quantitative characters (matrix 3) to observe the structuring of individuals in the ordination space based on the quantitative characters. A third analysis (PCA) was performed on matrix 4 containing populations as OTU. Before multivariate analyses, some quantitative characters were log-transformed. PCoA and PCA were run using the software Canoco 5.12 (ter Braak and Šmilauer 2012).

RESULTS

Cytotype diversity and population composition: chromosome numbers and flow cytometry

Chromosome numbers were obtained for six individuals from three populations (Online Resource 1). Three plants were diploids ($n = x = 20$), with one individual representing the fuchsii-2x group (pop. 28, Furth an der Triesting) and two individuals representing the sooana group (pop. 4, Hluboče; Fig. 1a). The other three plants were tetraploids ($n = 2x = 40$), belonging to a single population (27, Alland; Fig. 1b) and assigned to the fuchsii-4x group. Peak ratios for all of these reference individuals are shown in Online Resource 4.

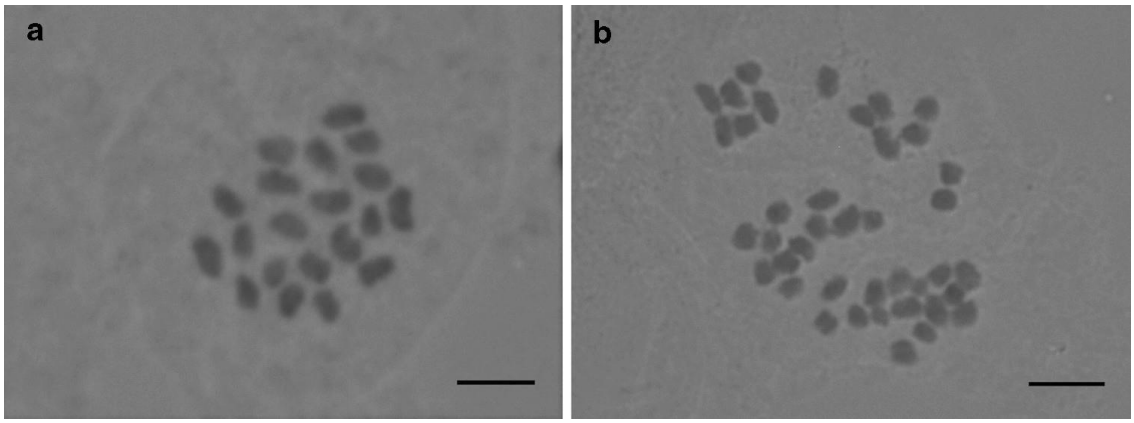

Figure 1. Meiotic metaphase chromosomes of (**a**) *Dactylorhiza maculata* subsp. *sooana* (n = 20; locality 4, Hluboče) and (**b**) *D. maculata* subsp. *fuchsii* (n = 40; locality 27, Alland). Bar = 5 µm.

One to 35 plants per (sub)population (mean ± SD; 8.2 ± 6.8) were analysed by FCM, accounting for a total of 738 plants representing 90 (sub)populations from 77 localities. Three DNA-ploidy levels were identified, corresponding to diploids, tetraploids, and a cytotype with a relative genome size intermediate between that of diploids and tetraploids, referred to as DNA-triploid (Suda et al. 2006). PPE was frequently observed. In leaf tissue, the non-replicated (2C) nuclei of tetraploids were detected, but endoreplicated (2C + P) nuclei prevailed in diploids, for which 2C peaks were not detectable on the FCM histograms. To avoid erroneous results, ovaries were used for all FCM analyses.

Within the analysed 140 individuals of the sooana group, only diploid plants were found. All three cytotypes were found in the fuchsii group. A majority of these plants corresponded to tetraploids (373; 62.4%), followed by diploids (196; 32.8%) and DNA-triploids (29 individuals; 4.8%). Most of the analysed populations of the fuchsii group (85.9%) were uniform in terms of ploidy level: 23 populations were exclusively diploid (37.7%) and 34 populations were tetraploid (55.7%). Only four mixed-ploidy populations were found in which diploids and tetraploids co-occurred with DNA-triploid individuals (30, Nasswald; 37, Weissenbach; 55, Zajačkova lúka; 74, Kramplje). DNA-triploids were also sporadically found as a minority cytotype in four predominantly diploid (8, Ransko; 14, Zakopane; 31, Fronbach; 65, Pârâul Rece) and one tetraploid (36, Postalm) populations. A higher proportion of DNA-triploids (5 out of 8 individuals) was found in only one population (74, Kramplje) comprising all three cytotypes.

Significant differences in relative genome size were found between all pairs of groups (Welch's test of means allowing for unequal variances; DAPI: $F_{3, 47.8} = 8829.0$, $P < 0.001$; PI: $F_{3, 58.0} = 6677.5$, $P < 0.001$), except for the sooana and fuchsii-2x groups with nearly the same genome size (Table 3). The genome size of polyploids was not additive compared to their diploid relatives. The average monoploid relative genome size of tetraploids corresponded to 88% of that of diploids, and that of DNA-triploids was exactly intermediate between the average monoploid relative genome sizes of diploids and tetraploids. PI and DAPI measurements yielded consistent results (Table 3).

Table 3. Relative DNA content (= fluorescence ratio between the positions of the sample and internal reference standard G_0/G_1 peaks) of the recognized groups assessed using flow cytometry; the stain was either DAPI or PI. All values are calculated relative to the *Pisum sativum* cv. 'Ctirad' as an internal reference standard. N = number of samples analysed; 1Cx = average monoploid relative genome size. Bonferroni multiple comparison test was used after a significant result of Welch's Test; different letters columnwise indicate significant differences at $P \leq 0.05$.

		Analysis with DAPI						**Analysis with PI**					
			Ratio to the standard						**Ratio to the standard**				
Group	2*n*	N	Min	Mean	SD	Max	1C*x*	N	Min	Mean	SD	Max	1C*x*
sooana	2*x*	28	0.63	0.68[a]	0.02	0.72	0.34	130	0.72	0.77[a]	0.02	0.84	0.39
fuchsii-2x	2*x*	115	0.64	0.68[a]	0.02	0.74	0.34	69	0.71	0.78[a]	0.04	0.87	0.39
fuchsii-3x	~3*x*	14	0.92	0.98[b]	0.04	1.05	0.33	15	1.03	1.10[b]	0.06	1.20	0.37
fuchsii-4x	4*x*	213	1.14	1.22[c]	0.04	1.38	0.31	167	1.28	1.37[c]	0.05	1.54	0.34

Absolute genome size was measured for five plants from two populations. Two individuals were diploids classified as fuchsii-2x (28, Furth an der Triesting), and three individuals were tetraploids classified as fuchsii-4x (27, Alland). The absolute genome size of diploids was estimated to be 2C = 6.55 and 6.64 pg, while the absolute genome size of tetraploids ranged from 2C = 11.89 to 12.22 pg (Online Resource 5). Chromosome number of $n = x = 20$ was counted for the diploid plant with 2C = 6.55 pg.

Morphological variation of *Dactylorhiza *fuchsii* populations

Only 13 out of 31 quantitative characters (42%) were significantly different at least between some of the groups (Table 1 and Online Resources 6). The majority of characters differing between groups were those recorded on flowers (A, B, C, E) or represented ratios (HH, A/D) derived from floral traits. The second set of characters differing among groups were related to plant habit, i.e. the length of internodes (in1, in2) and their ratios with plant height and length of leaf (e.g. plH/in1, plH/in2). However, just two characters (in2 and IL2/dBW) remained significant after the application of Bonferroni correction (Table 1 and Online Resource 6).

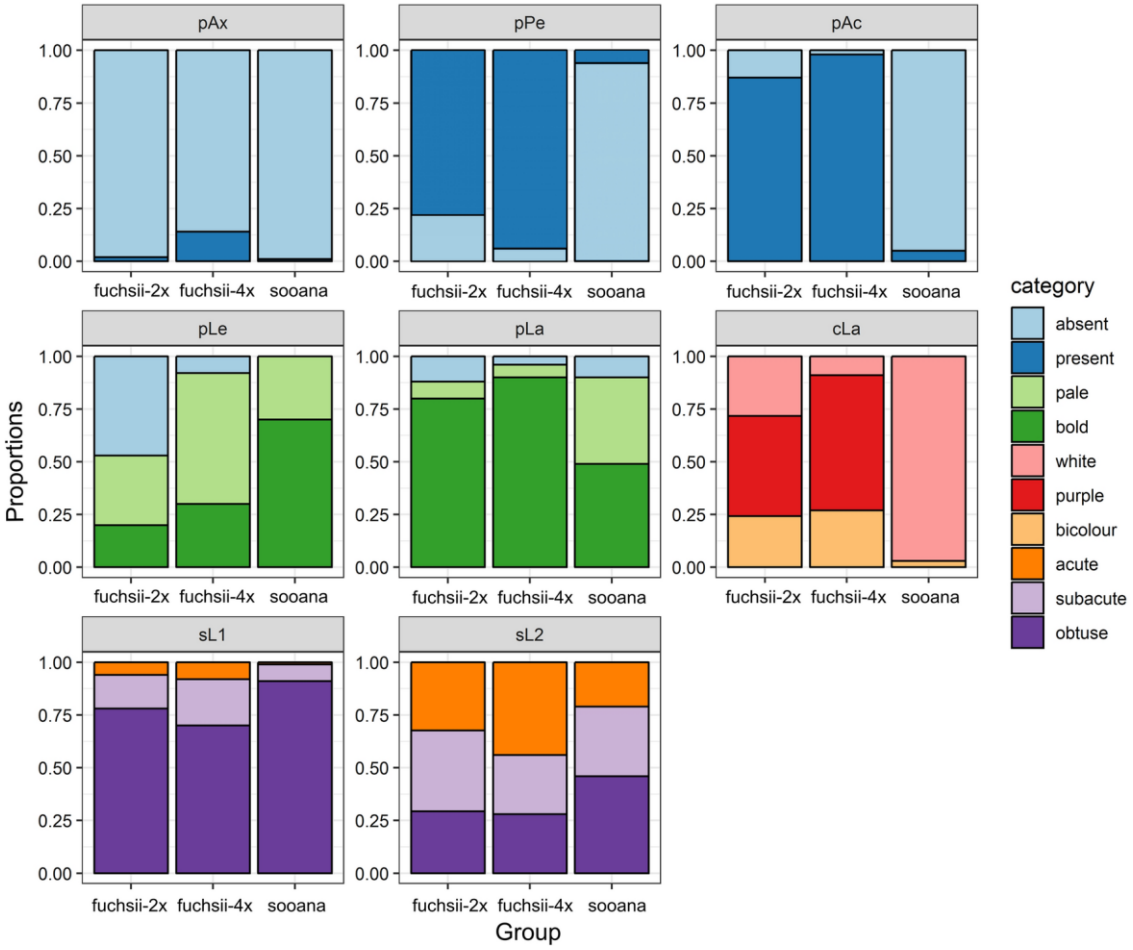

Figure 2. Stacked bar charts of eight qualitative characters in studied groups. Vertical axes represent proportions. The abbreviations of the characters see in Table 2.

Every binary character studied showed significantly different patterns at least between some groups (Fig. 2 and Table 2). Most plants of all three groups were without pigmentation on the inflorescence axis. Only fuchsii-4x plants had more frequently dark anthocyanin pigmentation on the inflorescence axis compared to the sooana group. The majority (94%) of sooana plants did not have pigmentation on the perianth (excluding

labellum), while the majority (90%) of fuchsii-2x and fuchsii-4x individuals had. Similarly, almost all fuchsii-2x and fuchsii-4x plants had anthocyanin pigmentation on the anther cap, while most of the sooana plants had anther caps without pigmentation.

Frequency distributions of the categories of every multistate categorical variable differed significantly among groups (Fig. 2 and Table 2). Intensity of spots on leaves increased in the direction fuchsii-2x → fuchsii-4x → sooana. While approximately half of the plants (47%) of the fuchsii-2x group were without spots on the leaves, 71% of sooana plants had bold spots on leaves. More than 75% of both fuchsii-2x and fuchsii-4x plants had bold labellum markings, while the sooana group had almost equal frequencies of plants with either bold or pale labellum markings. The sooana group also differed from both fuchsii groups in labellum colour, having a white labellum in most plants (97%), while both fuchsii-2x and fuchsii-4x groups had similar proportions of plants of three colour categories, with only predominantly purple labellum plants. All groups also differed in the shape of leaf apexes. Just a minority of plants in all groups possessed an acute leaf apex, with the highest proportion of such plants found in the fuchsii-4x and lowest in the sooana group.

The PCoA based on quantitative and qualitative characters (matrix 2; Fig. 3a, b) revealed a near complete separation of the fuchsii-4x and sooana groups along the first ordination axis, with just some fuchsii-4x individuals situated within the sooana cluster; most of these individuals belonged to one population (32, Giesshübl). On the other hand, the clump of fuchsii-2x individuals overlapped with the fuchsii-4x clump on the left part of the ordination diagram. Some fuchsii-2x individuals from two populations (1, Smutné udolí; 14, Zakopane) occurred in the right part of the ordination diagram where they overlapped with the sooana group (Fig. 3a). The observed pattern in the distribution of the groups along the first axis was almost completely caused by several qualitative characters related to labellum and anther cap colour and labellum marking. All these characters are tightly correlated with the first axis (Fig. 3b): cLaW (point biserial correlation coefficient; r = 0.67***), pLaP (0.39***), pAc (-0.75***), pLaB (-0.47***), and cLaP (-0.56***). It follows that the resemblance of some individuals of the fuchsii-2x, fuchsii-4x and sooana groups was due to sharing some of the diagnostic traits of the sooana group, particularly white flowers. Other characters, including all quantitative ones, did not significantly correlate with the first ordination axis; only some characters were related to the second ordination axis, suggesting phenotypic variation in size regardless of group identity (Fig. 3b).

After the removal of qualitative characters, incl. diagnostic traits of the sooana group, from the dataset (matrix 3), the PCA based on 22 quantitative characters (incl. their ratios) revealed no morphological differentiation among groups (Fig. 3c). Main gradient along the first axis was correlated with the size dimensions of the labellum and leaf width, irrespective of group identity (Fig. 3d).

The PCA based on a reduced set of 13 characters representing populations as OTUs (matrix 4) revealed a pattern of group distribution in the ordination space (Fig. 3e, f) similar to that in the PCoA analysis of matrix 2. The sooana group was nearly completely separated from the remaining groups; only two populations of fuchsii-2x (1, Smutné údolí; 14, Zakopane) were situated in an intermediate position between the sooana clump and fuchsii-2x clump. Both the fuchsii-4x and fuchsii-2x groups partly overlapped in the centre of the ordination diagram, but fuchsii-4x group also showed considerably higher

variability in the multivariate space than the fuchsii-2x group. Scores of populations along the first axis were significantly correlated with the following variables: pLaP (Pearson r = 0.73***), pLeB (0.58***), cLaP (-0.57**), plH/in1 (-0.78***), lL1/in1 (-0.71***) (Fig. 3f). Population 32, Giesshübl together with population 35, Sittersdorf were situated in the upper left part of the ordination diagram, in rather isolated positions from all remaining populations (Fig. 3e).

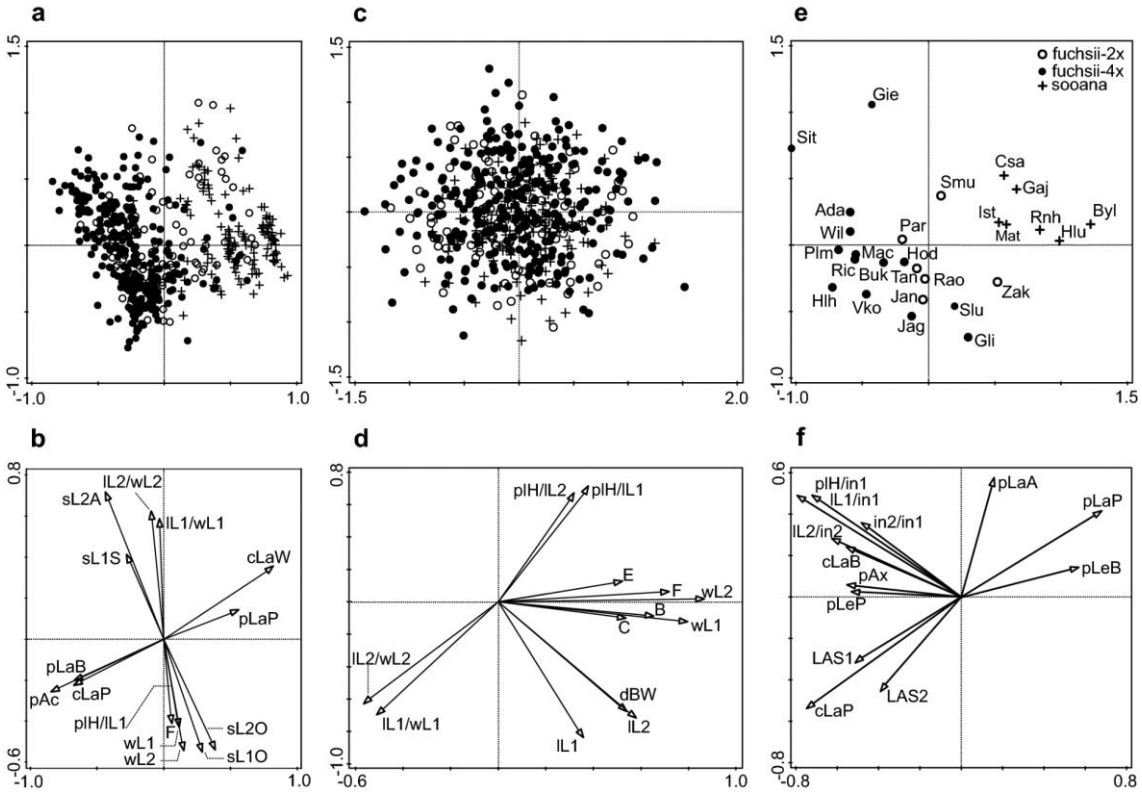

Figure 3. Results of multivariate analyses of morphological characters of *Dactylorhiza *fuchsii* plants. (**a**), (**b**) Principal coordinate analysis based on 32 quantitative and qualitative characters (matrix 2) with individual plants as OTUs. The first and second ordination axes explained 17.3% and 11.3% of the total variation, respectively. Characters, of which the larger absolute value of the two correlations with the ordination axes exceed 0.3, were shown in the diagram. (**c**), (**d**) Principal component analysis based on 22 quantitative characters (matrix 3) with individual plants as OTUs. The first and second ordination axes explained 23.4% and 17.2% of the total variation, respectively. Characters, whose individual fit on both displayed axes exceed 10%, were shown in the diagram. (**e**), (**f**) Principal component analysis based on 15 characters with populations as OTUs (matrix 4). The first and second ordination axes explained 30.4% and 14.0% of the total variation, respectively. Characters, whose individual fit on both displayed axes exceed 10%, were shown in the diagram. Symbols: fuchsii-2x – empty circle, fuchsii-4x – black circle, sooana – cross. The abbreviations of the characters see in Table 1 and Online Resource 3, the codes of populations see in Online Resource 1.

Distribution and cytogeography of the groups

Populations of the fuchsii group exhibited a clear geographical pattern in the distribution of their cytotypes throughout Central Europe (Fig. 4). Tetraploid populations (fuchsii-4x) prevailed in the Bohemian Massif, with only a single diploid population (58, Ranský brook) and one mixed-ploidy population with diploids and DNA-triploids (8, Ransko) found in this region (the Žďárské vrchy Mts). Solely three purely diploid populations (15, Tanew; 64, Cisnădioara; 66, Cheia) and one mixed-ploidy population of diploids with a single DNA-triploid plant (65, Pârâul Rece) were found in the Carpathians and peri-Carpathian region east and southeast of the Tatra Mts in Slovakia. The western half of the Western Carpathians, the Eastern Alps and Dinarides proved to be a transitional zone, where pure diploid, pure tetraploid, and mixed-ploidy populations containing all three cytotypes (30, Nasswald; 37, Weissenbach; 55, Zajačkova lúka; 74. Kramplje) were found. Several uniformly diploid populations (4, 5, 33, 47, 48, 49, 50, 51, 52, 53, 54, 59, 60; see Online Resource 2) corresponding to the sooana group were found in the Carpathian area of Northern Hungary, Southern Slovakia and Southeastern Czechia.

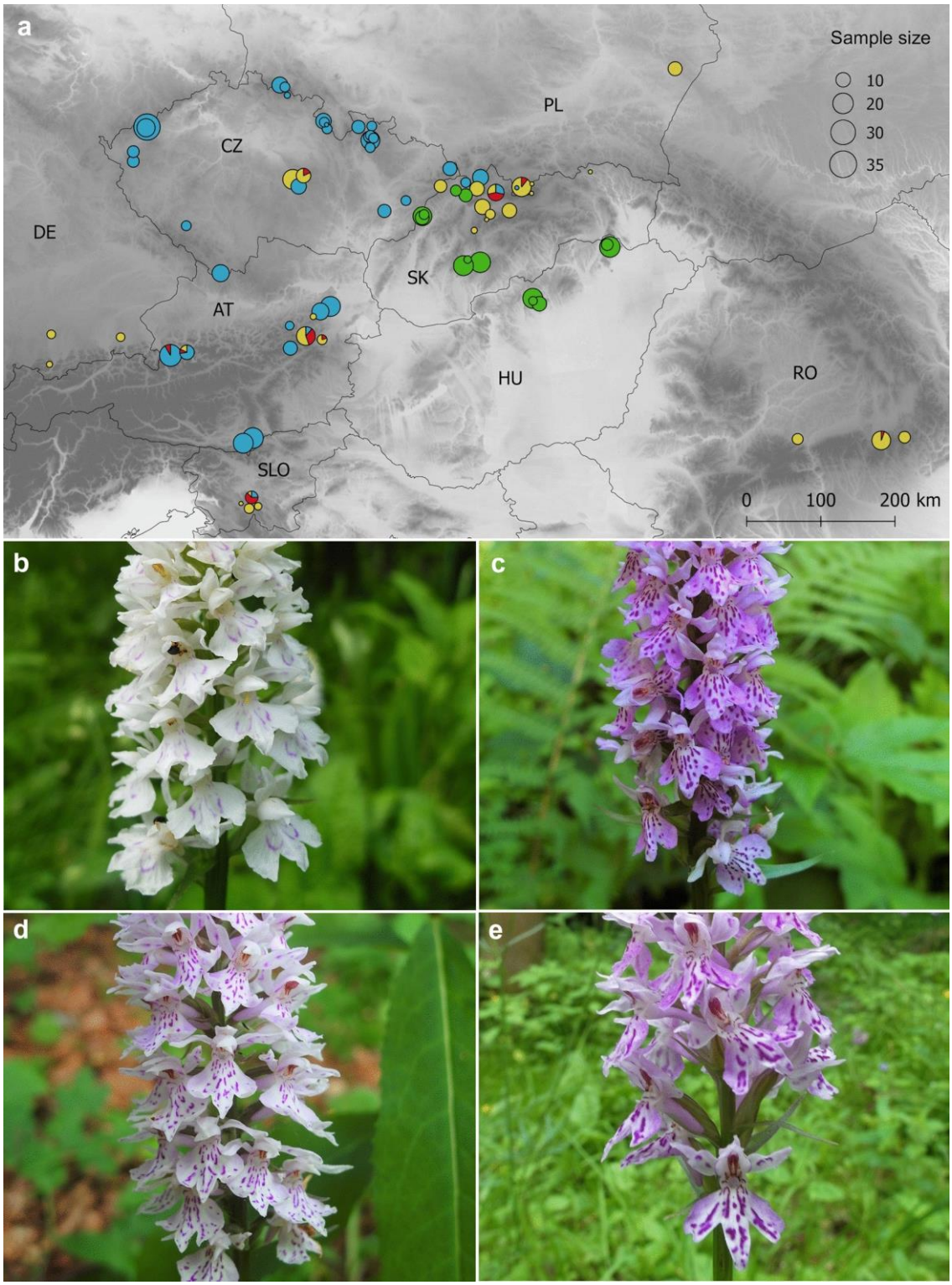

Figure 4. Groups and cytotypes of *Dactylorhiza *fuchsii* as recognized in this study. (**a**) Map of populations analysed by flow cytometry and proportions of diverse groups occurring at common localities: green = sooana, yellow = fuchsii-2x, red = fuchsii-3x, blue = fuchsii-4x. Symbol size is proportional to the sample size. Examples of plants belonging to different groups: (**b**) sooana (49, Mátraszentimre); (**c**) fuchsii-2x (58, Ranský brook); (**d**) fuchsii-3x (55, Zajačkova lúka); (**e**) fuchsii-4x (7, Adamova rokle). Photographs: V. Taraška.

DISCUSSION

A considerable cytotype diversity and morphological variation were found among populations of *D. *fuchsii* in Central Europe. Three cytotypes were identified, diploids, DNA-triploids and tetraploids. The distribution of these cytotypes was not even throughout Central Europe and regional cytotype diversity differed. The most important variation of phenotype concerned flower colouration and leaf spotting. Combination of morphological and karyological data allowed reliable delimitation of the fuchsii and sooana groups as two well-defined taxa.

Chromosome numbers and genome size

Three cytotypes were detected among populations of *D. *fuchsii* in Central Europe, diploid, DNA-triploid, and tetraploid. Chromosomal spreads confirmed the previously reported chromosome numbers, i.e. $2n = 2x = 40$ for diploids and $2n = 4x = 80$ for tetraploids (Heslop-Harrison 1951; Vöth and Greilhuber 1980; Jagiełło and Lankosz-Mróz 1988; Averyanov 1990; Amich et al. 2007). Progressively partial endoreplication (Bory et al. 2008; Trávníček et al. 2015) as well as genome downsizing in polyploids (Leitch and Bennett 2004; Parisod et al. 2010) occurred frequently. Relative genome size of some DNA-triploids was quite similar to the lowest values measured for plants considered to be tetraploids. However, DNA-triploids always co-occurred with plants of other cytotypes and their average relative genome size corresponded to the presumptive triploid genome size from the respective locality (with both PI and DAPI). Therefore, the three cytotypes were clearly distinguishable even despite some intracytotype variation of relative genome sizes.

This paper presents the first extensive ploidy level screening using FCM in the *D. maculata* s.l. taking into consideration the methodological task of PPE. Genome size, either absolute or relative, of the *D. maculata* group in Northern Europe has previously been investigated by using Feulgen-densitometry (Aagaard et al. 2005) and FCM (Ståhlberg and Hedrén 2008). However, FCM analyses relied on leaf tissue which could potentially obscure the results due to the incidence of PPE and should be regarded with caution (cf. Trávníček et al. 2015). The FCM estimate of the genome size of the *D. maculata* s.l. using ovaries was first presented by Šmarda et al. (2019), who analysed a single plant designated as *D. fuchsii* from the Hrubý Jeseník Mts (Bohemian Massif, Czech Republic), which was considered diploid, although its chromosomes were not counted. The genome size of this plant was estimated to be 2C = 10.83 pg, which is just a slightly lower value than the lowest estimates for tetraploids in the present study (2C = 11.89 pg), as well as the genome size of the tetraploid *D. maculata* investigated by Aagaard et al. (2005; 2C = 11.32 pg). Considering the lower estimates for internal standards by Šmarda et al. (2019), compared to Doležel et al. (1998) followed in this study, it may be suggested that the plant used in their analyses was rather tetraploid.

Morphological variability

Morphology may be strongly influenced by environmental factors, ontogenetic developmental stages, or interspecific interactions in orchids (Bateman and Denholm

1988, 1989). Similarly, the major part of the morphological variation among analysed groups recognized within *D. *fuchsii* is likely to be connected to environmental factors and the impact of local selection pressures, particularly concerning several quantitative traits of the flowers. High variation in floral traits in many orchid species is a consequence of a deceptive pollination system (Ackerman et al. 2011), where spatially and temporally variable selection pressures related to different pollinators or negative frequency-dependent selection (Gigord et al. 2001) or even non-adaptive processes (Vereecken and Schiestl 2009) might promote the persistence of phenotypic variance in floral traits (Ackerman et al. 2011). Flower characters were hypothesized not to be correlated with the phylogeny of the genus *Orchis* s.l. (Aceto et al. 1999), and these traits alone are probably unsuitable for taxonomic conclusions even in the genus *Dactylorhiza*. They may, however, be considered if they are correlated with other characters, ecological preferences, and/or patterns of geographical distribution (Pedersen 2009).

The most striking morphological differences were found between the sooana and fuchsii groups, the latter comprising both fuchsii-2x and fuchsii-4x individuals. These differences were connected to several qualitative traits related to leaf spotting and flower colouration, characters that were used for the classification of groups in this study. Importantly, these characters were also drivers of the main gradient of morphological variability among the analysed individuals as well as populations. There was just a slight overlap between the sooana group and the cluster formed by fuchsii-2x and fuchsii-4x plants in the PCA diagram based on individuals. This was caused by the presence of several albinotic individuals within both the fuchsii-2x and fuchsii-4x groups, which were similar to the sooana group in the flower colouration. Unlike sooana, such albinotic plants, however, lacked bold spots on their leaves. Furthermore, the sooana group was well-separated from the other groups in the PCA diagram based on populations. Therefore, the sooana group represented the most distinct, morphologically well-defined group within *D. *fuchsii* and it showed considerable dissimilarity from diploid as well as tetraploid fuchsii groups.

The fuchsii-2x and fuchsii-4x groups were similar to each other in their morphology. It was shown that autopolyploids in general may differ from their diploid progenitors in quantitative morphological traits; being more robust, possessing larger flowers, leaves, and stems (Parisod et al. 2010; Spoelhof et al. 2017). Only a few quantitative differences were detected between the diploid and tetraploid fuchsii groups, and significance was proved for just a single quantitative trait (length of the 2nd internode) after application of Bonferroni correction. Instead, the most apparent differences between these two groups were found in qualitative traits, i.e. leaf and labellum pigmentations. Diploid plants often lack spots on the leaves and their flowers are pale, with less conspicuous or even absent markings. Individuals with bold leaf spots and striking anthocyanin pigmentation of flowers are much more frequent among tetraploids. Notably, the intensity of leaf spotting is clearly correlated with the intensity of flower pigmentations in individuals of both fuchsii-2x and fuchsii-4x groups.

Populations of the fuchsii-4x group comprise larger morphological variability than those of the fuchsii-2x group. Some of the morphological differences observed between diploids and tetraploids may be also caused by putative gene introgression among tetraploid *D. *fuchsii* and other tetraploid taxa of the *D. maculata* group, as it was suggested by Jagiełło (1988) and later indicated by molecular markers (Ståhlberg and Hedrén 2010; Brandrud et al. 2020). Gene admixture could occur to various extents in

tetraploid populations of *D. *fuchsii*, which may verge to *D. maculata* s. str. in some morphological traits. Such a process may have affected the Heslop-Harrison index, which is slightly lower in the fuchsii-4x group, or the shape of the leaf apex, which is more frequently acute in the fuchsii-4x group compared to fuchsii-2x. Genetic structure of these tetraploids therefore requires further investigation.

Cytotype diversity and cytogeography

Diploid populations were found mainly in the Carpathians, Alps, and Dinarides, which is in congruence with previous karyological reports (e.g. Skalińska et al. 1957; Groll 1966; Vaucher 1966; Löve 1971; Májovský 1978; Vöth and Greilhuber 1980; Jagiełło and Lankosz-Mróz 1988; Uhríková 2007). Diploids have also been mentioned from Bohemian Massif (Potůček 1969; Kubát 2010), but most populations of *D. *fuchsii* from this region analysed in the current study were tetraploid. Žďárské vrchy Mts are the only region within the Bohemian Massif where a diploid population (58, Ranský brook) has been confirmed to date. Diploids were also reported from the vicinity of Jagniątków in the Karkonosze Mts (Poland; Jagiełło and Lankosz-Mróz 1988), but this population (9, Jagniątków) was shown to be tetraploid in the current analysis.

Diploid populations were found in both the fuchsii and the sooana groups. Unlike fuchsii, the sooana group was exclusively diploid. Both groups were also largely geographically separated: the sooana group was found in the southern part of Western Carpathians (i.e. Northern Hungary, Southern Slovakia, and the White Carpathians in the Czech Republic), while populations in other regions corresponded to the fuchsii group. The distribution areas of both groups slightly overlapped in Northwestern Slovakia. On the other hand, at least some literature records of *D. fuchsii* from Hungary may represent the sooana group, depicted under this name in the Atlas of Hungarian Orchids (Molnár et al. 2011). The sooana group is also the only one found in Hungary during our field survey.

Tetraploid populations were widespread in Bohemian Massif, as well as in the Alps and Western Carpathians, where they reached Tatra Mts as the easternmost region. Despite *D. *fuchsii* has been considered exclusively diploid by many authors (Heslop-Harrison 1951; Vöth and Greilhuber 1980; Kubát 2010), tetraploids were reported repeatedly (e.g. Jagiełło and Lankosz-Mróz 1988; Měsíček and Javůrková-Jarolímová 1992; Bertolini et al. 2000) from this area. Ståhlberg and Hedrén (2010) suggested that tetraploid populations of *D. *fuchsii* were geographically limited to Central Europe, which may be explained by the relatively recent origin of this evolutionary lineage, dated to Holocene. Nevertheless, sporadic records of tetraploid individuals were also published from Pyrenees (Cauwet-Marc and Balayer 1984) and Apennines (Bertolini et al. 2000), which points to ongoing recurrent polyploidization.

DNA-triploids together with diploid and/or tetraploid individuals, were found in the Western Carpathians (i.e. Northwestern Slovakia), the Eastern Alps (Austria) and the Northern Dinarides (Slovenia), which are putative contact zones between the diploid and tetraploid lineages of *D. *fuchsii*. They were also rarely found in the Žďárské vrchy Mts, where diploids and tetraploids also co-occur. Furthermore, DNA-triploids were found within a diploid population (65, Pârâul Rece) in Southern Carpathians, where tetraploids were not recorded. DNA-triploids always co-occurred with other cytotype(s) and never

formed a uniformly DNA-triploid population. Two different processes may have led to the establishment of ploidy-heterogeneous populations: (1) triploid formation within diploid populations via unreduced gamete formation in diploid individuals or (2) secondary contact of individuals of different ploidy levels (diploids and tetraploids) resulting in occasional hybridization giving rise to triploids (cf. Ramsey and Schemske 1998; Kolář et al. 2017; Popelka et al. 2019a, 2019b), which was observed in *D. maculata* s.l. in Scandinavia (Ståhlberg 2009). The DNA-triploids in the current study may have originated by either of these ways.

Taxonomic consequences

Using various approaches (morphological traits and ploidy level estimation) allows to delimit two groups of populations, representing two different taxa. The first consists of morphologically indistinguishable populations of fuchsii-2x and fuchsii-4x, but the fuchsii-3x group may be obviously included too, although its morphology was not evaluated. The other group comprises the populations here classified as the sooana group. These groups differ from each other in phenotypic variation, cytotype diversity and distribution patterns, but probably also in ecology, as populations of the sooana group are able to occupy more mesic habitats and avoid acidic substrates (V. Taraška et al., pers. observ.). Regarding all distinctions between these taxa, the rank of subspecies seems to be the most appropriate for them.

In the traditional view, they should be recognized as two subspecies of *D. fuchsii*. However, the taxonomic concept used by Scandinavian authors (Hedrén et al. 2001; Ståhlberg and Hedrén 2010) seems to be more appropriate, incorporating *D. *fuchsii* into the broadly interpreted species *D. maculata* s.l. Unlike the concept of two separate species, *D. maculata* s. str. and *D. fuchsii*, this approach is rather conservative and is applicable in the whole distribution area of both taxa, including Central Europe where they tend to merge secondarily. Consequently, the correct name for the subspecies represented by the fuchsii-2x, -3x and -4x groups is *D. maculata* subsp. *fuchsii* (Druce) Hyl. The other taxon, comprising populations of the sooana group, is being mentioned under various names based on the basionym *Dactylorhiza fuchsii* subsp. *sooana* Borsos (e.g. Vlčko et al. 2003; Kreutz 2004; Kubát 2010) and its taxonomic reassessment is discussed below.

Dactylorhiza maculata subsp. *fuchsii* is widely distributed throughout Europe and it includes diploids, DNA-triploids, and tetraploids. Nevertheless, these could be hardly classified as separate taxa, as they do not differ in morphology nor ecology, and they often co-occur. Furthermore, DNA-triploids may be involved in bidirectional gene exchange between diploids and tetraploids (Thórsson et al. 2001; Ståhlberg 2009). Relatively frequent occurrence of DNA-triploid individuals within diploid populations also indicates a recent polyploidization. Coexistence of multiple cytotypes should be regarded as a hidden intrapopulation diversity, with serious evolutionary potential and conservation importance (Soltis et al. 2007). Cytotype variation should be considered besides the population size when setting conservation priorities, as it was stated also for the closely related genus *Gymnadenia* (Trávníček et al. 2011). High cytotype diversity of *D. maculata* subsp. *fuchsii* was detected mainly in the Western Carpathians, Eastern Alps, and Northern Dinarides. These regions are situated in the contact zone of diploid and

tetraploid lineages (Ståhlberg and Hedrén 2010; Eccarius 2016). The Žďárské vrchy Mts must be regarded as one of the diversity hotspots of *D. maculata* subsp. *fuchsii* in the Bohemian Massif, because it is the only known place in that area where all three cytotypes co-occur. High morphological variability of Central European populations may be partly a consequence of recent or former hybridization and gene introgression between *D. maculata* subsp. *maculata* and *D. maculata* subsp. *fuchsii* at the tetraploid level (Ståhberg and Hedrén 2010). Genetic structure of tetraploid populations of *D. maculata* subsp. *fuchsii* therefore needs further investigation.

Several taxa are often mentioned to be derived from *D. *fuchsii* in Central Europe. Their taxonomic value as well as position within *D. maculata* s.l. however, remains unclear. Tetraploid plants from the population 12, Velká kotlina, are usually assigned to *D. fuchsii* var. *psychrophila* (Schltr.) Soó (cf. Kubát 2010; Bureš 2013; Kaplan et al. 2017). This name, however, relates to diploid taxon described from Northern Europe (Vermeulen 1947; Eccarius 2016). Taxonomic evaluation of this population thus requires a wider geographical and taxonomical context. Another noteworthy tetraploid population was that of the locality Giesshübl (32), which is *locus classicus* of the unclear taxon *D. maculata* subsp. *austriaca* Vöth. Although it was subordinated to *D. maculata* s. str. because of its tetraploid chromosome number, even the protologue admits that this taxon is morphologically close rather to *D. *fuchsii* (Vöth 1978). The most striking morphological characteristic of this population is a high proportion of individuals with low pigmentation of both flowers and leaves. Hypochromic individuals can be often found in populations of *D. maculata* subsp. *fuchsii*, although usually not in such a high proportion (Bateman and Denholm 1988; Pikner 2012). Locality Giesshübl consists of two small meadow enclaves in the forest, and the population is probably reproductively isolated. Various evolutionary processes, including stochastic events, could lead to increase in the number of the hypochromatic plants (Narbona et al. 2017). Recently, this taxon is usually not accepted (cf. Redl 2003; Fischer et al. 2008). Giesshübl is also probably the only locality from where *D. maculata* subsp. *austriaca* has been reliably reported. Ståhlberg and Hedrén (2010) mention this taxon also from the surroundings of the town of Furth an der Triesting, Lower Austria. The exact location is however not known (M. Hedrén, in litt.) and only diploid *D. maculata* subsp. *fuchsii* (28, Furth an der Triesting) was found in this area within our field work. Thus, *D. maculata* subsp. *austriaca* should be rather considered only a colour morph, which should not be recognized taxonomically (cf. Pedersen 1998).

Populations corresponding to the sooana group were found in several localities in hilly regions of the Western Carpathians, and they are usually mentioned under the name of *D. fuchsii* subsp. *sooana*. Some authors (Borsos 1961; Potůček 1969; Soó 1980; Vlčko et al. 2003) circumscribe this taxon solely based on the white colour of flowers; the flower colouration alone, however, cannot be used for its delimitation. These plants may be almost invariably characterized by white flowers with white anther caps and pale to bold spots on the leaves, and they are always diploid. In analogy to *D. maculata* subsp. *fuchsii*, the sooana group should be subordinated to *D. maculata* in the rank of subspecies. The oldest epithet related to this taxon at the subspecific level must be thus found.

The name *D. fuchsii* subsp. *sooana* commonly appears in the literature (Procházka 1979; Soó 1980; Batoušek 1995; Kubát 2010; Vlačiha 2013; Ponert 2019), but it is not valid, as no type specimen was stated for it in its protologue (cf. Borsos 1959), nor later. Thus, other names must be considered. In British Isles, plants with similar morphological

characteristics, i.e. white flowers with markings and spotted leaves, are recognized as *D. fuchsii* subsp. *okellyi* (Druce) Soó (e.g. Eccarius 2016). Bateman and Denholm (1988) stated that there are no differences between '*okellyi*' (recognized at variety level) and '*sooana*' that could justify their separation. Nevertheless, their description of *D. fuchsii* var. *okellyi* (Druce) Bateman et Denholm implies that British plants are considerably subtler than those from Central Europe. In addition, Harrap and Harrap (2009) mention that white-flowered individuals in British Isles represent only part of a population of plants which are more variable in flower colour. Even the distribution pattern suggests that the Carpathian populations and the populations from the British Isles represent separate evolutionary units of independent origin. Their similarity in some morphological traits is likely to be just a result of convergence, which is quite common in *Dactylorhiza* (Averyanov 1982; Delforge 2006; Efimov et al. 2016).

The high proportion of white-flowering individuals within the sooana group could indicate some relation with *D. maculata* subsp. *austriaca*; this name should also be applied if both taxa were found to be identical. The distribution areas of these taxa border on each other, as *D. maculata* subsp. *austriaca* is known from the Northeastern Alps (Vöth 1978). A considerable morphological overlap between *D. maculata* subsp. *austriaca* and the sooana group is also apparent in our data. However, unlike the sooana group, *D. maculata* subsp. *austriaca* is tetraploid. It is also improbable that *D. maculata* subsp. *austriaca* is a polyploid derivate of the sooana group, because its flower colouration is positively correlated with leaf pigmentation: white-flowered individuals typically lack spots on the leaves. This is not the case of the sooana group, and *D. maculata* subsp. *austriaca* seems to be derived rather from the tetraploid cytotype of *D. maculata* subsp. *fuchsii*.

According to our knowledge, there is no valid name available for the taxon represented by the sooana group at the subspecies level. With no doubt, the invalid name '*Dactylorhiza fuchsii* subsp. *sooana*' used by Borsos (1959) is related to this taxon. The epithet '*sooana*' (originally "*soóiana*", which is a typographical error) is thus adopted here, and a valid name of the subspecies is introduced, providing a diagnosis and stating the holotype.

Conlusions

Populations of *D. *fuchsii* in Central Europe are considerably variable both in morphology and ploidy level. Despite the commonly shared conviction that they are strictly diploid, a number of tetraploid populations was detected, as well as several DNA-triploids representing a minority cytotype within diploid or tetraploid, or even mixed ploidy populations. Tetraploid populations utterly prevail in the Bohemian Massif, while diploids are more common in the Carpathians, but all three cytotypes occur throughout Central Europe. This is the first large-scale screening of ploidy levels in *D. maculata* s.l. based on FCM considering PPE.

Based on the combination of phenotypic traits, ploidy level variation, and geographical distribution patterns, it is justifiable to separate a group of West Carpathian populations, which typically possess white flowers with white anther caps, pale to bold spots on the leaves, and strictly diploid chromosome numbers. In contrast, the other group of

populations, widespread in Central Europe, is more variable, characterized by white to purple flowers, spotted or unspotted leaves; but importantly, with a positive correlation between the intensity of leaves and flower pigmentation, and with purple anther caps even in plants with completely white flowers. All three cytotypes were found in this group, but they were morphologically indistinguishable. Following the more appropriate taxonomic concept, the latter of the groups should be recognized as *D. maculata* susbp. *fuchsii*, while the first is here described as *D. maculata* subsp. *sooana*, subsp. nova. A new combination of its hybrid with *D. majalis* subsp. *majalis* is also suggested, which is *D.* × *dinglensis* nothosubsp. *smitakii*, comb. nova.

TAXONOMIC TREATMENT

Dactylorhiza maculata* subsp. *sooana Borsos ex Batoušek, Taraška & Trávn., **subsp. nova**. [*Dactylorhiza fuchsii* subsp. *sooana* Borsos, nom. inval., Acta Bot. Acad. Sci. Hung. 5: 324, 1959 ('*soóiana*')]. — TYPE: 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 Jun 2017, leg. excursion group (holotype: OL 37871!; isotypes: OL 37872!, OL 37873!, BRNM 826419!) (photographs of the live holotype plant see Fig. 5, photograph of its herbarium specimen see Online Resource 7).

Etymology: The epithet '*sooana*' was adopted from Borsos (1959) and it refers to Károly Rezsö Soó (1903–1980), a Hungarian botanist and taxonomist with interest in genus *Dactylorhiza*.

Description: Herbaceous perennial plant with palmate tubers. Stem (26)37–61(67) cm high, with (4)5–9(13) leaves, often with brownish stripes. Lower 3–6 leaves with sheaths, upper leaves bract-like; at least lower leaves with bold or pale spots. Lowermost leaf obovate or oblong, usually obtuse at the apex, (46)74–141(180) × (14)18–35(52) mm, (2.3)3.1–5.6(7.5) times longer than wide. The 2nd lowermost leaf obovate, oblong or lanceolate, usually obtuse or subacute at the apex, (82)103–165(200) × (11)17–34(52) mm, (2.4)4.0–7.3(11.6) times longer than wide. Inflorescence a dense-flowered spike. Tepals white, sometimes with markings. Lip three-lobed, the Heslop-Harrison index (1.1)1.2–1.5(1.8), white with or without purple marking and white anther caps. Capsules cylindrical, seeds dust-like.

Diagnosis: *Dactylorhiza maculata* subsp. *sooana* differs from the type *D. maculata* subsp. *maculata* by broader, obtuse lower leaves, and deeply three-lobed lips of flowers (Heslop-Harrison index ≥ 1.3), as well as diploid chromosome number ($2n = 2x = 40$). These characteristics are mostly shared with *D. maculata* subsp. *fuchsii*, from which *D. maculata* subsp. *sooana* differs by a combination of several qualitative traits: white flowers, sometimes with purple markings and always with white anther caps, and spotted leaves, even in individuals with completely white flowers. Both taxa also differ in cytotype diversity, as *D. maculata* subsp. *sooana* is always diploid, while *D. maculata* subsp. *fuchsii* may be di-, tri- or tetraploid.

Chromosome numbers: $2n = 2x = 40$.

Habitats: Mesophilous to wet meadows, open broad-leaved (beech) forests.

Distribution area: Czech Republic, Slovakia and Hungary. Endemic to Western Carpathians.

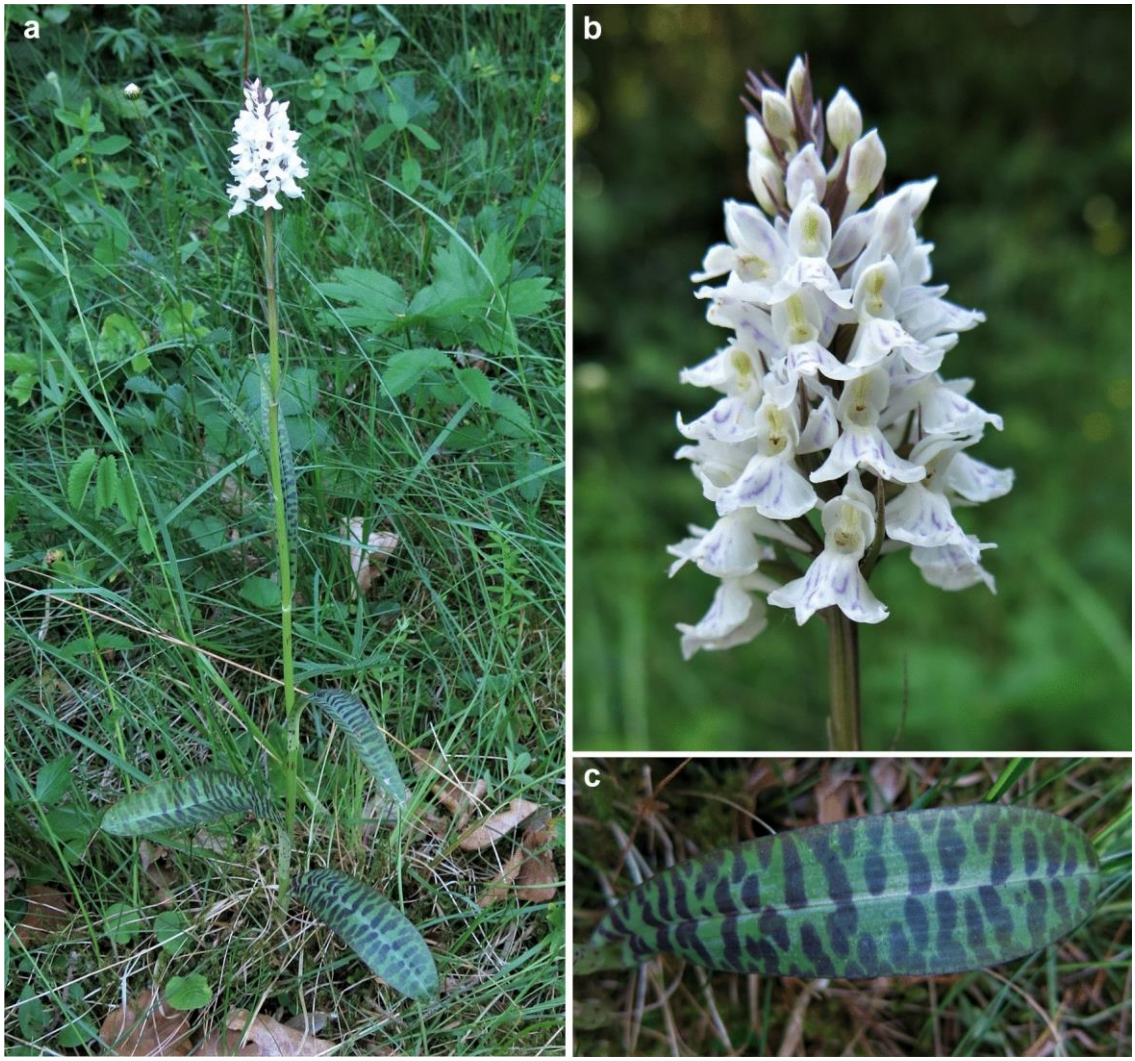

Figure 5. Plant selected as the holotype of *Dactylorhiza maculata* subsp. *sooana*: habitus (**a**), detail of inflorescence (**b**), and detail of the lowermost leaf (**c**). Photographs: B. Trávníček.

Dactylorhiza* × *dinglensis* nothosubsp. *smitakii (Batoušek) Batoušek, Taraška & Trávn., **comb. nova**. [*D. maculata* subsp. *sooana* × *D. majalis* (Rchb.) P.F.Hunt & Summerh. subsp. *majalis*]. ≡ *Dactylorhiza* × *braunii* nothosubsp. *smitakii* Batoušek, J. Eur. Orch. 29: 643, 1997. — HOLOTYPE: Moravia meridioorientalis, montes Bílé Karpaty, distr. Zlín: Nedašov, pratum clivis septentrionalis montis Cigán (744 m), 550 m a. s. l., 15 Jun 1980, *P. Batoušek* (GM 29845!).

Note: A hybrid of *D. maculata* subsp. *sooana* and *D. majalis* subsp. *majalis* was described by Batoušek (1997) as *D.* × *braunii* nothosubsp. *smitakii* Batoušek from Eastern Moravia (Czech Republic). The name *D.* × *braunii* (Halácsy) Soó is however applied to interspecific hybrids of *D. fuchsii* and *D. majalis*, where the first is recognized at the

species level. Following the here accepted taxonomic concept, in which *D. fuchsii* is considered as an infraspecific taxon of *D. maculata*, a new combination is required for the hybrid. The interspecific hybrids of *D. maculata* and *D. majalis* are recognized as *D.* × *dinglensis* (Wilmott) Soó, Nom. Nov. Gen. *Dactylorhiza* 10, 1962 based on the name of *Orchis* × *dinglensis* Wilmott, Proc. Linn. Soc. London 148: 128, 1936. This hybrid taxon was noted by us on the *locus clasicus* of *D. maculata* subsp. *sooana* (near Banský Studenec Village in Štiavnické vrchy Hills, Slovakia; photographs in Online Resource 8), as well as in further localities in Slovakia (54, Rudno nad Hronom), Czech Republic (5, Bylničky) and Hungary (48, Bohó-hegy). From Slovakian territory, this hybrid was reported by Vlčko et al. 2003: 97 (from the Biele Karpaty Mts).

SUPPLEMENTARY FILES

Supplementary files are available on the attached CD-ROM and online from https://doi.org/10.1007/s00606-021-01770-3.

Online Resource 1. Locality details of *Dactylorhiza *fuchsii*.

Online Resource 2. Details to (sub)populations of *Dactylorhiza *fuchsii*. Averaged relative DNA content (= fluorescence ratios between the positions of the sample and internal reference standard G_0/G_1 peaks) of investigated populations.

Online Resource 3. Explanations to quantitative characters used in the morphometrics.

Online Resource 4. Relative DNA content (= fluorescence ratios between the positions of the sample and internal reference standard G_0/G_1 peaks) of six plants with counted chromosome numbers; the stain was either DAPI or PI. All values are calculated relative to the *Pisum sativum* cv. 'Ctirad' as internal reference standard.

Online Resource 5. Absolute genome sizes (GS) of five individuals of *Dactylorhiza *fuchsii* estimated by flow cytometry.

Online Resource 6. Box plots of characters analysed for the fuchsii-2x, fuchsii-4x and sooana groups.

Online Resource 7. Holotype of *Dactylorhiza maculata* subsp. *sooana* Batoušek, Taraška & Trávn.

Online Resource 8. Images of *Dactylorhiza maculata* subsp. *sooana*, *D. maculata* subsp. *fuchsii* and *D.* ×*dinglensis* nothosubsp. *smitakii*.

CHAPTER 3:

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

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 (Hedrén 2001; Pillon et al. 2007; Devos et al. 2005; Hedrén et al. 2008; Nordström and Hedrén 2009; Balao et al. 2016; Brandrud et al. 2020).

The evolutionary history and phylogeny of the *D. maculata* agg. has been explored using allozymes (Hedrén 1996), AFLP (Hedrén et al. 2001), nuclear and plastid markers (Hedrén 2003; Devos et al. 2003, 2005; Ståhlberg and Hedrén 2008, 2010; Naczk 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 Hedrén 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 Hedrén 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 Hedrén 2010; Naczk 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) 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).

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 classification**	**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**
		Austria	**Slovakia**	**Europe**	**Europe, North Africa, Middle East**	**World**	**Czechia**	**Poland**	**Germany**	**Hungary**
maculata (mac)	*D. maculata* subsp. *maculata*	*D. maculata*	*D. maculata* subsp. *maculata*	*D. maculata* subsp. *maculata*	*D. maculata*	*D. maculata* subsp. *maculata*	*D. maculata* subsp. *maculata*	*D. maculata*	*D. maculata* subsp. *maculata*	–
fuchsii (fuc)	*D. maculata* subsp. *fuchsii*	*D. fuchsii* (incl. subsp. *psychrophila*)	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii*	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii*	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii*
sooana (soo)	*D. maculata* subsp. *sooana*	–	*D. fuchsii* subsp. *sooiana*	*D. fuchsii* var. *sooana*	?	*D. fuchsii* subsp. *fuchsii*	*D. fuchsii* subsp. *sooana*	–	–	?
elodes-WE (e-WE)	*D. maculata* subsp. *elodes*	–	–	*D. maculata* subsp. *elodes*	*D. maculata* var. *elodes*	*D. maculata* subsp. *maculata* '*elodes*'	–	–	*D. maculata* subsp. *elodes*	–
elodes-BM (e-BM)	*D. maculata* subsp. *averyanovii*	–	–	*D. maculata* subsp. *maculata*	?	?	*D. maculata* subsp. *elodes* '*averyanovii*'	?	–	–
elodes-CA (e-CA)	*D. maculata* subsp. *arcana*	–	*D. maculata* subsp. *elodes*	?	?	?	–	?	–	–

This work – analysed groups (abbreviation)	**This work – final classification**	**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**
		Austria	**Slovakia**	**Europe**	**Europe, North Africa, Middle East**	**World**	**Czechia**	**Poland**	**Germany**	**Hungary**
ericetorum (eri)	*D. maculata* subsp. *averyanovii*	–	*D. ericetorum*	?	?	?	–	–	–	–
transsilvanica (tra)	*D. maculata* subsp. *transsilvanica*	–	*D. maculata* subsp. *transsilvanica*	*D. maculata* subsp. *transsilvanica*	*D. maculata* var. *transsilvanica*	*D. maculata* subsp. *transsilvanica*	*D. maculata* subsp. *transsilvanica*	–	*D. fuchsii* var. *sudetica* (unclear)	*D. maculata* subsp. *transsilvanica*
psychrophila (psy)	*D. maculata* subsp. *sudetica*	*D. sudetica*	–	*D. fuchsii* subsp. *sudetica*	*D. sudetica*	*D. maculata* subsp. *sudetica*	*D. fuchsii* subsp. *psychrophila*	*D. sudetica*	*D. fuchsii* subsp. *psychrophila*	–

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. Bateman 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; Sczepanski 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 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.

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.	lL1 [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.	lL2 [mm]	length of the 2nd leaf
7.	wL2 [mm]	width of the 2nd leaf

No.	Character abbreviation [unit]	Numerical characters
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 – purplish, 3 – dark purple
		Categorial 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/lL1	hPl/lL1
26.	hPl/lL2	hPl/lL2
27.	hPl/nrL	hPl/nrL
28.	lL1/wL1	lL1/wL1
29.	lL2/mL2	lL2/mL2
30.	HH; Heslop-Harrison index	2A/(B + C)
31.	AD	A/(A – C)
32.	FE	F/E
33.	BBC	B/(B – C)

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	lL2/wL2	–	–	PCoA_1	–
Dataset 2b	58	1,195	individuals	–	lL2/wL2	–	–	PCoA_2	–
Dataset 3	28	544	individuals	agg, *fuchsii*, *sooana*	–	–	–	–	PLS-DA_1
Dataset 4	51	–	population	agg	IL2, wL2, C	–	CLUST_1, CLUST_2	PCA_1, PCA_2	–
Dataset 5	26	–	population	agg, *fuchsii*, *sooana*	IL2, wL2, C	–	–	PCA_3	–

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.

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 xlstat (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. Ctirad (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 G_0/G_1 peak (cf. 2C-peak; Trávníček et al. 2015) of *Dactylorhiza* and the mean position of the G_0/G_1 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 (hPI), 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.

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 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 (lSp/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 (lSp/A) and low Heslop-Harrison index (HH) and their leaves were widest in the basal part (lL2/mL2), while plants of the *elodes*-CA group had flowers with both absolutely and relatively long spur (lSp, lSp/A), and rather intensely pigmented both inflorescence (mLBb, ipInf) and leaves (spLb; Fig. 2d).

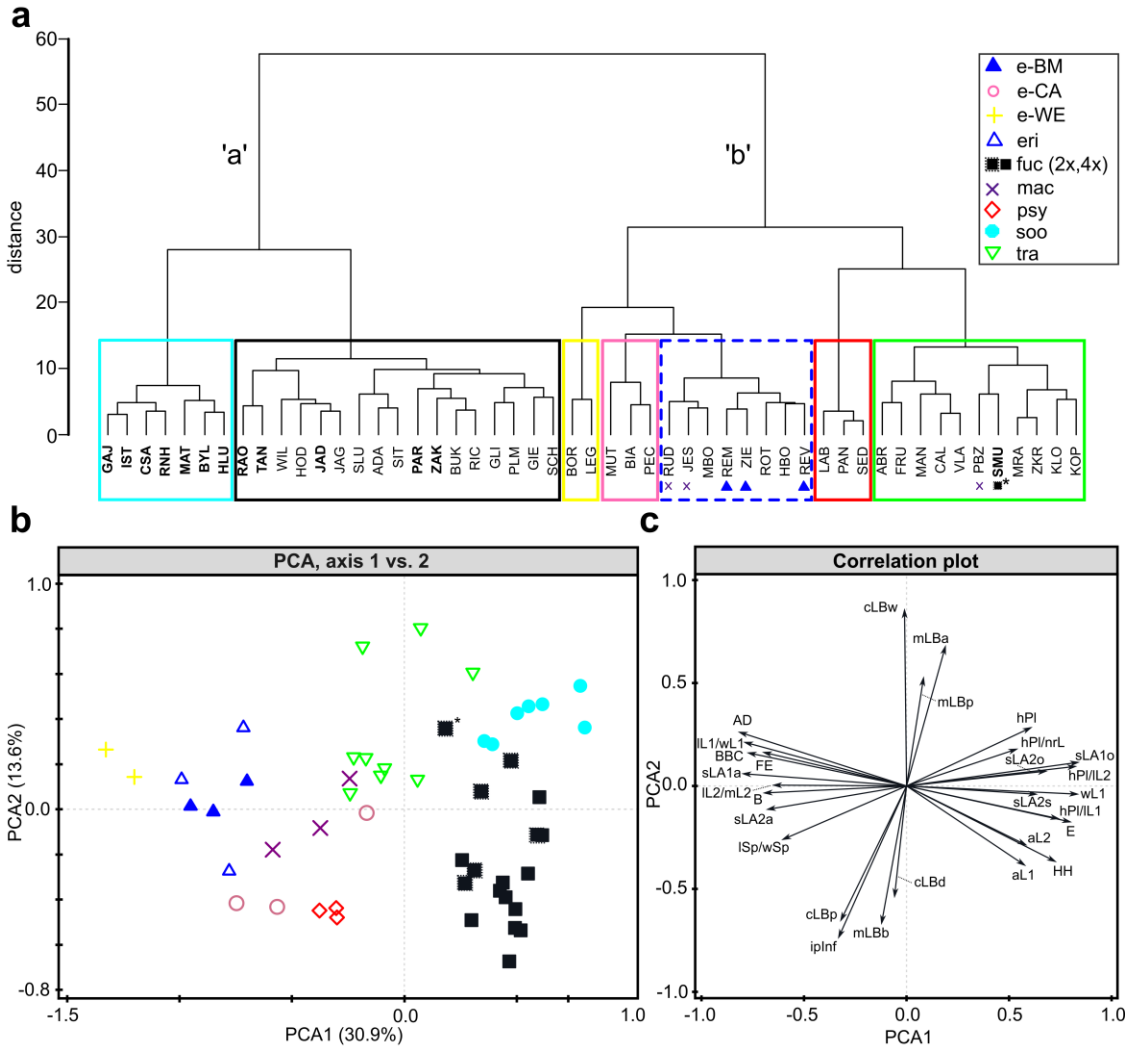

Figure 1. Multivariate analyses of morphological traits of *D. maculata* agg. populations as OTUs. Groups are identified by different colours and symbols. (**a**) Results of hierarchical cluster analysis using Ward's method (CLUST_1 analysis, Table 3) with resulting clusters 'a' and 'b'. Boxes demarcate 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.

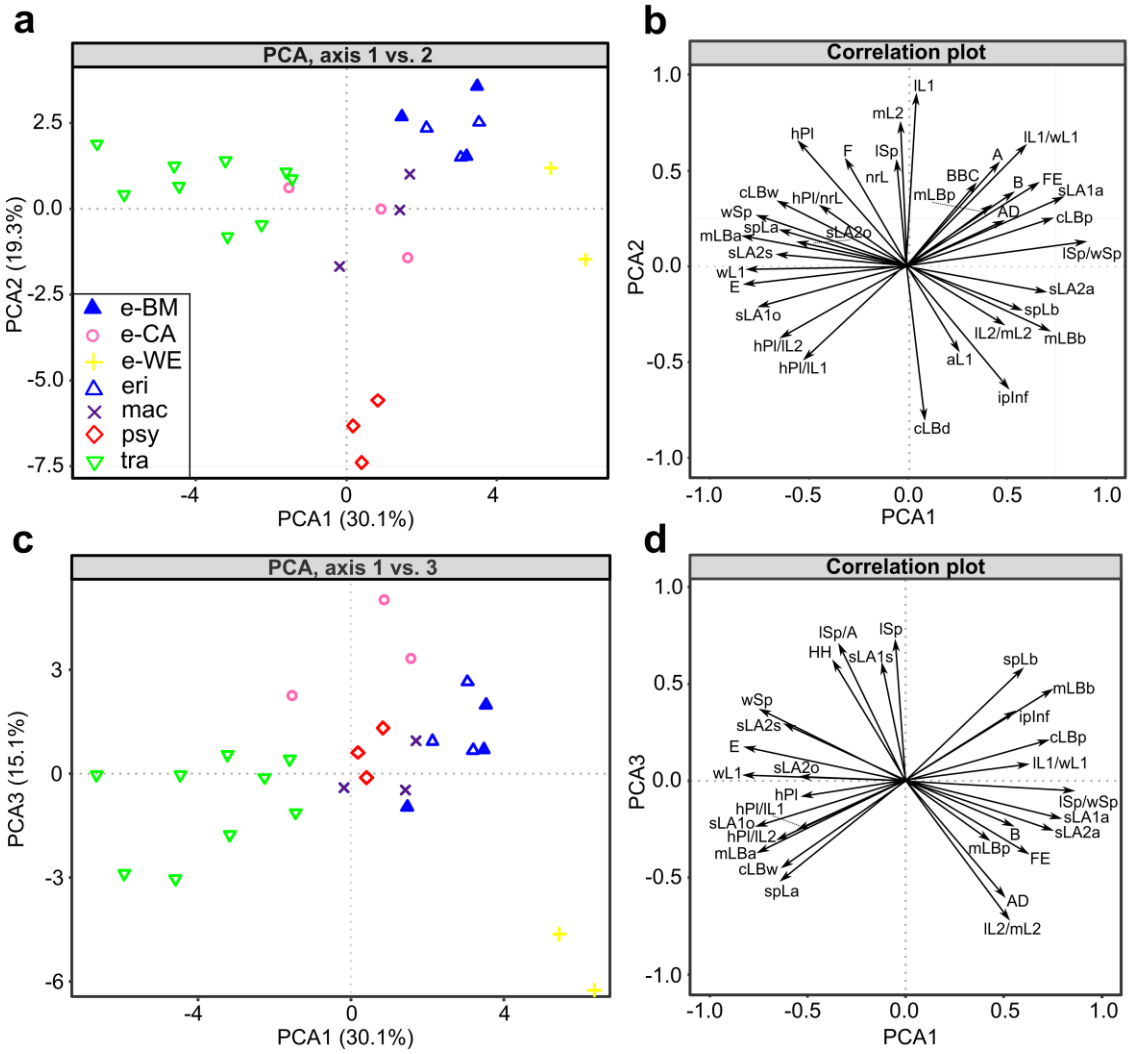

Figure 2. Multivariate analyses of morphological traits of *D. maculata* agg. populations as OTUs, with populations of the *fuchsii* and the *sooana* groups excluded (dataset 5, Table 3). Results of PCA_3 with (**a, b**) axes 1 and 2 and (**c, d**) axes 1 and 3, with sample plots and correlation plots. Variation explained by each axis is within parentheses. 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.

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 intesly 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.

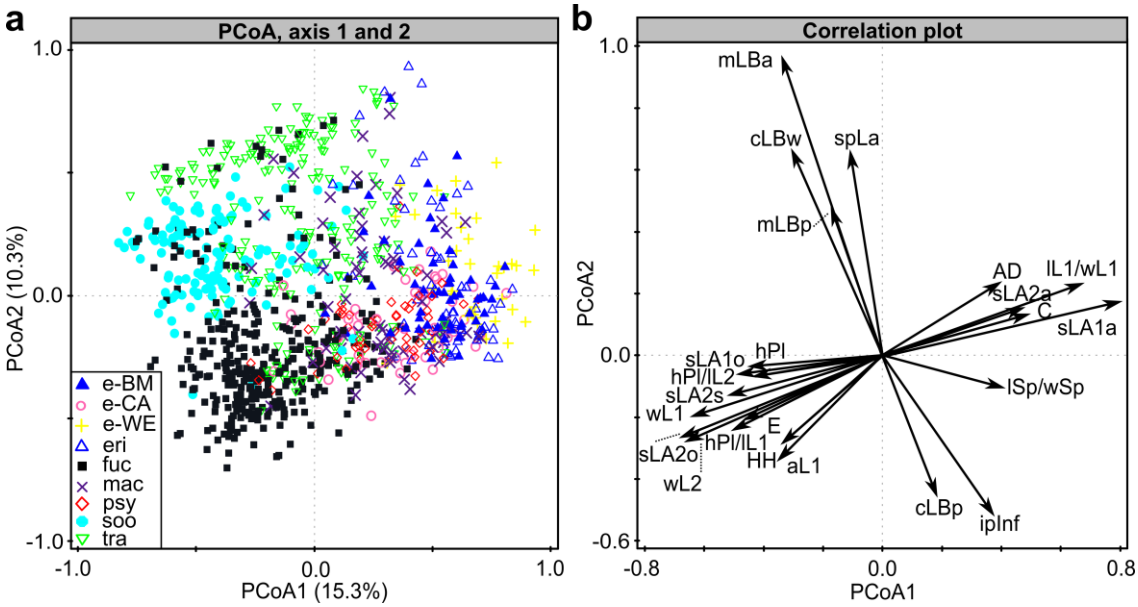

Figure 3. Multivariate analyses of morphological traits of *D. maculata* agg. individuals as OTUs, with populations of the aggregate group excluded (dataset 2a, Table 3). (**a**) Sample plot of the first two axes (PCoA1, PCoA2) of PCoA_1 (Table 3). Variation explained by each axis is within parentheses. (**b**) PCoA correlation plot of characters analysed. Only variables whose correlations exceed |0.40| with at least one axis are displayed in the plot. Group abbreviations are explained in Table 1 and character abbreviations in Table 2.

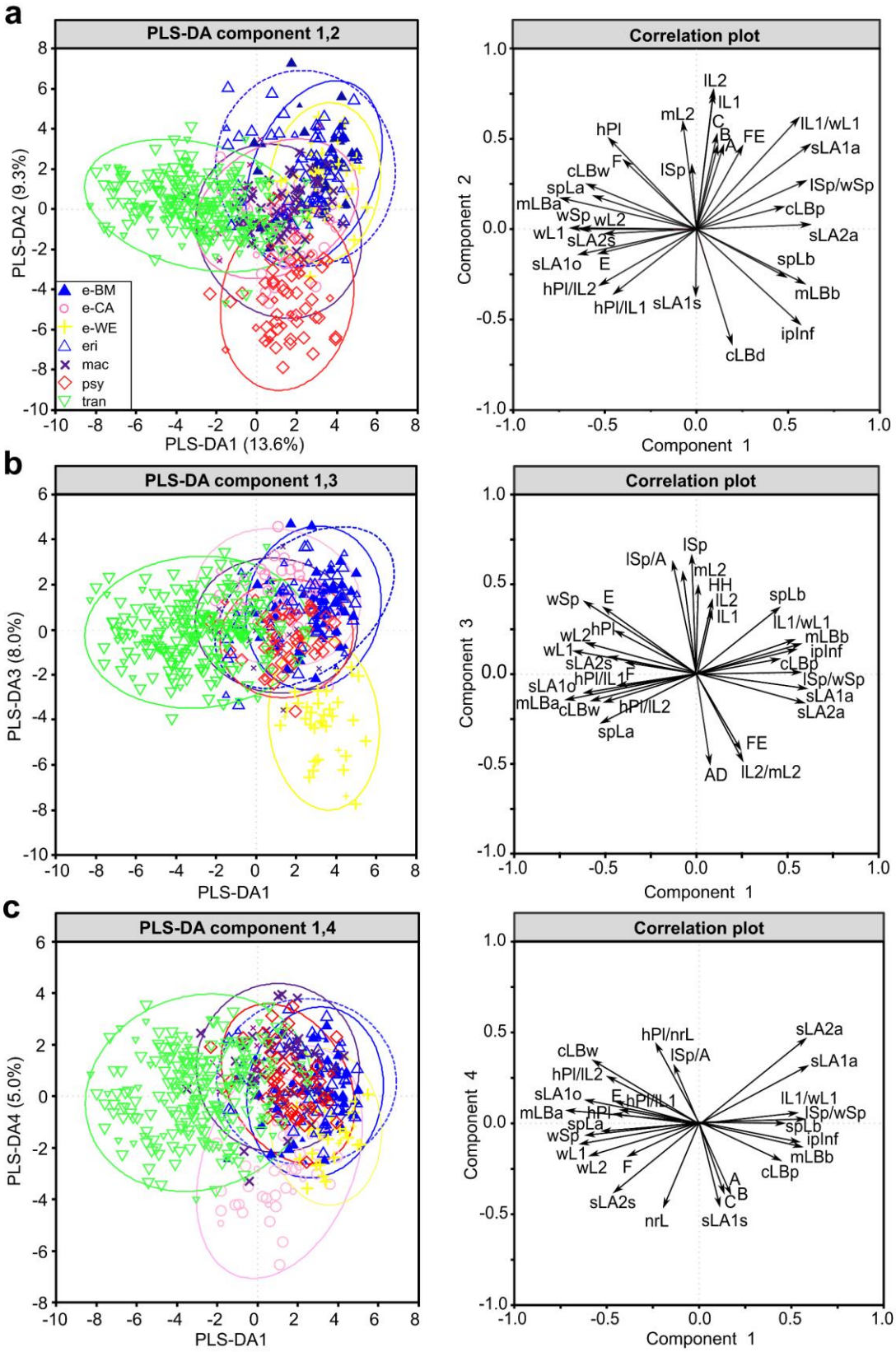

Figure 4. Sample plots and correlation plots from partial least squares discriminant analysis (PLS-DA_1) of individuals of seven taxonomic groups of *D. maculata* agg. (dataset 3, Table 3), divided into training (75% of dataset) and validation (25%) samples and balanced across the groups. The first four 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-DA_1 analysis, Table 3) estimated the number of 8 predictive components to be optimal for the final model, with $R^2X_{cum} = 0.570$, $R^2Y_{cum} = 0.451$, and Q^2 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 (lSp, wSp, lL1/wL1, lSp/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).

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	***elodes*-BM**	***elodes*-CA**	***elodes*-WE**	***ericetorum***	***maculata***	***psychrophila***	***transsilvanica***	**Total**	**% Correct (training)**	**% Correct (validation)**
elodes-BM	28 (11)	1 (0)	0 (1)	2 (2)	2 (1)	1 (0)	7 (1)	41 (16)	68.29	68.75
elodes-CA	1 (1)	28 (7)	0 (0)	3 (0)	1 (0)	1 (0)	4 (2)	38 (10)	73.68	70.00
elodes-WE	0 (0)	0 (0)	29 (8)	0 (0)	0 (0)	0 (0)	0 (0)	29 (8)	100.00	100.00
ericetorum	7 (4)	1 (2)	2 (0)	26 (7)	2 (1)	0 (1)	6 (3)	44 (18)	59.09	38.89
maculata	1 (1)	1 (1)	1 (1)	5 (1)	27 (11)	1 (0)	16 (6)	52 (21)	51.92	52.38
psychrophila	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	41 (13)	2 (0)	43 (13)	95.35	100.00
transsilvanica	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

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).

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

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, 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 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.

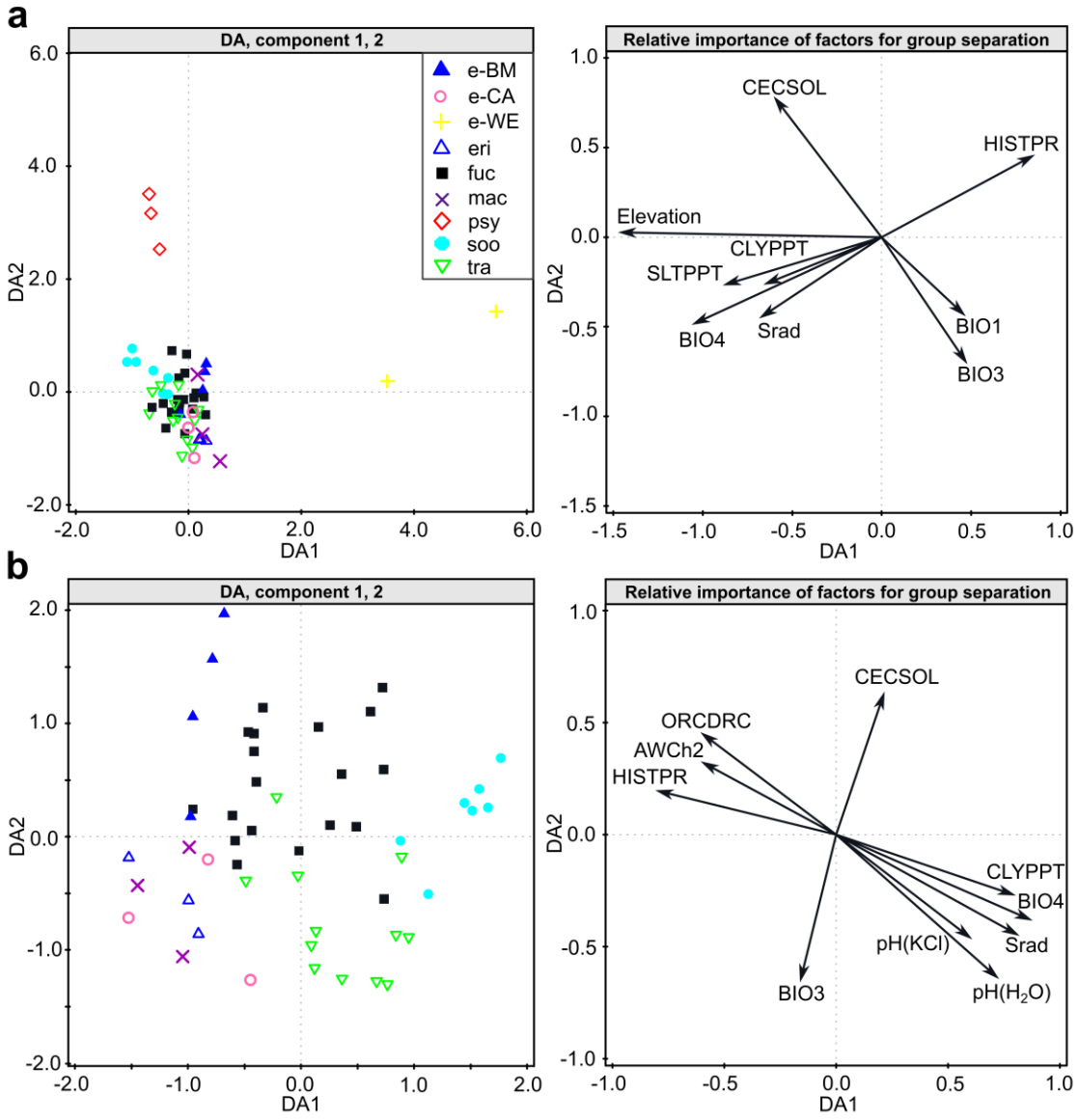

Figure 5. Sample plots and plots of relative importance of factors for group separation from discriminant analysis (DA) of environmental conditions extracted from the WorldClLim and SoilGrid databases for the sites of groups of *D. maculata* agg. studied (abbreviations explained in Table 1). The first 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) (×100); BIO4 – temperature seasonality (standard deviation ×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.

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 *transilvanica* groups, while these vegetation types were only rarely recorded in connection with some of the other groups.

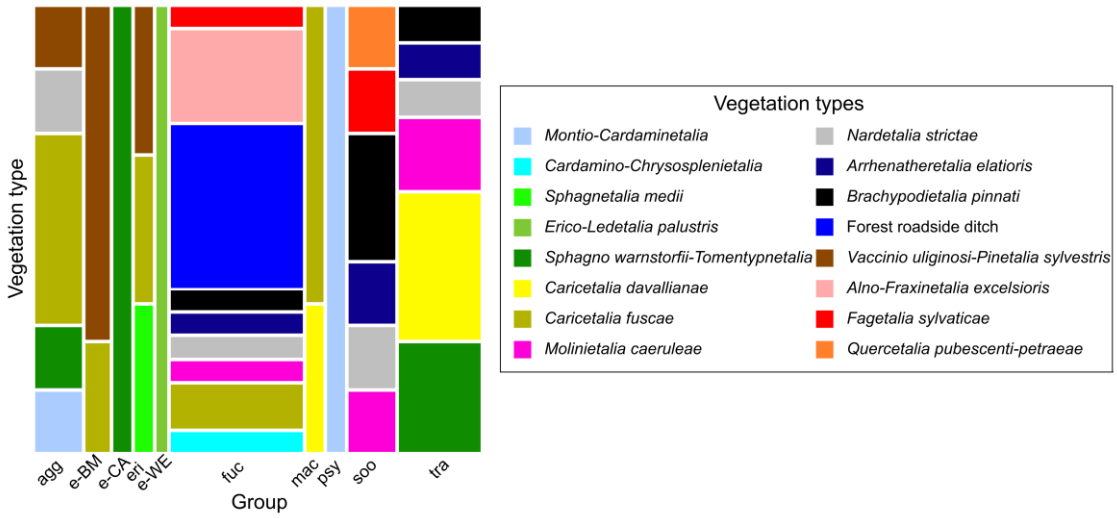

Figure 6. Mosaic plot of the frequencies of vegetation types (phytosociological orders *sensu* Mucina et al. 2016 plus an additional type 'forest roadside ditch') in ten study groups of *D. maculata* agg. (abbreviations are explained in Table 1).

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.

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

Taxon	IUCN Red List Category for Czechia
D. maculata subsp. *averyanovii*	CR B1ab(iii)+2ab(iii)
D. maculata subsp. *fuchsii*	NT
D. maculata subsp. *maculata*	EN B1ab(ii,iii,iv)+2ab(ii,iii,iv)
D. maculata subsp. *sooana*	EN B1ab(ii,iii,iv)+2ab(ii,iii,iv); C2a(i)
D. maculata subsp. *sudetica*	EN B1ab(iii)+2ab(iii)
D. maculata subsp. *transsilvanica*	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 Naczk 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. 2019a; 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. Kobrlová 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; Naczk 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 hybridogeneous 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; Naczk 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; Naczk 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* (Jagiełło 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).

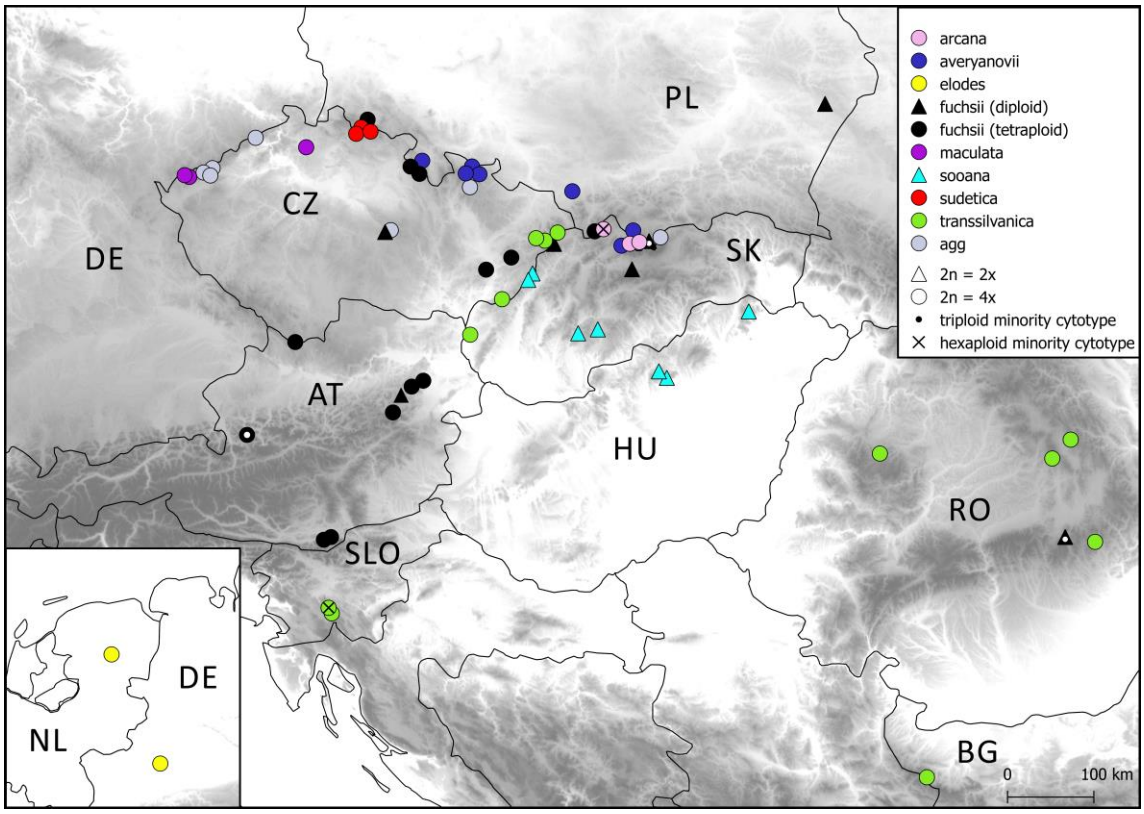

Figure 7. Map of the locations of the sample populations, classified as subspecies following the here accepted taxonomic concept. The symbol shapes indicate the ploidy levels, and the colours indicate subspecies identity.

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 genetic variation (Ståhlberg and Hedrén 2010; Naczk 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. 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 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; Sczepanski 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 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 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; 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. Vlačiha and Dundr 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 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.

Figure 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.

Figure 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.

Figure 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.

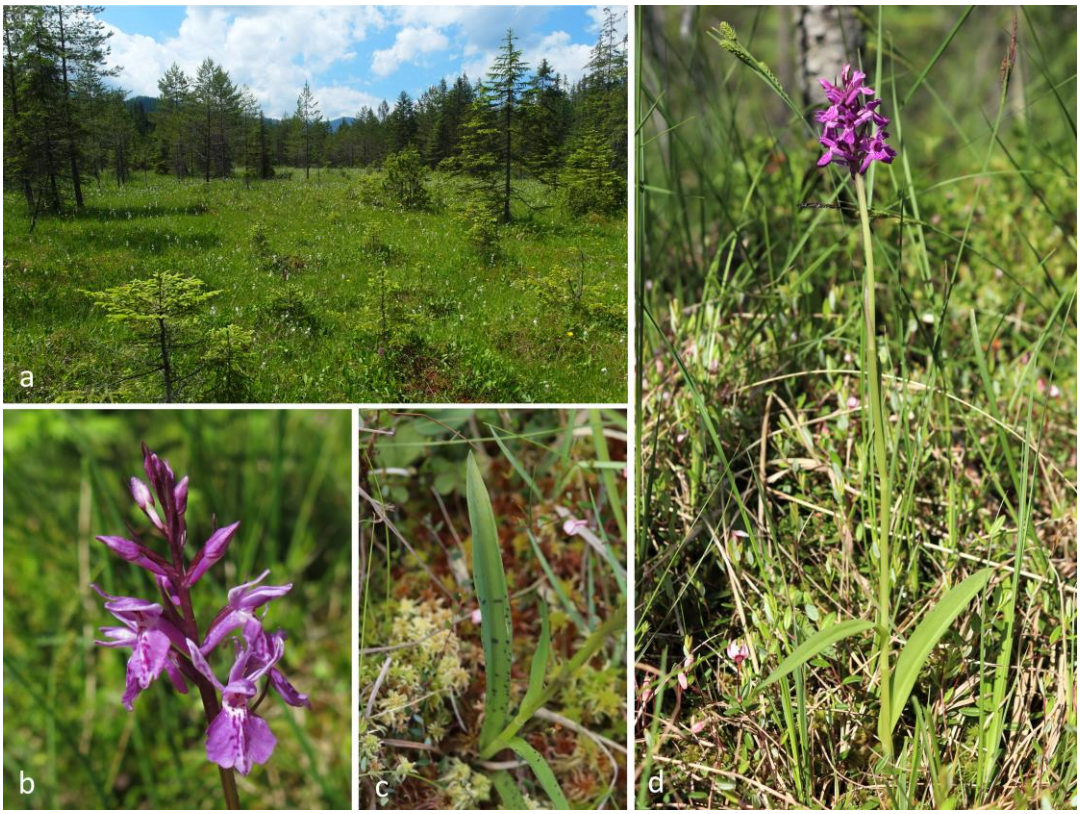

Figure 11. *Dactylorhiza maculata* subsp. *arcana*: (**a**) habitat; (**b**) inflorescence; (**c**) leaves; (**d**) whole plant; all photographs are from loc. 3, Biały potok.

Figure 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.

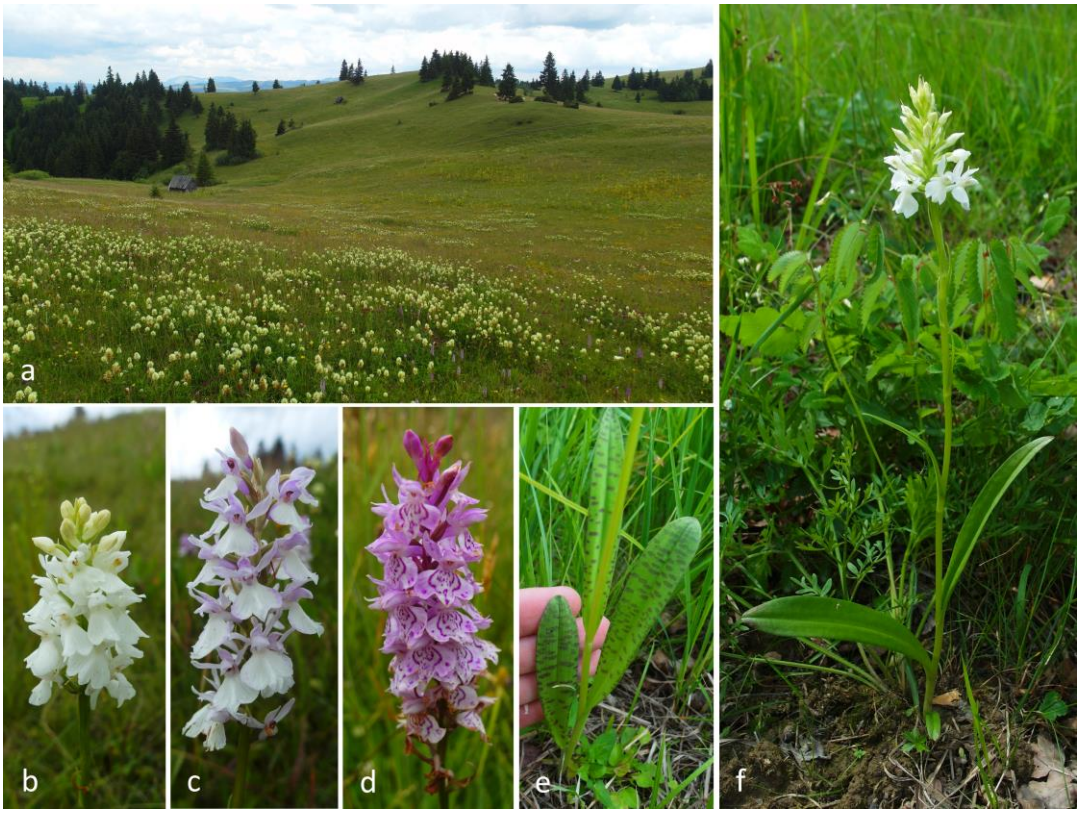

Figure 13. *Dactylorhiza maculata* subsp. *transsilvanica*: (**a**) habitat, loc. 10, Frumoasa; (**b, c**) inflorescence, loc. 10. Frumoasa; (**d**) inflorescence, loc. 28. Mânăstirea Suzana; (**e**) leaves, loc. 10. Frumoasa; (**f**) whole plant, loc. 21, Jazevčí.

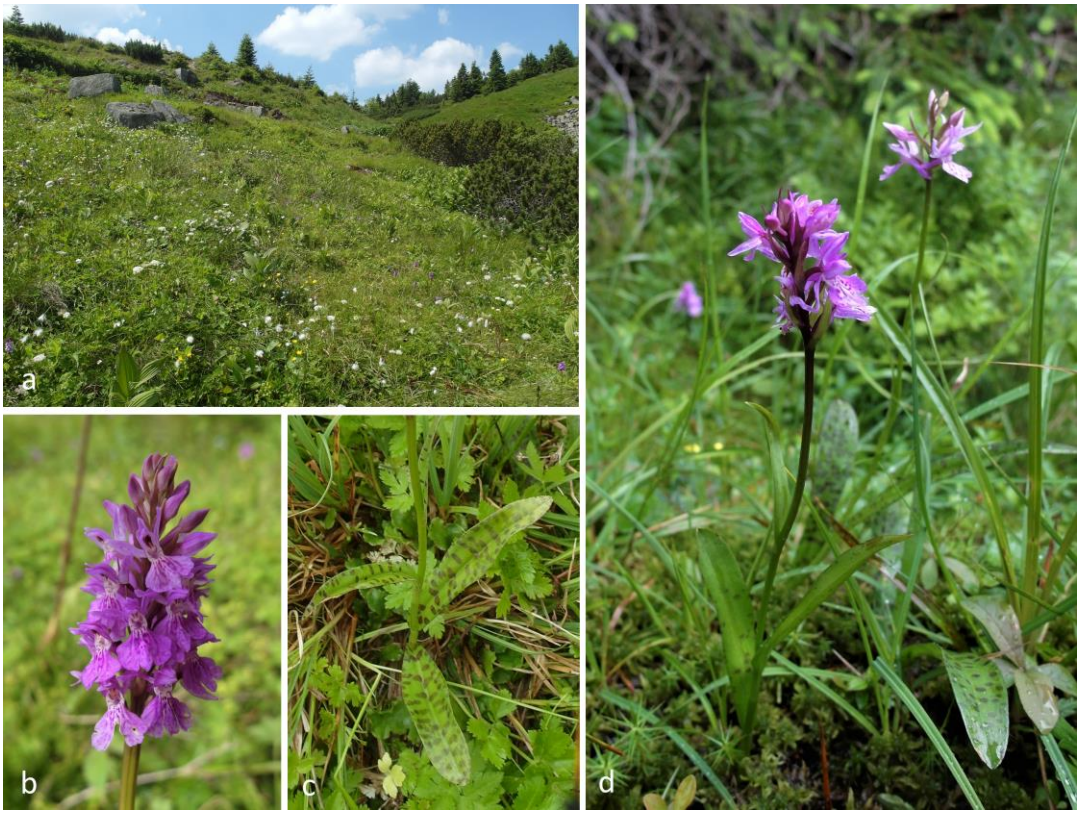

Figure 14. *Dactylorhiza maculata* subsp. *sudetica*: (**a**) habitat, loc. 25, Labský vodopád; (**b**) inflorescence, loc. 25, Labský vodopád; (**c**) leaves, loc. 25, Labský vodopád; (**d**) whole plant, loc. 33, Pančava.

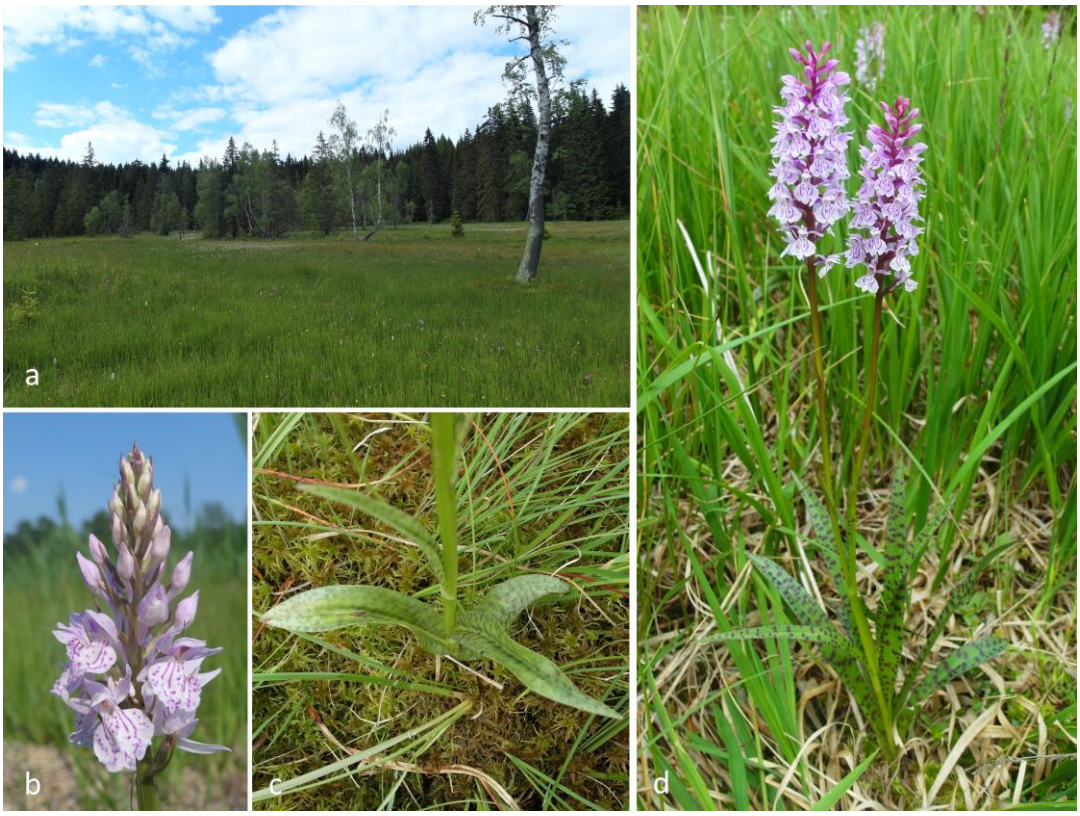

Figure 15. *Dactylorhiza maculata* subsp. *maculata*: (**a**) habitat, loc. 45, Rudné; (**b**) inflorescence, loc. 22, Jestřebí; (**c**) leaves, loc. 45, Rudné; (**d**) whole plant, loc. 40, Přebuz.

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

– *D. 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.

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 Szeląg 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-Harison 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 Szeląg 2006). Slovakia: NT (Eliáš et al. 2015).

Dactylorhiza maculata (L.) Soó **subsp. *maculata***

= *Orchis maculata* subsp. *ericetorum* E. F. Linton, Fl. Bournemouth 208 (1900) ≡ *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 Szeląg 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., Pl. 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)

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

≡ *Dactylorhiza fuchsii* subsp. *sudetica* (Poech ex Rchb.f.) Verm., Orchideeën 37(3): 78 (1975)

≡ *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 Szeląg 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 (Eliáš 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)× 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)× 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 ± 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***

SUPPLEMENTARY FILES

Supplementary files are available on the attached CD-ROM and online from https://doi.org/10.1007/s12224-024-09441-0.

Table S1. Details on sample populations of the *D. maculata* agg.

Table S2. Schematic illustration, list of characters and descriptive statistics for morphological traits.

Table S3. Supplementary material to morphometric analysis.

Table S4. Chromosome count details for the *D. maculata* agg.

Table S5. Supplementary material to the analysis of environmental characteristics.

Table S6. Type series of *Dactylorhiza maculata* subsp. *arcana* Trávn., Taraška, Batoušek et Lamla, subsp. nov.

CHAPTER 4:

Distribution of the *Dactylorhiza maculata* agg. in the Czech Republic

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INTRODUCTION

Geographical distribution is an important characteristic of plant species (cf. Chytrý et al. 2021). In the past, there were several attempts to process the distribution data of various plant species in the territory of current Czechia (e.g. Slavík 1971). Grid-based distribution maps of all orchids in eastern part of the Czech Republic (i.e. Moravia and Silesia) were published by Jatiová and Šmiták (1996). It their work, maps for *D. fuchsii* subsp. *fuchsii, D. fuchsii* subsp. *sooana* and *D. maculata* subsp. *transsilvanica* (names as stated by the authors) were included, while maps for other *Dactylorhiza* taxa as well as western part of the country (i.e. Bohemia) were missing. Relatively detailed information on distribution of most *Dactylorhiza* taxa is also available in Flora of the Czech Republic (Kubát 2010). New opportunities for processing and further employment of the distribution data were triggered by the progress in development of information technologies. Distribution data of vascular plants for the Czech Republic were integrated in the Pladias database (Wild et al. 2019), which resulted in number of grid maps published in a series of papers (Kaplan et al. 2015, and further). Following parts of this chapter were adopted from the fifth part of this series (Kaplan et al. 2017), in which maps for genus *Dactylorhiza* were included. The paper had been compiled previous to the taxonomic revision in Chapter 3. This is partly reflected in comments on particular taxa, but the nomenclatoric suggestions given in Chapter 3 could not be taken into consideration in the paper. Avoiding changes in the once published text, the nomenclature in this chapter follows Danihelka et al. (2012), and it thus differs from the rest of the thesis. The names used in this chapter may be substituted as follows:

D. fuchsii var. *fuchsii* → *D. maculata* subsp. *fuchsii*

D. fuchsii var. *psychrophila* → *D. maculata* subsp. *sudetica*

D. fuchsii subsp. *sooana* → *D. maculata* subsp. *sooana*

The taxonomic circumscriptions of mapped taxa are however compatible with the other chapters of this thesis, with the exception of *D. maculata* subsp. *sudetica* (see Addendum at the end of this chapter).

MATERIALS AND METHODS

Taxonomic scope

The following groups of vascular plants are mapped: native taxa, naturalized aliens, most casuals and certain hybrids. Distribution maps are produced for species and subspecies, and in exceptional cases also for varieties or infrageneric taxa (e.g. sections). Plants of species groups that are difficult to assign to species may be mapped as species aggregates. Field crops and plants deliberately cultivated in gardens and parks are not included in the mapping project. Nomenclature, taxonomic concepts and delimitation of species aggregates mostly follow Danihelka et al. (2012), with differences indicated where necessary. For taxa not included in that checklist, a taxonomic reference is given. Publication of maps does not follow any alphabetical or systematic order but mainly the maps that resulted from recent revisions are printed.

Data sources

All relevant floristic data sources are used. Major national herbaria and some local and foreign collections, incl. BRNL, BRNM, BRNU, CB, CELM, CESK, CHEB, CHOM, GM, HOMP, HR, KMKV, LIM, MJ, MMI, MP, MZ, NJM, OL, OLM, OP, OSM, PL, PR, PRA, PRC, ROZ, VM, W, WU and ZMT (acronyms follow Thiers 2017), were consulted as the main source of taxonomically revised records. Most records for maps of common and easy-to-identify taxa came from the recently developed Pladias database (hosted at the Institute of Botany, Průhonice), which has integrated all the available records on the distribution of vascular plants in the Czech Republic. Among the most important incorporated databases are: the Database of the Distribution of Vascular Plants in the Czech Republic (FLDOK), the Czech National Phytosociological Database (CNPD), plant records from the Floristic Summer Schools and other activities of the Czech Botanical Society, the Species Occurrence Database of the Nature Conservation Agency of the Czech Republic (NDOP), the Database of Forest Typology of the Forest Management Institute of the Czech Republic (DLT) and the Floristic Database of the South Bohemian Branch of the Czech Botanical Society (JCP CBS). Unpublished field records previously entered into the Pladias database by the authors of maps or regional contributors were also considered.

Mapping procedure

All records used for mapping are entered into the Pladias database and geographically sorted according to the traditionally used CEBA (Central European Basic Area) grid template (Niklfeld 1999) divided into quadrants of 5 × 3 arc minutes (corresponding to approximately 5.5 × 5.9 km). The territory of the Czech Republic is covered by 2551 quadrants, of which 2181 are completely within the borders of this country. Individual records and the whole distribution of each taxon are checked and evaluated by the author of a particular map in a web-based mapping interface of the Pladias database. Maps of taxonomically critical groups are based solely or mainly on herbarium records revised by taxonomic experts; these cases are indicated in the text accompanying the particular map. Maps of all other taxa are based on records from databases, literature and herbaria, which were scrutinized by the authors of the respective maps. Records used for producing maps are listed in Electronic Appendices 1–6. Draft distribution maps and the background records are released in a web-based review process for scrutiny by field botanists, regional collaborators and members of the Czech Botanical Society. Their comments and additional records are collected in the database and returned to the responsible specialists for consideration before producing the distribution maps.

Final maps and comments

The treatment of each taxon consists of a grid distribution map and accompanying text; authors of the maps are indicated in the figure captions, who also had a major role in preparing the first drafts of the respective texts. Maps are displayed using a spherical

Mercator projection (EPSG:3857) in which meridians and parallels appear as straight lines, and the fields of the mapping grid are thus displayed as squares. The background relief was derived from SRTM data (http://www2.jpl.nasa.gov/srtm/, the version provided by http://srtm.csi.cgiar.org) and the river network was adapted from data provided by CENIA (www.cenia.cz). In the caption to each map, counts of occupied quadrants are indicated according to the symbols used in the map; uncertain occurrences are not included in the counts. The accompanying text includes the accepted scientific name, a brief outline of the total distribution, information on habitats occupied by the species and a description of its distribution in the Czech Republic. Where appropriate, comments on the taxonomy, biology and details of the spatial and temporal dynamics of the distribution are given.

DISTRIBUTION MAPS AND COMMENTS

Dactylorhiza maculata agg. (Fig. 1)

Dactylorhiza fuchsii subsp. *fuchsii* var. *fuchsii* (Fig. 2), *D. fuchsii* subsp. *fuchsii* var. *psychrophila* (Fig. 3), *D. fuchsii* subsp. *sooana* (Fig. 4), *D. maculata* subsp. *maculata* (Fig. 5) and *D. maculata* subsp. *transsilvanica* (Fig. 6)

Dactylorhiza maculata agg. is a taxonomically critical complex of diploid and polyploid taxa. It is widely distributed from the Atlantic regions in Europe to Central Asia and from the northern coasts of Africa to northernmost Scandinavia and the Kola Peninsula (Delforge 2006; Eccarius 2016). Two species are usually recognized in the Czech Republic: *D. fuchsii* and *D. maculata* s. str. They were suggested to differ in their morphology, as well as ploidy level, since the former was considered to be diploid while the latter tetraploid. However, a number of studies indicate that the morphology is not always associated with the ploidy level, as the plants morphologically corresponding to *D. fuchsii* are often tetraploid, especially in central Europe (Jagiełło and Lankosz-Mróz 1988; Ståhlberg and Hedrén 2010). The same ploidy level very probably allows gene-flow between the two taxa. As a result, many tetraploid populations are probably of more complex origin and vary morphologically between *D. fuchsii* and *D. maculata* s. str. Moreover, a large number of taxa have been described within the *D. maculata* agg., of which some cannot be clearly assigned either to *D. maculata* s. str. or *D. fuchsii*. Because of these taxonomic ambiguities and until a comprehensive taxonomic revision is done, we maintain the concept of the two species traditionally used in Czech literature (Kubát 2010; Danihelka et al. 2012), although the whole complex may be better treated as a single species with several infraspecific taxa. Because of frequent misidentifications, all maps of both species and their infraspecific taxa are based solely on revised herbarium specimens. An additional map of *D. maculata* agg. was prepared based on both herbarium and non-herbarium records. Herbarium specimens that could not be reliably classified to subspecies or variety level were also included in this map. Nevertheless, most of these records probably belong to *D. fuchsii* var. *fuchsii*, which is by far more widespread in the Czech Republic than the other taxa in this complex. The entire complex occurs from the lowlands to the subalpine vegetation belt, but mainly in the mountains.

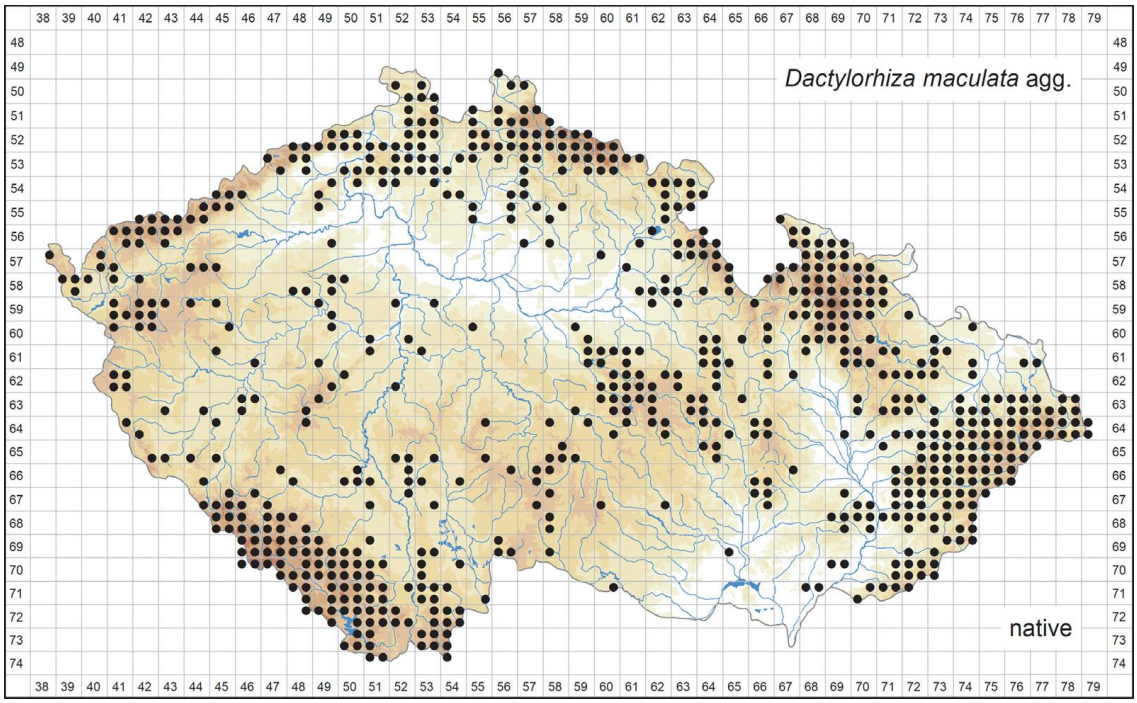

Figure 1. Distribution of *Dactylorhiza maculata* agg. in the Czech Republic (705 occupied quadrants).

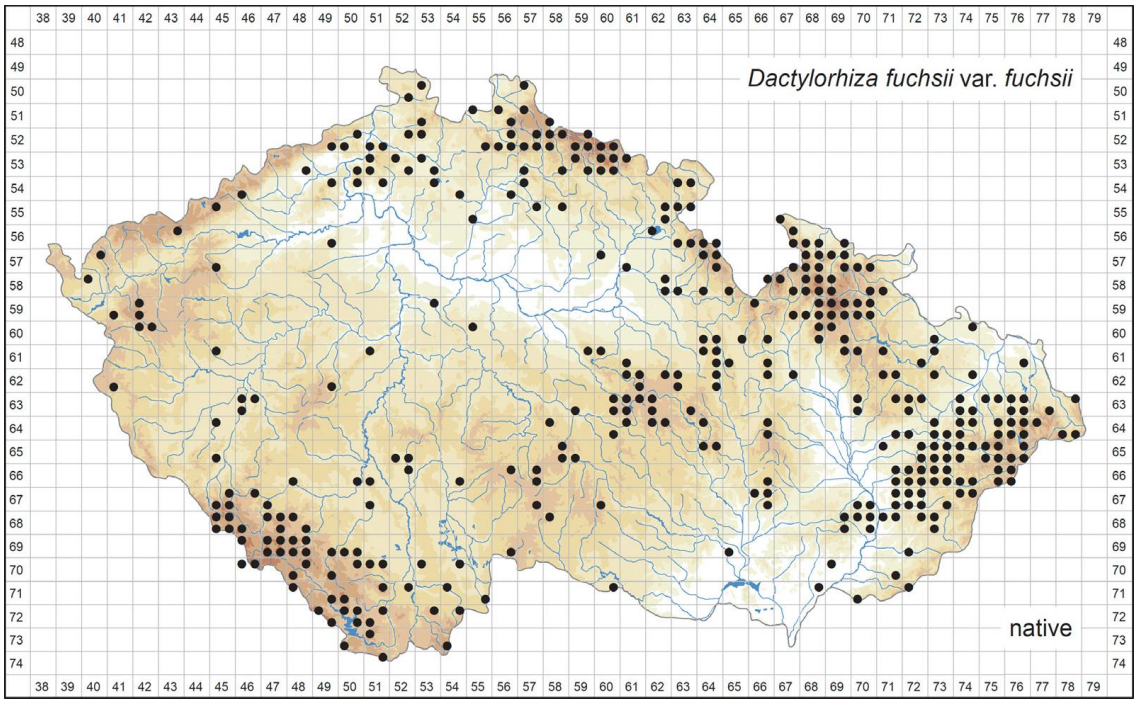

Figure 2. Distribution of *Dactylorhiza fuchsii* (subsp. *fuchsii*) var. *fuchsii* in the Czech Republic (374 occupied quadrants).

Dactylorhiza fuchsii var. *fuchsii* is widely distributed across temperate and boreal zones in Europe and Asia. It occurs in most of Europe, being absent from northern Scandinavia and the warm southern parts of Europe, only reaching the northern part of the Iberian

Peninsula. The southern border of its range in the Balkan Peninsula is not clear because of the confusion with *D. saccifera*. In Asia it occurs in the Caucasus Mts, Central Asia and southern Siberia eastwards as far as Lake Baikal (Delforge 2006; Eccarius 2016). Overall, its distribution is poorly known because of confusions with other taxa of the *D. maculata* agg. It inhabits a wide range of natural and semi-natural habitats, preferably on wet soils. It grows in forests, forest edges, fringes of mountain brooks, both forest and non-forest springs, marshes, peat bogs, moss-rich fens, wet to mesophilous meadows and pastures, road ditches etc. It grows mainly on alkaline to slightly acidic soils. In the Czech Republic it occurs from the colline to supramontane belt, more frequently in the mountains up to 1,250 m a.s.l. Since the map of *D. fuchsii* var. *fuchsii* is based solely on revised herbarium specimens, the taxon is probably more widespread than indicated by the map. A lot of the specimens revised as *D. maculata* agg. are likely to be just atypical individuals of *D. fuchsii* var. *fuchsii*; these records are not included in the map for this variety. Also, most of the earlier non-herbarium records of "*D. maculata*" probably refer to *D. fuchsii* (var. *fuchsii*), because the two species were not distinguished until the second half of the 20th century. Although *D. fuchsii* var. *fuchsii* is still the second most common *Dactylorhiza* (after *D. majalis* subsp. *majalis*) in the Czech Republic, the number of its localities has recently declined (Jatiová and Šmiták 1996) and this variety is thus classified as of lower risk – near threatened (Grulich 2012).

The total distribution of *D. fuchsii* var. *psychrophila* is impossible to assess because of its taxonomic ambiguity. In the Czech Republic this name is traditionally used for populations known from the subalpine vegetation belt in the Krkonoše Mts and one locality in the Hrubý Jeseník Mts. In addition, this variety was reported to occur in the Krušné hory and Orlické hory Mts and Mt Králický Sněžník. However, our field experience indicates that the populations in the Krušné hory and Orlické hory Mts are not the same as those in the Krkonoše and Hrubý Jeseník Mts. We have not seen any herbarium specimens resembling *D. fuchsii* var. *psychrophila* from Mt Králický Sněžník. The type of *D. psychrophila* is from northern Finland (Vermeulen 1947) and represents a taxon that is widely distributed in northern Scandinavia. However, the Czech populations probably differ in their morphology, as well as ploidy level: *D. psychrophila* is reported to be diploid and closely related to *D. fuchsii* var. *fuchsii* (Averyanov 1982, Eccarius 2016), while the Czech plants are tetraploid (Krahulcová 2003; Taraška, Batoušek and Trávníček unpubl.) and their taxonomic position is uncertain, as they are often assigned to *D. maculata* s. str. (e.g. Eccarius 2016; Jagiełło 1988). According to Devillers and Devillers-Terschuren (2000), the populations in the Sudetes Mts might be an independent, local evolutionary unit. In this case their correct name should be based on the basionym *Orchis maculata* var. *sudetica* Rchb. f., described from the Krkonoše Mts. According to some authors, these populations should be considered to be only an ecomorphosis of *D. fuchsii* var. *fuchsii*, adapted to the extreme environmental conditions in the subalpine belt (Potůček 1969). Since this taxonomic riddle remains unresolved, here we consider these populations to be a unique evolutionary lineage, for which we provisionally use the name *D. fuchsii* var. *psychrophila*, following the current Czech plant checklist (Danihelka et al. 2012). In the Krkonoše Mts this variety is abundant in subalpine springs, marshes and cirque vegetation, where it reaches an elevation of 1,350 m. However, it also occurs in wet meadows at lower elevations, particularly close to mountain huts. In the Hrubý Jeseník Mts it was recently confirmed only in cliff vegetation in the Velká kotlina cirque

but in the past it also occurred in the Malá kotlina cirque. Although its populations are abundant and not directly threatened, it is classified as endangered because of its overall rarity (Grulich 2012).

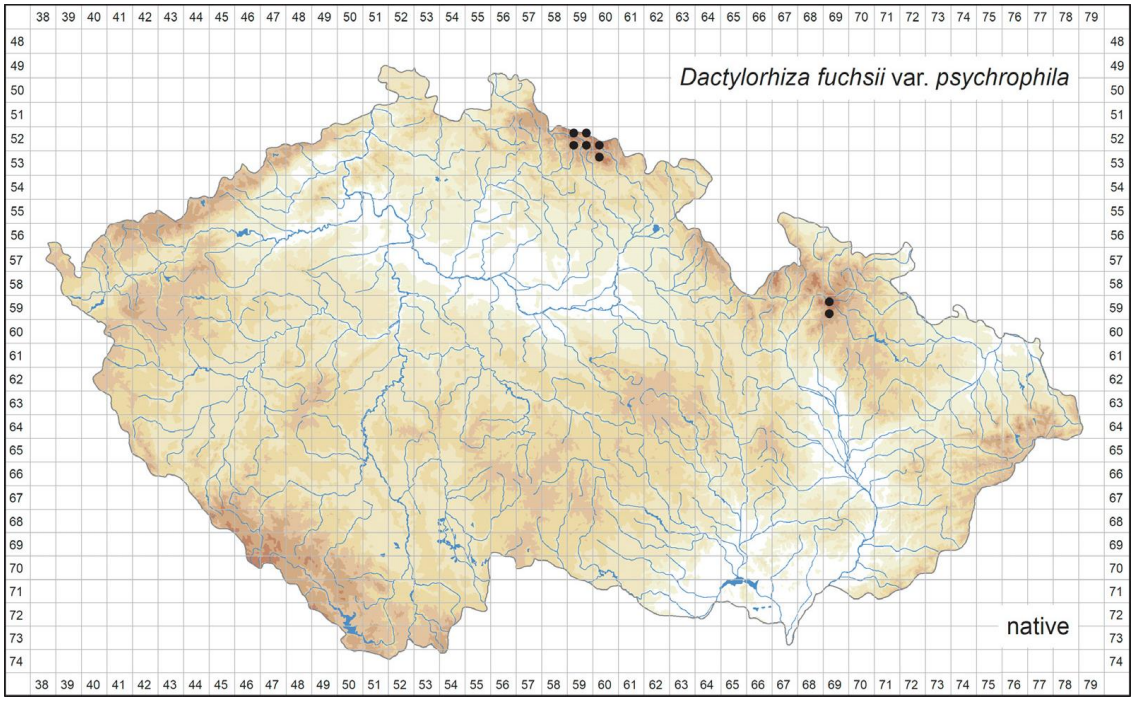

Figure 3. Distribution of *Dactylorhiza fuchsii* (subsp. *fuchsii*) var. *psychrophila* in the Czech Republic (8 occupied quadrants).

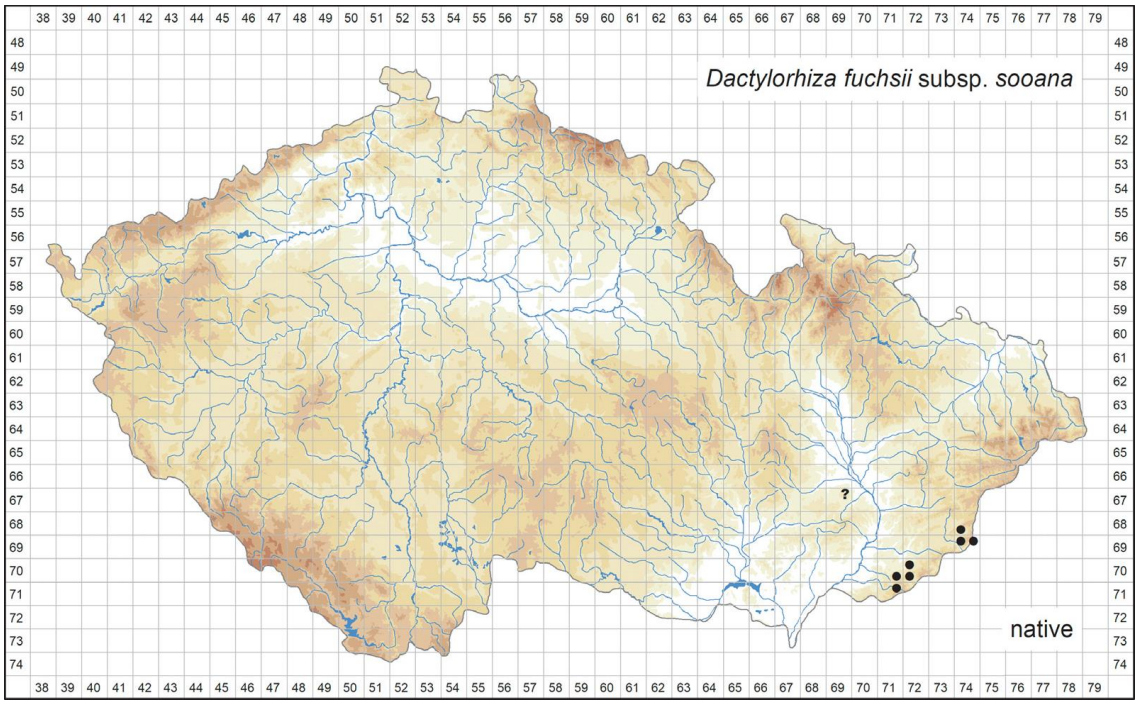

Figure 4. Distribution of *Dactylorhiza fuchsii* subsp. *sooana* in the Czech Republic (7 occupied quadrants).

Dactylorhiza fuchsii subsp. *sooana* occurs in Hungary, Slovakia and the Czech Republic (Batoušek 1995), where it was recently recorded at several localities in the Bílé Karpaty Mts. In addition, there is an old herbarium specimen from the eastern part of Litenčické vrchy hills, which might also belong to this taxon. This subspecies grows in wet and mesophilous meadows, spring fens and edges of forests in the supracolline belt, usually on fresh, slightly acidic to slightly alkaline soils. This subspecies is classified as critically threatened (Grulich 2012).

The total distribution of *D. maculata* subsp. *maculata* is difficult to estimate because of confusion with *D. fuchsii* var. *fuchsii*, which was not reliably distinguished until recently and has never been accepted as a separate species by many authors (e.g. Buttler 2000; Ståhlberg & Hedrén 2010). *Dactylorhiza maculata* subsp. *maculata* is considered to occur in temperate and boreal zones in Eurasia. It is widespread in Atlantic and northern Europe, including Scandinavia and the Baltic countries. In central and eastern Europe it is rather scattered. Its range extends as far as central Siberia (Delforge 2006; Eccarius 2016). It occurs mainly in fens and mires, peat bogs and wet meadows, mainly on acidic to neutral soils with a permanent water supply. In the Czech Republic this subspecies is known from the Jestřebské slatiny fens near the town of Doksy and from the Krušné hory Mts in northern Bohemia. It is classified as critically threatened (Grulich 2012).

Dactylorhiza maculata subsp. *transsilvanica* is recorded mainly in the mountains in central and south-eastern Europe: the Carpathians and adjacent areas (Czech Republic, Slovakia, north-eastern Hungary, Romania), north of the Balkan Peninsula (Bosnia and Herzegovina, Bulgaria) and the Eastern Alps (Slovenia) (Eccarius 2016). Nevertheless, its taxonomy has not yet been resolved and the name may be used for various evolutionary lineages in different countries. *Dactylorhiza maculata* subsp. *transsilvanica* grows in spring fens, wet to mesophilous meadows, mountain meadows and pastures. In the Czech Republic it has been recorded in the Bílé Karpaty Mts, Hostýnské vrchy hills, Javorníky Mts and the Moravskoslezské Beskydy Mts. In addition, there is a single collection from the Dúbrava forest near the town of Bzenec, which probably belongs to this subspecies. In the Moravskoslezské Beskydy Mts it was believed to form mixed populations with *D. fuchsii* var. *fuchsii* and their hybrids, with the main discriminating traits being the colour of their flowers and the occurrence of the spots on the leaves (Batoušek 2010; Vlačiha 2013). However, our research indicates that these populations do not comprise two distinct species (all plants are uniform in terms of quantitative traits and ploidy level) and they should be considered to be two forms of a single taxon. Thus, *D. maculata* subsp. *transsilvanica* in our concept includes both of these colour forms, as well as transitional individuals. Populations in the Bílé Karpaty Mts are rather uniform in terms of flower and leaf pigmentation. The occurrence of this subspecies in the Javorníky Mts has not been recently confirmed. The relationships between populations from various parts of this subspecies' range and their relationships to the type population from Transylvania need further investigation. In the Czech Republic this subspecies is classified as critically threatened (Grulich 2012).

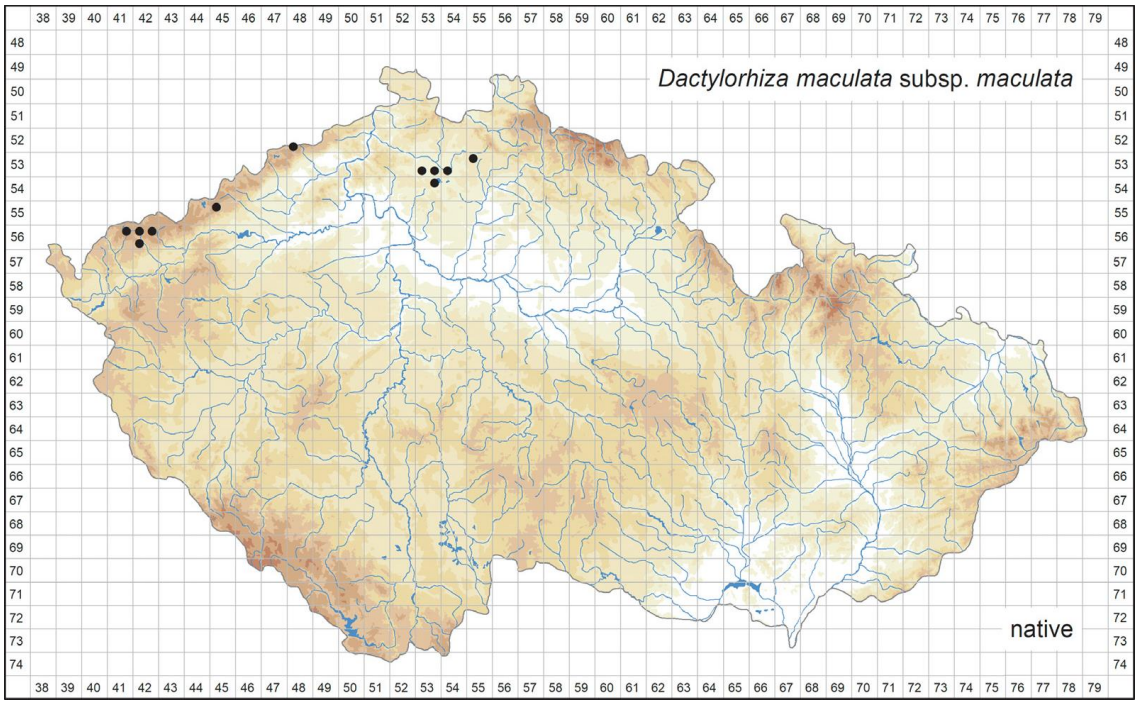

Figure 5. Distribution of *Dactylorhiza maculata* subsp. *maculata* in the Czech Republic (11 occupied quadrants).

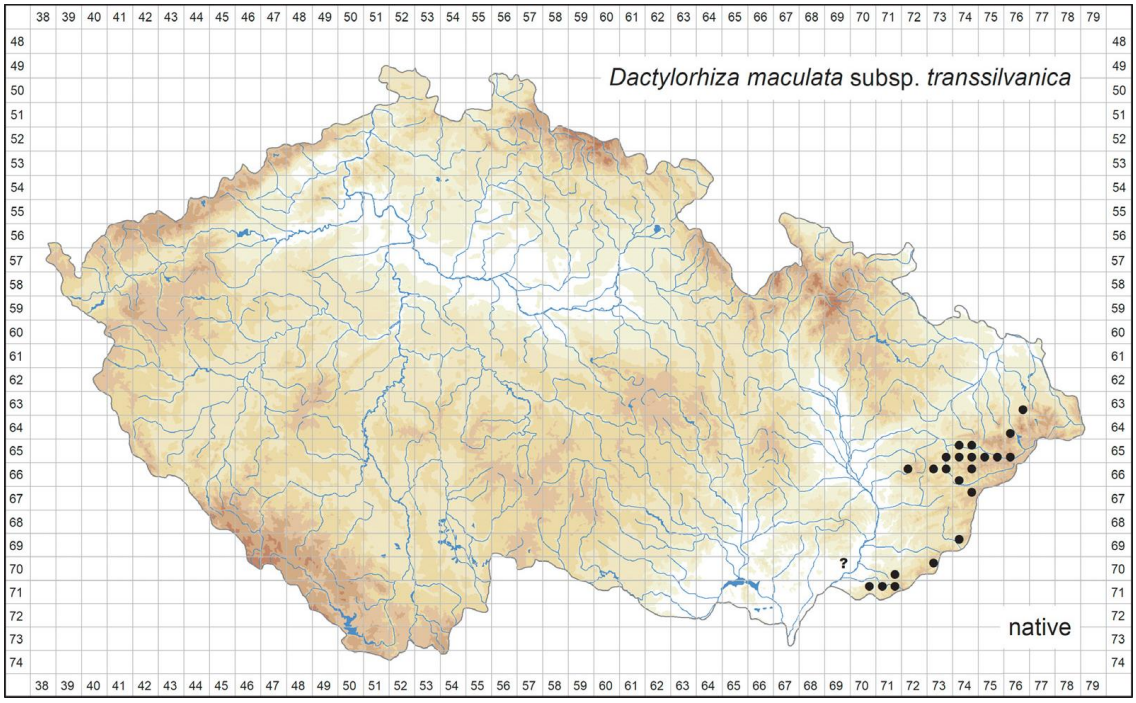

Figure 6. Distribution of *Dactylorhiza maculata* subsp. *transsilvanica* in the Czech Republic (22 occupied quadrants).

ADDENDUM

Two emendations are needed in the light of new findings since the original paper was published.

(1) *D. maculata* subsp. *sudetica* (here referred to as *D. fuchsii* var. *psychrophila*) should be regarded as missing from the quadrants 5969a and 5969c. The records from the Jeseníky Mts are based on plants morphologically similar (convergent) to 'true' subsp. *sudetica*, which is now regarded as an endemic to the Krkonoše Mts.

(2) A map of *D. maculata* subsp. *averyanovii* is missing, because this taxon was out of the taxonomic scope of the paper (cf. Danihelka et al. 2012). This subspecies is known to occupy only the quadrant 5769d in the Czech Republic.

SUPPLEMENTARY FILES

Supplementary files are available on the attached CD-ROM and online from the provided links. They may also be searched out in https://www.preslia.cz/map.

Electronic Appendix 1. Distribution of *Dactylorhiza maculata* agg. in the Czech Republic. https://www.preslia.cz/map/pdf?id=323

Electronic Appendix 2. Distribution of *Dactylorhiza fuchsii* var. *fuchsii* in the Czech Republic. https://www.preslia.cz/map/pdf?id=320

Electronic Appendix 3. Distribution of *Dactylorhiza fuchsii* var. *psychrophila* in the Czech Republic. https://www.preslia.cz/map/pdf?id=321

Electronic Appendix 4. Distribution of *Dactylorhiza fuchsii* subsp. *sooana* in the Czech Republic. https://www.preslia.cz/map/pdf?id=319

Electronic Appendix 5. Distribution of *Dactylorhiza maculata* subsp. *maculata* in the Czech Republic. https://www.preslia.cz/map/pdf?id=324

Electronic Appendix 6. Distribution of *Dactylorhiza maculata* subsp. *transsilvanica* in the Czech Republic. https://www.preslia.cz/map/pdf?id=325

CHAPTER 5:
Summary and conclusions

Vojtěch Taraška

Similarly to many other orchids, members of the *D. maculata* agg. are considered as threatened in Central European countries. However, insufficient knowledge on their overall variability and taxonomic diversity is an obstacle in their effective protection. Morphological and ploidy level variability, cytogeography, phytosociology and environmental traits in Central European populations of the *D. maculata* agg. were investigated in this thesis. The aim was to eliminate ambiguities resulting from different taxonomic approaches in particular countries, and to provide a revised taxonomic concept as a tool for nature conservation authorities, field botanists as well as researchers focused on biology and ecology of this group.

Special attention was paid to *D. maculata* subsp. *fuchsii*, which was traditionally considered a diploid member of the group (Heslop-Harrison 1951; Averyanov 1990; Kubát 2010), despite an increasing number of karyological investigations suggesting that its Central European populations may be tetraploid (e.g. Vermeulen 1968; Vöth & Greilhuber 1980; Jagiełło 1988; Měsíček and Javůrková-Jarolímová 1992). It was demonstrated in this thesis that both diploids and tetraploids occur in Central Europe, either in pure-ploidy or mixed-ploidy populations. Moreover, DNA-triploids have been found to co-occur with the other cytotypes. Particular cytotypes lack any clear morphological distinctions, although tetraploids exhibit larger variability, probably as a result of gene introgression from other tetraploid taxa. A significant cytogeographical pattern was found in subsp. *fuchsii*: all cytotypes seem to be common in the Alps, West Carpathians and probably also northern Dinarides, while populations in the Bohemian Massif are almost exclusively tetraploid. Variation in ploidy levels was also revealed in some other taxa, namely *D. maculata* subsp. *arcana* and subsp. *transsilvanica*, which are typically tetraploid, but DNA-hexaploid individuals were sporadically detected. These are undoubtedly a result of recent polyploidization with participation of unreduced gametes. This may also be the case of some DNA-triploids found in diploid populations of subsp. *fuchsii*, but hybridization between diploid and tetraploid plants is more likely to occur in areas of their sympatry.

As indicated above, the polyploid system of the *D. maculata* agg. is obviously more complicated than previously assumed, which has serious evolutionary consequences with implications for taxonomy. Two widely distributed taxa, *D. *maculata* and *D. *fuchsii*, were traditionally recognized as distinct species best delimited by their ploidy levels, the first considered to be tetraploid and the latter diploid (e.g. Heslop-Harrison 1951). In Central Europe, both these taxa may be tetraploid, which facilitates the admixture of their genomes. This may also explain the observed pattern in morphological variability. Eight peculiar morphotypes may be distinguished based primarily on the leaf and lip shape, flower colouration, and leaf pigmentation. However, overall variability of the *D. maculata* agg. is rather continuous at both population and individual levels. The single species concept is thus advocated here, regarding the whole aggregate as *D. maculata* s. lat. (see also Ståhlberg and Hedrén 2010; Naczk et al. 2015).

Taxonomic status of various taxa recognized in Central European literature was reassessed based on integrated morphological, karyological and eco-environmental data, and a total of eight subspecies were recognized within *D. maculata* in the studied area. Two of them, namely *D. maculata* subsp. *arcana* and subsp. *sooana*, were formally described by us as new to science, although they had previously been recognized under incorrect or invalid names. *Dactylorhiza maculata* subsp. *averyanovii*, formerly described from Poland (Jagiełło 1990), was resurrected and found as new to floras of the Czech Republic and Slovakia. More attention was paid to the Czech Republic. Revised specimens from major public herbaria as well as other floristic data integrated in the

Pladias database (Wild et al. 2019) were utilised to build grid maps for particular taxa occurring in this country. Comprehensive knowledge of their past and present distributions also allowed for evaluation of these taxa against IUCN Red List criteria, and their threat status could be successfully assessed at the national level.

Several outputs of this thesis may be mentioned to have further implications for nature conservation. Except for subsp. *fuchsii*, all other subspecies occurring in the Czech Republic should be considered as threatened, and they are likely to deserve some threat status also in other countries. Although information on their distributions and abundances outside this country are usually incomplete, some general conclusions may be provided. For example, subsp. *maculata* appeared to be much rarer than presumed in Central Europe. Three taxa require the status of endemics of relatively narrow areas, namely subsp. *arcana* (foothills of the Tatry Mts), subsp. *sooana* (West Carpathians) and subsp. *sudetica* (Krkonoše Mts). The circumscription of subsp. *transsilvanica* must be broadened so that it also includes plants previously mentioned as other sympatrically growing taxa, which virtually increases the total abundance of subsp. *transsilvanica* in some countries. In contrast, subsp. *elodes* must be eliminated from national floras of all Central European countries except for Germany. Ploidy level variation appeared to be a hidden source of variability, which is particularly true for subsp. *fuchsii*; in this light, rare and inconspicuous diploid populations should be regarded as of much higher conservation importance than other populations in the area of Bohemian Massif. On the other hand, areas where more cytotypes co-occur may be valuable as venues of ongoing evolutionary process in the *D. maculata* agg.

A unified taxonomic concept and determination key to *D. maculata* agg. subspecies in whole Central Europe are introduced here, which have promise to facilitate the future transfer of knowledge across the state borders. Anyway, further taxonomic research is required to elucidate some questions not answered in this thesis. The evolutionary history, phylogenetic relationships and origins of taxa recognized in this work must be scrutinised by advanced methods in plant systematics. Evidence from morphometrics and genome size analyses suggest that some taxa may have originated via merging of distinct evolutionary lineages, which would definitely challenge the traditional taxonomic concept of two species. On the other hand, the rank of subspecies may be lowered for some taxa if their distinctions are not confirmed. The geographic limits of tetraploid subsp. *fuchsii* are unknown. It is not even excluded that this cytotype occurs outside Central Europe, including Fennoscandia where previous flow cytometric measurements (Ståhlberg and Hedrén 2008; Ståhlberg 2009) may have been biassed due to endoreplication (cf. Trávníček et al. 2015). There is no doubt that *D. maculata* agg. still remains a challenging group. Hopefully, this thesis will be beneficial at least for the conservation of some endangered and so far overlooked taxa, so that the never-ending disputations on their taxonomic status may be held even by further generations of botanists.

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Biosystematic and chorological study of *Dactylorhiza maculata* agg. in Central Europe

Summary of the PhD thesis

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The oral defence will take place on 11 June 2024 in front of the Commission for PhD thesis of the study programme Botany at the Department of Botany, Šlechtitelů 27, Olomouc, auditorium 51.105.

The PhD thesis will be deposited in the Biology Branch Library of the Faculty of Science at Palacký University, Šlechtitelů 27, Olomouc.

Table of content

1. Introduction

Many European species of the orchid family underwent a serious decline during the 20th century (Štípková and Kindlmann 2021). Orchids are known to enter complex interactions with other organisms (Fay and Chase 2009), which makes them popular umbrella and flagship species in nature conservation. However, effective protection may be complicated in some extremely variable groups with unresolved taxonomy due to uncertainties in delimitation of particular taxa.

This is also the case of the *Dactylorhiza maculata* agg. comprising several diploid and autotetraploid evolutionary lineages, whose taxonomic treatment is still not consensual. Two species are traditionally recognized in Central Europe, namely *D. fuchsii* (Druce) Soó and *D. maculata* (L.) Soó (e.g. Kubát 2010; Eccarius 2016). They have been supposed to differ from each other in morphology, ecology, and ploidy levels, as the first was considered to be diploid, while the latter tetraploid (Heslop-Harrison 1951). Distinctions between these taxa were also confirmed in recent phylogenetic studies (Ståhlberg and Hedrén 2010; Brandrud et al. 2020). While they seem to be well distinguishable in North and West Europe (e.g. Tyteca and Gathoye 2003; Bateman and Denholm 2003), their delimitation based on morphology is tricky in Central Europe (Procházka 1979; Kubát 2010). Moreover, tetraploid plants of *D. fuchsii* have been reported from that area (Vermeulen 1968; Vöth 1978; Jagiełło 1988), where reciprocal gene flow between both abovementioned taxa may occur via homoploid hybridization (Ståhlberg and Hedrén 2010; Naczk et al. 2015). The frequency of occurrence and geographic distribution of both *D. fuchsii* cytotypes are still poorly known. In addition, several enigmatic taxa of the *D. maculata* agg. are mentioned from Central Europe, which further complicates understanding of the variability of the group.

As a result of these ambiguities, very different taxonomic concepts and nomenclatoric solutions are applied in various Central European countries. Their unification is thus needed, which must be based on analysis of its variability in this whole area. This is also essential for assessment of threat statuses of individual taxa, as well as taking measures to their protection.

2. Aims of the thesis

The main aim of this thesis was to reveal the variability and taxonomic diversity of the *D. maculata* agg. in Central Europe. This could be accomplished by resolving following questions:

1) What is the ploidy level variation of populations recognized as *D. fuchsii*? Is there some geographic pattern in distributions of its cytotypes? And is the ploidy level connected with morphological variability?

2) What is the overall morphological and ploidy level variation within the *D. maculata* agg.? Which taxa may be delimited within the group based on their morphology, ploidy level variation, and ecological characteristics in Central Europe?

3) Which taxa occur in the Czech Republic? Where are they distributed in this country? And what is their threat status?

3. Material and methods

Plant material

Plant material and data were collected primarily in Central European countries, including Austria, Czechia, Hungary, Germany, Poland, and Slovakia. Populations from Bulgaria, the Netherlands, Romania, and Slovenia were also included, if it was necessary for taxonomic assessment of the Central European populations. Each population was preliminarily assigned to either of nine taxonomic groups delimited based on previous studies and literature, or it was marked as unclassifiable ('aggregate'). Most data were collected directly in the field to minimize the damage of the plants. Only one flower per individual was picked for morphometrics, and several ovaries or flower buds were needed for determination of ploidy level in laboratory.

Ploidy level determination and genome size

Genome size was analysed by flow cytometry (FCM) following the protocol by Doležel et al. (2007). Either 4,6-diamidino-2-phenylindole (DAPI, 4 μg/ml) or propidium iodide (PI, 50 μg/ml) were used as fluorescent dyes. Internal standardization was ensured with *Pisum sativum* cv. Ctirad (2C = 9.09 pg) or, in DNA-triploid samples, *Zea mays* cv. CE-777 (2C = 5.43 pg; Temsch et al. 2021). Fresh ovaries of *Dactylorhiza* were used as sample tissue in order to avoid results biased by endoreplication (Trávníček et al. 2015). The analyses were conducted in following instruments: 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. For most accessions, only relative genome size was estimated, expressed as the ratio of the mean position of G_0/G_1 peak of *Dactylorhiza* and the mean position of the G_0/G_1 peak of the internal standard. Absolute genome size [pg] was stated for two diploid and three tetraploid individuals.

In order to calibrate the relative genome sizes with chromosome numbers, metaphase chromosome plates were prepared for 10 individuals previously analysed by FCM, comprising both diploids and tetraploids. Flower buds were fixed in an ethanol : acetic acid (3 : 1) solution and stored at −20°C until used. Haplophasic chromosome numbers were counted from immature pollinaria following the protocol by Weiss et al. (2003). Flower buds were hydrolysed in 5 N HCl for 30 min and stained with Schiff's reagent (Sigma, Vienna, Austria) for 1–2 hours. Pollinaria were extracted and squashed in 60% acetic acid. Chromosome spreads were observed under 1,000× magnification.

Morphometric analysis

Morphological characters were measured on living plants, with exception of several traits assessed from digitized image of flattened lip. Both floral and vegetative traits were examined, of which many have been used in previous studies by other authors (e.g. Heslop-Harrison 1951; Bateman and Denholm 1988; Jagiełło1988; Tyteca and Gathoye 2003). These traits included numerical, ordinal and categorical variables, but also several variables calculated from two or more measured traits.

Morphological variability was assessed using univariate and multivariate statistics, revealing patterns at the levels of individuals, populations, taxonomic groups, or distinct cytotypes of the same group. Agglomerative hierarchical clustering (Ward's and UPGMA methods), PCA and PCoA were employed to identify main clusters (or morphotypes) and uncover their positions within the *D. maculata* agg. given by their morphological dis/similarities. PLS discriminant analysis was used to examine the differentiation between taxonomic groups, and to identify traits contributing to their distinguishing. Kruskal-Wallis and chi-square tests were also used to test these differences. Separate analyses were carried out for plants assigned to *D. fuchsii* to find distinctions between its diploid and tetraploid populations, which were also analysed using ANOVA and GLMM. Basic descriptive statistics for taxonomic groups and both analysed cytotypes of *D. fuchsii* are provided. Analyses were performed using R 4.0.4 (R Core Team 2021), Canoco 5.12 (ter Braak and Šmilauer 2012), XLSTAT (Addinsoft 2022), and NCSS 9 (NCSS 2013) softwares.

Ecological and environmental traits

Bioclimatic variables with spatial resolution of ca 1 km from WorldClim 2.1 (Fick and Hijmans 2017) and soil variables with spatial resolution of 250 m from SoilGrid 2.0 (Hengl et al. 2017) were gathered for a set of populations. Discriminant analysis (DA) was performed using Canoco 5.12 to test environmental differentiation among taxonomic groups. Vegetation inhabited by these populations was classified following the Hierarchical floristic classification system of European vegetation (Mucina et al. 2016) into the level of phytosociological order.

Distribution maps and threat statuses

Distribution maps were prepared only for the territory of the Czech Republic and for taxa included in the national checklist of vascular plants of this country (Danihelka et al. 2012). Facilities of the Pladias database (Wild et al. 2019) were employed, which integrates floristic data from various sources. Specimens of genus *Dactylorhiza* were revised in all major and several regional public herbaria in the Czech Republic, label data were digitized and uploaded into the Pladias database. Maps were created on CEBA (Central European Basic Area) grid template (Niklfeld 1999). Exclusively the revised herbarium material was accepted in maps of five species and subspecies, while an additional map of *D. maculata* agg. took into consideration also the other floristic records (literary, database). Once the distribution data were available, all taxa occurring in the Czech Republic could also be evaluated against the IUCN Red List criteria (IUCN 2012), and current threat status at the national level was assessed for all of them.

4. Survey of results

Three cytotypes were found in populations traditionally recognized as *D. fuchsii*, namely diploids ($2n = 2x = 40$), DNA-triploids ($2n \sim 3x$), and tetraploids ($2n = 4x = 80$). Only di- and tetraploids formed pure ploidy populations, while DNA-triploids always co-occurred with one or both of the other cytotypes. Major cytotypes were not distributed evenly (Fig. 1). Diploids were almost completely absent from the Bohemian Massif. They were only found in two nearby populations in this area, which is predominantly occupied by tetraploids. In contrast, no tetraploid individuals of *D. fuchsii* were found east and south-eastwards from the Tatry Mts. All three cytotypes occurred in the Alps, West Carpathians and northern Dinarides.

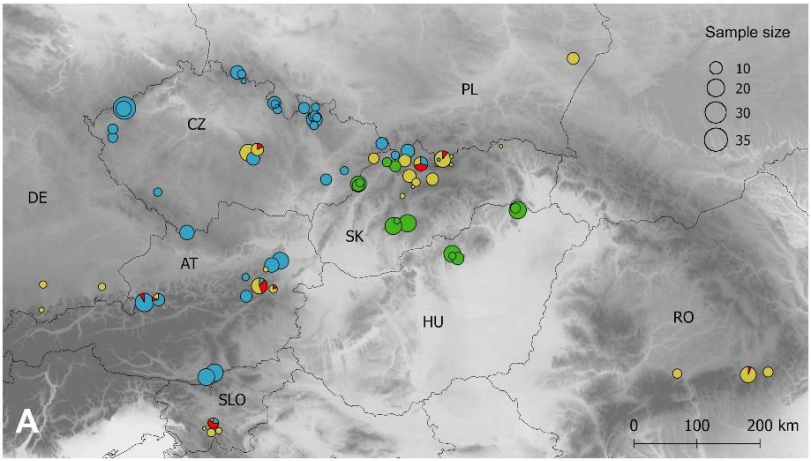

Figure 1. Cytotype distributions of *D. maculata* subsp. *fuchsii* (yellow – diploids; red – DNA-triploids; blue – tetraploids) and *D. maculata* subsp. *sooana* (green, all diploids). Symbol size is proportional to sample size.

A distinctive group of strictly diploid populations could be delimited, having white background colour of the lip, including anther caps, but heavily spotted leaves, which is an unusual combination in other diploids. They also grew in habitats with higher soil pH and solar radiation, and they occurred in a

specific area of West Carpathians where 'typical' *D. fuchsii* is probably absent. This allowed for taxonomic evaluation (including formal description) of these plants which had previously been either omitted or rejected in taxonomic literature, or they had been recognized under an invalid name *D. fuchsii* subsp. *sooana*, nom. inval. introduced by Borsos (1959). The rest of populations traditionally recognized as *D. fuchsii* (subsp. *fuchsii*) represented highly variable but coherent group. Different cytotypes were not distinguishable in morphology, but tetraploids exhibited larger extent of variability compared to diploids.

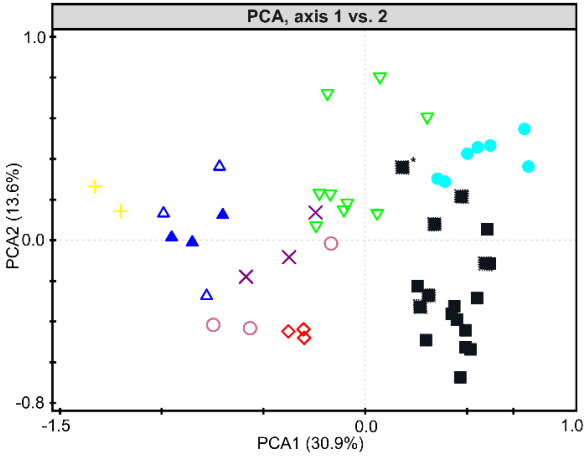

Figure 2. PCA of morphological traits with *D. maculata* agg. populations. Legend to subspecies: yellow cross – *elodes*; blue triangles – *averyanovii*; violet cross – *maculata*; violet circles – *arcana*; red diamonds – *sudetica*; green triangles – *transsilvanica*; blue circles – *sooana*; black squares – *fuchsii*.

Analysis of morphological traits in the whole *D. maculata* agg. revealed rather continuous variability at both population and individual levels. The main gradients were found in the shape of leaves, shape of lip, and the flower colouration. Agglomerative hierarchical clustering using the Ward's method best fitted to previously revealed phylogeny of the group (Brandrud et al. 2020), as it divided the whole dataset into two main clusters. The first comprised *D. *sooana* (the asterisk denotes taxa regardless of their taxonomic rank) and both major cytotypes of *D. *fuchsii*, while the latter contained all other taxa, incl. *D. *maculata* (s. str.). Such a clear division was however not apparent in clustering by UPGMA, nor in the PCA (Fig. 2) and PCoA diagrams. Several taxa recognized from Central Europe in literature could be delineated as peculiar morphotypes, though being interconnected by

transitional populations or individuals. The only exceptions were plants mentioned as *D.* **ericetorum* from Slovakia and *D.* **elodes* from Czechia, which proved to be identical and had to be merged. PLS discriminant analysis showed that *D.* **maculata* (i.e., *D. maculata* s. str.) represents the worse differentiated morphotype, being more or less average in morphological traits.

Diploids and tetraploids were confirmed as major cytotypes in the *D. maculata* agg. Diploids were concentrated within *D.* **sooana* and part of *D.* **fuchsii*, while tetraploids covered almost all morphotypes. DNA-triploids were only found in *D.* **fuchsii*. In addition, two DNA-hexaploid ($2n \sim 6x$) individuals were found within tetraploid populations of various groups. Chromosome counts were obtained for three diploid ($n = 20$) and seven tetraploid ($n = 40$) individuals representing five taxonomic groups. The absolute genome size of diploids was estimated to be 2C = 6.55 and 6.64 pg, while the absolute genome size of tetraploids ranged from 2C = 11.89 to 12.22 pg.

Environmental traits partly contributed to delimitation of some taxonomic groups. Those with larger areas were able to grow in more diverse environmental conditions and occupy a wider range of habitats. In contrast, rare morphotypes were usually confined to specific habitats, possessing a narrow ecological amplitude. However, possible causation between ecology and morphology, as well as the role of epigenetics (Paun et al. 2010) were not scrutinized.

A total of eight groups were successfully delineated based on morphology, ploidy level and ecology, and they could be thus taxonomically classified. Six taxa recognized in this work were also found to occur in the Czech Republic. Out of them, only populations corresponding to *D.* **fuchsii* are distributed throughout the country, while the other groups are geographically restricted. Therefore, *D.* **fuchsii* is assessed to be near-threatened (NT), while the other taxa meet criteria of endangered (EN) or critically endangered (CR) in national Red List of the Czech Republic.

5. Conclusions

Morphological variability and cytotype diversity of *D. maculata* agg. in Central Europe was revealed. The variability in morphological traits appeared to be rather continuous, with serious overlaps between studied taxonomic groups. The main gradient of morphological variability was similar as in many previous studies, but it was not unequivocally related to the ploidy level, as reported from the other parts of Europe (e.g. Bateman and Denholm 2003; Tyteca and Gathoye 2003; Ståhlberg and Hedrén 2010).

Polyploid system of the *D. maculata* agg. appeared to be more complex than previously believed. DNA-triploids within diploid populations, and DNA-hexaploids within tetraploid populations point to recurrent polyploidization. DNA-triploids may also arise from hybridization between di- and tetraploids. Heteroploid hybridization enables gene flow across ploidy levels, while homoploid hybridization breaks the reproductive barriers among particular taxa in the *D. maculata* agg.

Setting aside the extremely rare DNA-hexaploids, the only taxonomic group variable in its ploidy level was that corresponding to *D. *fuchsii*, which comprised both diploid and tetraploid populations, as well as all detected DNA-triploid individuals. Complex distribution pattern of its cytotypes was revealed. Areas predominantly occupied by diploids (Eastern, Southern and Inner Western Carpathians), tetraploids (Bohemian Massif), or sharing all three cytotypes (Alps, Slovenian Dinarides, Outer Western Carpathians) may be demarcated.

Populations of *D. *fuchsii* were also extremely variable in their morphology. This may be related to their considerable genetic variability reported in previous studies (Ståhlberg and Hedrén 2010; Naczk et al. 2015; Brandrud et al. 2020), but also a wide range of habitats occupied by them. Anyway, some tetraploid populations of *D. *fuchsii* possessed features of *D. maculata* s. str., which implies the gene introgression from the other tetraploid taxa. On the other hand, plants recognized as *D. **maculata* represented the least differentiated group in this work, despite a very strict criteria to its delimitation.

The observed patterns in morphological and ploidy level variability did not allow for separation of *D.* **maculata* and *D.* **fuchsii* at the level of species; instead, it supported the single-species concept. However, a total of eight infraspecific taxa may be recognized within *D. maculata* s. lat. in Central Europe using the combination of morphological, karyological and ecological traits. They are here classified as subspecies, and their nomenclature is resolved. As a result, two subspecies had to be described as new to science, namely *D. maculata* subsp. *arcana* Trávn. et al., and *D. maculata* subsp. *sooana* Batoušek et al. In addition, *D. maculata* subsp. *averyanovii* Jagiełło was resurrected. The complete overview of subspecies recognized in Central Europe includes:

D. maculata (L.) Soó

- subsp. *arcana* Trávn., Taraška, Batoušek et Lamla
- subsp. *averyanovii* Jagiełło
- subsp. *elodes* (Griseb.) Soó
- subsp. *fuchsii* (Druce) Hyl.
- subsp. *maculata*
- subsp. *sooana* Borsos ex Batoušek, Taraška et Trávn.
- subsp. *sudetica* (Poech ex Rchb.f.) Vöth
- subsp. *transsilvanica* (Schur) Soó

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8. Souhrn (Summary in Czech)

Biosystematická a chorologická studie *Dactylorhiza maculata* agg. ve střední Evropě

Ochrana vzácných a mizejících druhů středoevropských orchidejí často naráží na nedořešené fylogenetické vztahy a nejasné vymezení jednotlivých taxonů. To je také případ okruhu prstnatce plamatého (*Dactylorhiza maculata* agg.), jenž zahrnuje několik diploidních a autotetraploidních linií. Ve střední Evropě bývají tradičně rozlišovány dva druhy s různým počtem vnitrodruhových taxonů, přičemž stěžejní význam je přikládán právě ploidii: diploidní rostliny jsou označovány jako *D. fuchsii* (Druce) Soó, zatímco tetraploidní jako *D. maculata* (L.) Soó. Oba druhy se dále mají odlišovat též morfologicky a charakterem preferovaných stanovišť. V posledních desetiletích však přibývá studií, které tento koncept problematizují, a to zejména poukázáním na existenci morfologických přechodů, stanovením tetraploidního počtu chromozomů u *D. fuchsii*, nebo prokázáním genového toku mezi oběma domnělými druhy.

Cílem této práce bylo (i) prozkoumat morfologickou a ploidní variabilitu středoevropských populací *D. maculata* agg., (ii) zjistit vzájemné korelace mezi morfologickými a karyologickými znaky i jejich souvislosti s geografií a stanovištními poměry, a (iii) na základě těchto poznatků pak navrhnout taxonomický koncept uplatnitelný ve všech středoevropských zemích. (iv) Pro území České republiky byla dále provedena kritická revize rozšíření jednotlivých taxonů, na základě čehož byly stanoveny kategorie jejich ohrožení.

Morfologická variabilita byla analyzována na úrovni jedinců i populací, jež byly pracovně klasifikovány do devíti skupin odpovídajících jednotlivým taxonům rozlišovaným v současné literatuře. Ani v jednom případě nebylo možné vymezit ostré linie mezi těmito skupinami. Zatímco variabilita na úrovni jedinců byla taxonomicky zcela neuchopitelná, na úrovni populací bylo možné vymezit určité koherentní morfotypy, byť někdy propojené morfologicky přechodnými populacemi. Některé morfotypy navíc vykazovaly vazbu na určitá, poměrně specifická stanoviště, což svědčí o jejich ekologické diferenciaci.

Metodou průtokové cytometrie, doplněné o počítání chromozomů z roztlakových preparátů, byly u studovaných rostlin zjištěny čtyři ploidní stupně: diploidní, DNA-triploidní, tetraploidní a DNA-hexaploidní. Převážná většina studovaných skupin byla tvořena pouze tetraploidními populacemi (event. s ojedinělým výskytem DNA-hexaploidů), jedna skupina byla striktně diploidní. Významnější variabilita ve velikosti genomu byla zjištěna pouze u populací odpovídajících *D. fuchsii* subsp. *fuchsii*, jež byly buď diploidní, tetraploidní, nebo smíšené, v nichž se nezřídka uplatňovali též DNA-triploidi. Jednotlivé cytotypy jsou morfologicky nerozlišitelné, avšak jejich rozšíření má jistou geografickou vazbu: v České vysočině zcela převažují tetraploidní populace, které naopak nebyly vůbec zaznamenány na východě Slovenska a Polska, ani v Rumunsku, kde dominují diploidi. V Západních Karpatech, Alpách a slovinských Dinaridech pak koexistují všechny tři cytotypy, mezi nimiž zjevně dochází ke genovému toku. V oblastech s výskytem tetraploidního cytotypu *D. fuchsii* lze navíc předpokládat relativně častou hybridizaci s ostatními tetraploidními taxony.

Kombinace morfologických znaků a ploidie neumožňuje rozdělit středoevropské populace do dvou skupin odpovídajících samostatným druhům. To by ostatně ani nebylo v souladu s předpokladem takřka neomezeného genového toku v rámci celého okruhu. V této práci je proto přijat jednodruhový koncept, v němž jsou všechny taxony studovaného okruhu spojovány do široce pojatého druhu *D. maculata*. Jednotlivé morfologicky, karyologicky a ekologicky vyhraněné skupiny jsou pak rozlišovány na infraspecifické úrovni, zde v kategorii poddruhu. Kompletní výčet taxonů vyskytujících se v zemích střední Evropy obsahuje *D. maculata* subsp. *arcana, averyanovii, elodes, fuchsii, maculata, sooana, sudetica* a *transsivanica* (zkratky autorských jmen viz str. 13).

V České republice se vyskytuje šest poddruhů. Nejhojnějším zástupcem je zde *D. maculata* subsp. *fuchsii*, které v národním červeném seznamu náleží kategorie téměř ohroženého taxonu (NT). Za ohrožené (EN) je třeba považovat subsp. *maculata* vyskytující se v severozápadních Čechách, subsp. *sudetica* endemickou pro Krkonoše, a také subsp. *transsilvanica* a subsp. *sooana*, obě zasahující na české území pouze v oblasti Karpat. Na jediné (makro)lokalitě v Hrubém Jeseníku se pak vyskytuje subsp. *averyanovii*, která je proto hodnocena jako kriticky ohrožená (CR).