Annals of Botany **131**: 71–86, 2023
https://doi.org/10.1093/aob/mcab123, available online at www.academic.oup.com/aob

ANNALS OF BOTANY
Founded 1887

PART OF A SPECIAL ISSUE ON POLYPLOIDY IN ECOLOGY AND EVOLUTION

Morphological and environmental differentiation as prezygotic reproductive barriers between parapatric and allopatric *Campanula rotundifolia* agg. cytotypes

Kristýna Šemberová[1,2,*] Marek Svitok[3,4] Karol Marhold[1,5] Jan Suda[†] and Roswitha E.Schmickl[1,2]

[1]Faculty of Science, Department of Botany, Charles University, Benátská 2, 12843, Prague, Czech Republic, [2]Czech Academy of Sciences, Institute of Botany, Department of Evolutionary Plant Biology, Zámek 1, 25243, Průhonice, Czech Republic, [3]Faculty of Ecology and Environmental Sciences, Technical University in Zvolen, T. G. Masaryka 24, 96001, Zvolen, Slovakia, [4]Faculty of Science, Department of Ecosystem Biology, University of South Bohemia, Branišovská 1760, 37005, České Budějovice, Czech Republic, and [5]Institute of Botany, Plant Science and Biodiversity Centre, Slovak Academy of Sciences, Dúbravská cesta 9, 84523 Bratislava, Slovakia
** For correspondence. E-mail Kristyna.semberova@seznam.cz*
[†]Deceased.

Received: 21 April 2021 Returned for revision: 24 August 21 Editorial decision: 20 September 2021 Accepted: 21 September 2021
Electronically published: 4 October 2021

• **Background and Aims** Reproductive isolation and local establishment are necessary for plant speciation. Polyploidy, the possession of more than two complete chromosome sets, creates a strong postzygotic reproductive barrier between diploid and tetraploid cytotypes. However, this barrier weakens between polyploids (e.g. tetraploids and hexaploids). Reproductive isolation may be enhanced by cytotype morphological and environmental differentiation. Moreover, morphological adaptations to local conditions contribute to plant establishment. However, the relative contributions of ploidy level and the environment to morphology have generally been neglected. Thus, the extent of morphological variation driven by ploidy level and the environment was modelled for diploid, tetraploid and hexaploid cytotypes of *Campanula rotundifolia* agg. Cytotype distribution was updated, and morphological and environmental differentiation was tested in the presence and absence of natural contact zones.
• **Methods** Cytotype distribution was assessed from 231 localities in Central Europe, including 48 localities with known chromosome counts, using flow cytometry. Differentiation in environmental niche and morphology was tested for cytotype pairs using discriminant analyses. A structural equation model was used to explore the synergies between cytotype, environment and morphology.
• **Key Results** Tremendous discrepancies were revealed between the reported and detected cytotype distribution. Neither mixed-ploidy populations nor interploidy hybrids were detected in the contact zones. Diploids had the broadest environmental niche, while hexaploids had the smallest and specialized niche. Hexaploids and spatially isolated cytotype pairs differed morphologically, including allopatric tetraploids. While leaf and shoot morphology were influenced by environmental conditions and polyploidy, flower morphology depended exclusively on the cytotype.
• **Conclusions** Reproductive isolation mechanisms vary between cytotypes. While diploids and polyploids are isolated postzygotically, the environmental niche shift is essential between higher polyploids. The impact of polyploidy and the environment on plant morphology implies the adaptive potential of polyploids, while the exclusive relationship between flower morphology and cytotype highlights the role of polyploidy in reproductive isolation.

Key words: *Campanula rotundifolia* agg., polyploidy, cytotype distribution, reproductive isolation, contact zone, diploid, tetraploid, hexaploid, morphological differentiation, environmental niche shift, parapatry, allopatry.

INTRODUCTION

Polyploidy, the possession of more than two complete chromosome sets gained through whole-genome duplication (WGD), is present in all living angiosperms (Jiao *et al.*, 2011) and is widely considered one of the most important and common modes of plant speciation (Weiss-Schneeweiss *et al.*, 2013; Wendel, 2015; Landis *et al.*, 2018). Understanding the indisputable evolutionary role of polyploidy and the ubiquity of polyploids in nature (Soltis and Burleigh, 2009; Mandáková *et al.*, 2017) is challenging because newly formed polyploids as a minority cytotype face frequency-dependent selection that can lead to their extinction (Levin, 1975). The complex physiological processes needed for the successful survival and establishment of polyploids and the necessity of reproductive isolation from their diploid progenitors thus raised the hypothesis of polyploids as dead ends (Soltis *et al.*, 2009; Mayrose *et al.*, 2015; Levin, 2019). Reproductive barriers are crucial to reduce the risk of insufficient fertilization (López-Jurado *et al.*, 2019) and/or the production of unfit hybrids (Hopkins, 2013). Polyploidy itself instantly creates an effective postzygotic reproductive

barrier between diploids and related polyploids (tetraploids) by producing undeveloped, less viable or sterile triploid offspring (triploid block; Köhler *et al.*, 2010; Hülber *et al.*, 2015). However, the triploid block is probably not sufficient to prevent ineffective hybridization, resulting in partially fertile triploids (triploid bridge; Mandáková *et al.*, 2013; Suda and Herben, 2013; Lafon-Placette *et al.*, 2017; Mao *et al.*, 2020) or tetraploids formed by fusions of reduced and unreduced gametes (Sutherland and Galloway, 2016). Therefore, prezygotic reproductive barriers are important because they promote assortative mating within each cytotype (Kolář *et al.*, 2017; Castro *et al.*, 2020).

Altering genome dosage, WGD immediately impacts cell size (nucleotypic effect; Levin, 1983) and plant phenotype (Gigas effect; Ramsey and Schemske, 1998; Simón-Porcar *et al.*, 2017). The morphology often changes predictably, especially in synthetic neopolyploids, which may, however, differ morphologically from polyploids already established in natural populations under selection (Husband *et al.*, 2016). Altered floral reproductive traits often do not have an impact on pollinator behaviour or plant self-incompatibility (Porturas *et al.*, 2019; Castro *et al.*, 2020). Thus, cytotype spatial segregation often accompanied by environmental niche differentiation explains most patterns of reproductive isolation (López-Jurado *et al.*, 2019; Castro *et al.*, 2020).

Polyploid niche evolution is limited by available niche space (Brochmann *et al.*, 2004) and may act not merely at the ploidy level but also at different lineages within the same cytotype (López-Jurado *et al.*, 2019). Despite a possible lack of niche differentiation between diploids and polyploids due to phylogenetic niche conservatism (Glennon *et al.*, 2014) or their recent origin and ongoing gene flow (Wos *et al.*, 2019), niche expansion is frequent for triploids and tetraploids. As the first polyploids formed, they fill the niche space unoccupied by diploids, thus avoiding competition and interploidy mating. For subsequent polyploids, however, environmental divergence may be necessary to find available niches, and the remaining niche space is often at the extremes of environmental gradients. Higher ploidies may then encounter constraints to the range due to limitations in their environmental tolerances (Laport *et al.*, 2013; Muñoz-Pajares *et al.*, 2017; López-Jurado *et al.*, 2019). When compared to their diploid or lower polyploid ancestors, polyploids show niche contraction because they are locally adapted to narrower and marginal niches in specific habitats (Sonnleitner *et al.*, 2016; López-Jurado *et al.*, 2019).

Extreme climatic conditions and environmental stress may also enhance the formation of unreduced gametes (Ramsey and Schemske, 1998; Wilson *et al.*, 2020), leading to recurrent polyploid formation. Local adaptations of multiple lineages within the same cytotype (Parisod and Besnard, 2007; Castro *et al.*, 2018) often lead to a wider polyploid distribution (López-Jurado *et al.*, 2019; Castro *et al.*, 2020; Wilson *et al.*, 2020). Subfunctionalization and neofunctionalization of the duplicated genes (Moore and Purugganan, 2005; Flagel and Wendel, 2009) may be essential for local adaptation and allow for colonization of new habitats, which may lead to invasiveness (te Beest et al., 2012).

Successful polyploid establishment often requires more than just size-related changes in plant phenotype (Porturas *et al.*, 2019). Phenotypic changes driven by the local environment promote higher competitive abilities or habitat displacement (Balao *et al.*, 2011; Laport *et al.*, 2017). However, they also mask the morphological changes induced by polyploidization. Distinguishing the effect of polyploidization from the effects of selection and local adaptation that follow the reproductive isolation of polyploids from their diploid progenitors is challenging and crucial for studies on plants from natural populations.

Heteroploid taxa provide useful insight into the evolution of different ploidy levels (Kolář *et al.*, 2017) and allow the assessment of whether differentiation in environmental requirements and morphology proportionally increases with increasing ploidy level, following the hypotheses of niche expansion and the Gigas effect, or is individual for each cytotype. Since ploidy level and cytotype spatial isolation may influence the strength of the prezygotic reproductive barriers, we compared cytotype pairs based on their ploidy level and geographical distribution. We hypothesize that cytotypes in contact (sympatry, parapatry) have higher morphological and ecological differentiation driven by selection for assortative mating (reinforcement; Hopkins, 2013). On the other hand, morphological and environmental differentiation between populations with limited contact (parapatry or allopatry) is hypothesized to be driven by local ecological conditions in different areas.

Three cytotypes (2*x*, 4*x*, 6*x*) from a polyploid complex of *Campanula rotundifolia* agg. co-occur at different levels of contact in Central Europe (the Bohemian Massif, the Pannonian Basin and the Western Carpathians; Mráz, 2005). A contact zone is either present within a population (sympatry, mixed-ploidy populations of 2*x*+4*x* and 4*x*+6*x*; Kovanda, 1967, 1970*a*, 2002) or between uniform-ploidy populations of each cytotype (parapatry; Kovanda, 1966; Mráz, 2005; Rauchová, 2007; Šemberová, 2013). The known cytotype distribution pattern shows a longitudinal shift from diploids in the west (the Bohemian Massif) to tetraploids in the east (2*x*–4*x* mixed ploidy populations in the Bohemian Massif and a continuous range of tetraploids in the Pannonian Basin and the Western Carpathians) (see first figure in Supplementary Data Material S1). Hexaploids are reported mainly from 4*x*–6*x* mixed ploidy and a few uniform ploidy populations almost exclusively in the Pannonian Basin (Gadella, 1964; Kovanda, 1967, 1970*a*, *b*, 1983, 2002; Mráz, 2005).

We aimed: (1) to revise and complement existing data on the distribution of 2*x*, 4*x* and 6*x* cytotypes; (2) to detect differences and variation in environmental requirements and morphological differentiation between parapatric and allopatric cytotype pairs and to test whether the changes proportionally increase with each ploidy level, following the hypotheses of niche expansion and the Gigas effect, or are individual for each ploidy level; (3) to quantify the extent of morphological variability accounted for by the local environmental conditions and cytotype effects; and (4) to infer the contribution of environmental and morphological differentiation to prezygotic reproductive barriers between the three cytotypes.

MATERIALS AND METHODS

Study group and study area

The three cytotypes ($2n = 2x = 34$ chromosomes, $2n = 4x = 68$ chromosomes, and $2n = 6x = 102$ chromosomes) of *Campanula rotundifolia* agg. (harebells, Campanulaceae, also

referred to as *Campanula* sect. Heterophylla *sensu* Mansion *et al.*, 2012) are widely distributed in Central Europe, an area where mixed-ploidy, as well as uniform-ploidy, populations are described (Supplementary Data Material S1; Gadella, 1964; Kovanda, 1967, 1970*a*, 1977, 1983, 2002; Mráz, 2005; Rauchová, 2007). While diploids were identified in the Bohemian Massif (western part of the Czech Republic), tetraploids inhabit the broadest geographical range consisting of two disjunct areas, one in the Bohemian Massif and the other in the Western Carpathians (north-eastern part of the Czech Republic, central and eastern Slovakia), with overlap into the northernmost part of the Pannonian Basin (south-eastern part of the Czech Republic, north-eastern Austria, southern Slovakia). Hexaploids are found in the north-western part of the Pannonian Basin and the adjacent part of the Western Carpathians.

Central European harebells are usually considered autopolyploids (Kovanda, 1970*b*; Laane *et al.*, 1983; Sutherland and Galloway, 2018) with complicated interspecific relationships, unresolved phylogeny (Mansion *et al.*, 2012; but see also Nierbauer *et al.*, 2017; Sutherland *et al.*, 2018; Wilson *et al.*, 2020) and unclear taxon delimitation (Kovačić, 2004), often based on ploidy-related changes in size-affected phenotypic traits (stomata, pollen). However, the trend of size increase is not beyond doubt (Kovanda, 2002; Rauchová, 2007), and inferring polyploid origins from morphology only or combined with niche or distributional range has pitfalls in merging evolutionarily independent lineages (Segraves *et al.*, 1999; Doyle and Sherman-Broyles, 2017). Thus, similar to works in other polyploid complexes (Sonnleitner *et al.*, 2016; Hanušová *et al.*, 2019; López-Jurado *et al.*, 2019), we studied cytotype environmental and morphological differentiation while omitting taxonomic entities (diploids and tetraploids in the Bohemian Massif are represented by *C. rotundifolia* L. and *C. gentilis* Kovanda, tetraploids from the Western Carpathian and hexaploids by *C. moravica* (Spitzner) Kovanda; Supplementary Data Fig. S1). In the study region, diploid and tetraploid cytotypes are also present in endemic taxa. These taxa were omitted from the study because of their restricted distribution and strict ecological requirements [subalpine tetraploids: *C. bohemica* Hruby: Krkonoše Mts, *C. gelida* Kovanda: Jeseníky Mts, *C. rotundifolia* subsp. *sudetica* (Hruby) Sóo: Krkonoše Mts and Jeseníky Mts, *C. tatrae* Borbás: Tatry Mts, and diploids: *C. serrata* (Kit. ex. Schult.) Hendrych: Carpathian Mts, *C. cochleariifolia* Lam.: alpine zone in most European mountains and diploid *C. xylocarpa* Kovanda from Slovakian karst].

Sampling

Plant material from 231 localities was sampled between 2012 and 2017 (Fig. 1; Supplementary Data Table S1), with a particular focus on localities with published chromosome counts and mixed ploidy localities (Supplementary Data Material S1; Gadella, 1964; Kovanda, 1967, 1970*a*, 1977, 1983, 2002; Mráz, 2005; Rauchová, 2007, Šemberová, 2013). At each locality, a GPS coordinate was saved, and plants for further analyses were randomly sampled to cover the entire morphological and habitat spectra (e.g. plant height, number of flowers, shaded vs. light stands) at a minimum of 15-m spans to avoid clones (Stevens *et al.*, 2012). On average, 14 ± 18 individuals and 34 ± 19 individuals were sampled on localities without and with previously published chromosome counts, respectively (Table S1 and Supplemetary Material S1). Selected mixed-ploidy localities were subsequently studied to obtain higher resolution data regarding cytotype spatial distribution.

DNA ploidy (relative genome size) estimation

All individuals were analyzed by flow cytometry (FCM) to estimate the ploidy level following the best practice recommendation (Sliwinska *et al.*, 2021). Sample preparation followed a simplified two-step procedure (Doležel *et al.*, 2007): an appropriate amount of leaf tissue for both *Campanula* and internal standard (*Bellis perennis*, 2C = 3.38 pg, Schönswetter *et al.*, 2007*b*) was chopped together by a razor blade in 0.5 mL of ice-cold Otto I buffer (0.1 M citric acid, 0.5 % Tween 20) in a plastic Petri dish. The suspension of nuclei was filtered through a 42-µm nylon mesh. For ploidy level estimation, 1 mL of staining solution, containing Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12H_2O$), 4 µg·mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) and 25 µL·mL^{-1} β-mercaptoethanol, was added to the suspension of nuclei. The solution of stained nuclei was analysed using a Partec CyFlow ML cytometer (Partec GmbH, Münster, Germany) equipped with a 365-nm UV-LED as a source of UV light for DAPI excitation. The fluorescence intensity of at least 3000 particles was recorded for further data processing. Up to 10 individuals were pooled, and samples with a CV (coefficient of variation) >3 % were re-analysed. Altogether, 6011 individuals were screened for DNA ploidy level.

Environmental data

The environmental niches of the cytotypes were defined using 36 variables (Supplementary Data Table S2). The data were acquired from climatic and topographic GIS layers preprocessed by GeoModel Solar (Bratislava, Slovakia), developer and operator of the SolarGIS service. Air temperatures at 2 m were derived from the Climate Forecast System Reanalysis (National Centres for Environmental Prediction, USA) for the period from 1990 to 2009. The data were spatially enhanced to 30 arc-sec resolution by disaggregation based on the correlation between terrain altitude and temperature. Precipitation data were processed from the database of the Global Precipitation Climatology Centre project (Schneider *et al.*, 2014) for the period from 1951 to 2000. Source data resolution was increased to 2 arc-minutes by disaggregation based on the focal correlation of precipitation with SRTM 30 elevation data and cloudiness (clear-sky index) derived from the SolarGIS database. Topographic data (altitude, slope and aspect) were obtained from the terrain elevation model (The Shuttle Radar Topography Mission data – SRTM3) at 15 arc-sec resolution. Photosynthetically active radiation (PAR) was calculated by the SolarGIS model for the period 1994–2013 at 15 arc-sec resolution.

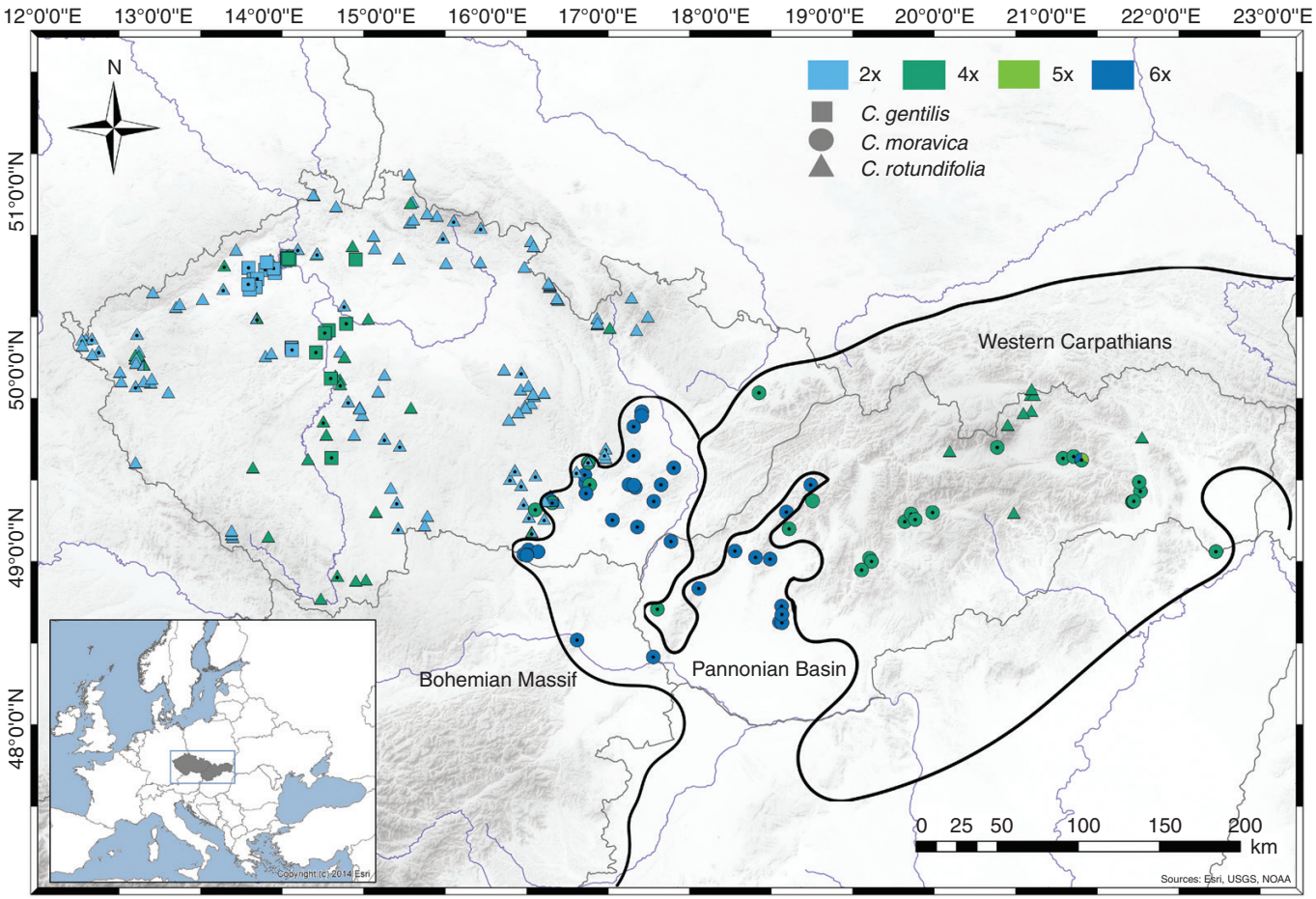

FIG. 1. Cytotype distribution of *Campanula rotundifolia* agg. in the investigated area of Central Europe inferred from flow cytometry analyses of individuals from 231 localities in the Czech Republic, Slovakia and Austria. Black dots mark the populations used for morphometric analysis. Geographical features were adapted from Mráz (2005) and simplified.

For environmental modelling, long-term yearly and monthly averages of air temperatures and precipitation were used. As a measure of climatic variability, the average annual standard deviations of temperatures and precipitation were calculated. The aspect values were linearized and rescaled to range from 0 to 4 (0 = south, 1 = south-east and south-west, 2 = east and west, 3 = north-east and north-west, 4 = north). Finally, information on geological bedrock was added from online maps (https://mapy.geology.cz/geocr50/, http://apl.geology.sk/gm50js/), the intensity of human influence was estimated using personal observations in the field (human-influenced vs. natural), and habitat type was ranked from rocks (0) through grasslands (1) to forests (2).

Plant morphological data

A subset of 1157 individuals was selected from the sampled material based on the presence of 19 primary characters (Supplementary Data Material S2) chosen according to previous studies (Kovanda, 1970*b*; Rauchová, 2007). Morphological characteristics were measured on material sampled in July–August 2012 and 2013 using a digital calliper, and the outer part of the ovary was checked for the presence or absence of the papilla by using an Olympus SZ51 stereomicroscope with a magnification of 8–10× (Olympus Corp., Tokyo, Japan).

Data analysis

Environmental niche shifts and morphological differentiation among the cytotypes were assessed using discriminant analysis. However, a high number of environmental variables (36) and morphological variables (29) and strong correlations among them (environmental data: Pearson's r = –0.937–0.998, morphological data: r = –0.745–0.885) pose severe limitations on the use of traditional methods. Under these circumstances, classical linear discriminant analysis can result in a solution with unstable coefficients or even in the inability to determine the optimal discriminant function (Wehrens, 2011). An ideal way of solving this problem would be to consider only a subset of uncorrelated proximal variables (i.e. those directly influencing the distribution of the cytotypes or describing morphological differentiation). Unfortunately, we had little prior knowledge of the direct effects of habitat characteristics on the cytotype distribution or the biologically meaningful morphological differences between the cytotypes. Moreover, there is no guarantee

that individual proximal variables would bear independent (uncorrelated) information (Jiménez-Valverde *et al.*, 2009). Another possibility would be to exclude correlated environmental and morphological variables prior to the analysis. De Marco and Nóbrega (2018) advise against subjective dropping of variables and advocate the use of latent variable methods such as principal component analysis (PCA). The latent variable approach is easily implemented and commonly used in environmental niche modelling (e.g. Broennimann *et al.*, 2012; De Marco and Nóbrega, 2018, and references therein) and multivariate morphometrics (e.g. Blackith and Reyment, 1971; Claude, 2008). We adopted a partial least square (PLS), supervised latent variable method that has an advantage over traditional PCA since it takes the dependent variable into account when defining ordination scores and loadings, whereas PCA captures variance in predictors only. The general idea of PLS is to construct a few latent variables while maximizing the covariance between a predictor and response matrices. PLS in discrimination analysis provides a dimension reduction technique that finds the optimal group separation while being guided explicitly by between-group variability (Barker and Rayens, 2003). However, structured noise may complicate the interpretability of PLS models, particularly when there are many components (Eriksson *et al.*, 2006). To remove such undesirable systematic variation in the data, we employed PLS with orthogonal projection to latent structures (OPLS) (Trygg and Wold, 2002). The objective of OPLS in discriminant analysis (OPLS-DA) is to divide the systematic variation in the predictor matrix into two parts: the predictive part, which models the relationships between variables and groups, and the orthogonal part, which captures the systematic variation in variables that is linearly unrelated to group separation, which generally leads to improved model interpretability.

The OPLS-DA models of environmental and morphological differences among the cytotypes were built with two predictive components to efficiently separate the three ploidy levels. The environmental and morphological data were standardized to *z*-scores to equalize the weight of variables in the analysis. Since the PLS methods are prone to overfitting, we used cross-validation to select the optimal number of components and to assess the model performance on independent data (Westerhuis *et al.*, 2008). We iteratively fitted OPLS-DA models with increasing complexity (up to 10 components), trained them on all the data except for one observation at a time and made predictions for those data points left out of the training sets (leave-one-out cross-validation). In both environmental and morphological datasets, models with more than two components provided little or no additional predictive power (Supplementary Data Fig. S2). Moreover, the relative importance of the components was assessed using randomization tests where variance explained by the components was compared with its null distribution generated from data randomly reshuffled 1000 times (Manly, 2006; Westerhuis *et al.*, 2008). To facilitate interpretation of the results, OPLS-DA score plots with 95 % confidence ellipses were displayed. Variable weights for the significant components were plotted to assess the relative influence of environmental and morphological characteristics on the discrimination of ploidy levels. Using an orthogonal projection to latent structures, these weights are primarily related to differences between cytotypes and their interpretation is quite straightforward (Wold *et al.*, 2001).

Environmental niche breadth and morphological variation were compared among the three cytotypes using distance-based tests of homogeneity of multivariate dispersions (Anderson, 2006). Environmental heterogeneity was quantified by calculating pairwise Euclidean distances among sampling sites from the matrix of standardized habitat characteristics. For morphological analyses, characters measured across multiple individuals were averaged within sites to cope with autocorrelation in the repeated measurements. The spread of sites from their medians in principal component space was used as a measure of heterogeneity. Equality of multivariate dispersion was tested using a randomization test based on 1000 permutations of the least absolute deviation residuals.

In addition to the overall comparison, cytotypes were divided into subsets based on their geographical distribution, and the differences in environmental niche and morphological traits were compared between parapatric and allopatric populations. In parapatry, we compared 2*x*–4*x* from the Bohemian Massif, 6*x*–4*x* from the Western Carpathians and 2*x*–6*x* from the Bohemian Massif and the Pannonian Basin. In allopatry, we compared 2*x*–4*x* from the Western Carpathians, 6*x*–4*x* from the Bohemian Massif and 4*x*–4*x* from the Bohemian Massif and the Western Carpathians. In selected sympatric populations, detailed cytotype distribution was displayed to assess cytotype microhabitat preferences.

Plant morphology is affected by both cytotype and environment, and the environment may also relate to cytotype distribution. The evidence for potential synergies was explored using a causal network of piecewise structural equation models (SEMs; Shipley, 2009). To gain more insight, the environmental data were split into two subsets specifying local habitat conditions and climate (Supplementary Data Table S2), and the morphological data were split into three subsets consisting of variables characterizing the morphology of the leaf, flower and shoot (Supplemetary Material S2). These multivariate datasets were standardized and subjected to PCA to reduce their dimensionality. The first components for each subset accounted for 24–75 % of the variance in the datasets and were used in the SEM to represent original multivariate data. The variables were arranged in a directed acyclic graph, and a series of regression models was fitted to integrate plant morphology with ploidy levels and environment. Linear mixed models (LMMs; Pinheiro and Bates, 2000) were used to test the effect of cytotype and environment on plant morphology while accounting for multiple plants measured at the same sites. The structure of the LMMs involved random intercepts of sites and fixed effects of cytotype, habitat and climate. The models were screened for normality, homoscedasticity and spatial autocorrelation using standard diagnostic plots of residuals and spline correlograms (Bjørnstad and Falck, 2001). The LMMs for leaf and shoot morphology showed heterogeneous error variances, and thus the original models were reformulated by including an exponential variance function structure to fix the heteroscedasticity. No other violations of model assumptions were detected. To investigate the influence of the environment on the distribution of cytotypes, we fitted the ordinal outcomes of ploidy levels by using a cumulative logit mixed model with the Gauss–Hermite

quadrature approximation (Tutz and Hennevogl, 1996). Likelihood-ratio tests were used to assess the overall significance of the models and the significance of individual terms. Marginal determination coefficients (R^2_m) were calculated to quantify the proportion of the total variance explained by the models (Nakagawa *et al.*, 2017). The relative contribution of individual variables was assessed using semi partial marginal determination coefficients (sR^2_m) derived from commonality analysis (Ray-Mukherjee *et al.*, 2014). Correlative paths in the SEM were estimated using the Pearson correlation coefficient.

All analyses were performed in R language version 3.6.0 (R Core Team, 2019) using the libraries ellipse (Murdoch and Chow, 2018), ggplot2 (Wickham, 2016), lme (Pinheiro *et al.*, 2019), ordinal (Christensen, 2019), ncf (Bjørnstad, 2019), performance (Lüdecke *et al.*, 2020), pls (Mevik *et al.*, 2019) and vegan (Oksanen *et al.*, 2019).

RESULTS

Cytotype spatial distribution, mixed-ploidy populations and potential DNA aneuploids

A survey of 6011 individuals from 231 localities in Central Europe analysed by flow cytometry revealed three main ploidy levels (2*x*, 4*x*, 6*x*; Fig. 1, Supplementary Data Table S1), one odd ploidy level (5*x* in the locality Dreveník; site ID 14, Supplementary Data Material S3 and Supplementary Data Fig. S3) and several irregularities in the relative fluorescence suggesting differences in relative genome size and interpreted as potential DNA aneuploids (for all three ploidy levels from 17 localities; six, three and eight localities for 2*x*, 4*x* and 6*x* potential DNA aneuploids, respectively; Supplementary Data Material S3). Potential DNA-aneuploids were detected in samples where up to 10 plants were pooled, with the CV varying from 1.18 to 2.14 %.

Cytotypes were spatially clustered with parapatric distribution (Fig. 1, Supplementary Data Material S1). Hexaploids exclusively dominate the Pannonian Basin with a warm and continental climate and the westernmost part of the Western Carpathians (site IDs: 32, 33). Hexaploids share a long contact zone with tetraploids at the borders between the Western Carpathians and the Pannonian Basin, and another large area of tetraploids was found in the Bohemian Massif (i.e. Southern and Central Bohemia). Additional populations of tetraploids were scattered in South Moravia where the Bohemian Massif, the Pannonian Basin and the Western Carpathians are in contact (site IDs: 57, 66, 90, 97, 101) and in relict-like habitats such as serpentinite outcrops and rocky river valleys within the diploid range (Western and Central Bohemia: site IDs: 128, 129, 139, 273, 274, 469) and the hexaploid range (Southern Slovakia, site ID: 30). Diploids are widespread but almost exclusively in the Bohemian Massif, where they occupy a large spectrum of habitats (ruderal roadsides, pastures, meadows, forest edges, rocks and castle ruins).

A comparison of our flow cytometry data with previously published chromosome counts from the same locality showed discrepancies in ploidy level in 38 % of populations (Supplementary Data Material S1; Gadella, 1964; Kovanda, 1967, 1970*a*, 1977, 1983, 2002), including mixed-ploidy populations (Kovanda, 1967, 1970*a*). All revised mixed-ploidy populations were found to be of uniform ploidy, with only the higher ploidy present (Supplementary Data Material S1). However, eight mixed-ploidy populations (2*x*–4*x*, 4*x*–5*x*–6*x*, 2*x*–6*x*) were detected elsewhere, with higher ploidies always a minority except for the 2*x*–6*x* mixed-ploidy population with an almost equal proportion of 2*x* and 6*x* (Fig. 1, Supplementary Data Table S1 and Fig. S3).

Environmental niche breadth and niche divergence among cytotypes

OPLS-DA revealed significant shifts in environmental niches among the cytotypes (Table 1). The cytotypes were separated along with the first predictive component that significantly accounted for almost 52 % of the variability in the dataset (Fig. 2A). The second component was statistically non-significant. The first discriminant function represents the main climatic gradient, the contrast between calcareous and siliceous bedrocks and, to some extent, the intensity of human pressure (Fig. 3; Supplementary Data Table S3). Hexaploids prefer hotter and drier habitats with calcareous bedrock, and diploids occupy sites with lower temperatures and higher humidity often situated on siliceous bedrock with higher anthropic impact. Tetraploids typically dwell in intermediate conditions, which is also apparent from their central position in the ordination space of the OPLS-DA (Fig. 2A). Considering pairwise differences, the environmental niche of hexaploids differed significantly from both diploids (variance explained by the first component = 57.7 %, $P = 0.027$) and tetraploids (61.2 %, $P = 0.002$). The niches of diploids and tetraploids overlapped considerably and were statistically indistinguishable, either between parapatric or between allopatric cytotype pairs (Table 1).

The environmental niche breadth differed significantly ($F = 11.2$, $P < 0.001$), and the environmental space occupied by the cytotypes decreased not gradually but in the following direction: 4*x* > 2*x* > 6*x* (Table 1). The same trend was recorded for parapatric and allopatric pairwise comparisons, although niche breadth between parapatric 2*x*–4*x* in the Bohemian Massif and the allopatric tetraploids did not differ significantly.

Morphological differences and variation among cytotypes

While cytotype morphological variation did not increase with increasing ploidy levels, their morphology differed significantly (Table 1). The cytotypes were gradually separated along with the first OPLS-DA component, which significantly accounted for more than 22 % of the variability in the morphological dataset (Figs 2B and 3; Supplementary Data Table S4). Diploid plants differed significantly from both tetraploids (variance explained by the first component = 19.9 %, $P < 0.001$) and hexaploids (25.7 %, $P < 0.001$) in vegetative characters. The latter cytotypes also differed, mainly in generative characters, although marginally non-significantly (14.5 %, $P = 0.072$). The density of the papillae on the ovary, the ratio of the length/width of the leaves in the middle and upper parts of the stem, the length of the leaves in the middle part of the stem, and other characteristics continually increased from diploids to hexaploids, while the width of the leaves in the middle

TABLE 1. *Summary of the tests for differences in environmental niche shifts, environmental niche breadth, morphology and morphological variability among three cytotypes (2x, 4x and 6x) of* Campanula rotundifolia *agg. in Central Europe (tetraploids are divided into two groups based on their geographical occurrence: Western Carpathians = WC, Bohemian Massif = BM). Tests of niche shifts and morphological differences are based on OPLS-DA, and the table shows variance explained by the predictive components (comp 1, comp 2) along with probabilities in parentheses. Tests of differences in niche breadth and morphological variability are based on the homogeneity of multivariate dispersion, and the table shows F statistics (F) and respective probabilities in parentheses. Results significant at α = 5 % are highlighted in bold. Pairwise comparisons of the significant results are given in the contrast column. The type of cytotype spatial isolation (parapatry, allopatry) is indicated for pairwise comparisons.*

Model	Contact zone	Environmental niche shift			Environmental niche breadth		Morphological differences			Morphological variability	
		Comp 1 (%)	Comp 2 (%)	Contrast	F	Contrast	Comp 1 (%)	Comp 2 (%)	Contrast	F	Contrast
All (2*x* vs. 4*x* vs. 6*x*)		**52 (0.027)**	15 (0.517)	2*x* ≠ 6*x*, 4*x* ≠ 6*x*	**11.2 (< 0.001)**	6*x* < 2*x* < 4*x*	**22 (< 0.001)**	15 (0.181)	2*x* ≠ 6*x*, 2*x* ≠ 4*x*	0.4 (0.691)	–
2*x* vs. 4*x* BM	para	35 (0.475)	–	–	< 0.1 (0.888)	–	14 (0.331)	–	–	0.7 (0.417)	–
2*x* vs. 4*x* WC	allo	31 (0.555)	–	–	**5.7 (0.018)**	2*x* < 4*x* WC	**22 (< 0.001)**	–	2*x* ≠ 4*x* WC	0.8 (0.370)	–
6*x* vs. 4*x* BM	allo	**52 (0.029)**	–	6*x* ≠ 4*x* BM	**11.5 (0.001)**	6*x* < 4*x* BM	**20 (0.014)**	–	6*x* ≠ 4*x* BM	1.2 (0.287)	–
6*x* vs. 4*x* WC	para	**65 (0.003)**	–	6*x* ≠ 4*x* WC	**14.6 (< 0.001)**	6*x* < 4*x* WC	11 (0.466)	–	–	0.6 (0.457)	–
2*x* vs. 6*x*	para	**58 (0.027)**	–	2*x* ≠ 6*x*	**10.2 (0.002)**	6*x* < 2*x*	**26 (< 0.001)**	–	2*x* ≠ 6*x*	0.3 (0.577)	–
4*x* WC vs. 4*x* BM	allo	31 (0.485)	–	–	2.7 (0.108)	–	**19 (0.012)**	–	4*x* WC ≠ 4*x* BM	1.9 (0.174)	–

and upper parts of the stem and the filament length continually decreased (Fig. 3; Supplementary Data Table S4). Comparisons of parapatric and allopatric cytotype pairs generally corroborated these results; however, two specific findings emerged (Table 1; Supplementary Data Table S4). First, allopatric tetraploids differed significantly in generative characters (shorter filaments and anthers and smaller ratios of the corolla length to calyx or corolla lobe lengths). Second, there were no differences between parapatric 2*x*–4*x* in the Bohemian Massif and 6*x*–4*x* from the Western Carpathians.

Environmental and ploidy level effects on morphology

Structural equation modelling showed that cytotype distribution was influenced by local habitat conditions (e.g. bedrock, PAR and altitude) rather than climatic characteristics, although both groups of environmental features were positively correlated (Fig. 4). Ploidy level significantly affects the shape of leaves ($F = 22.1$, $P < 0.001$), flowers ($F = 19.3$, $P < 0.001$) and, to a lesser extent, the overall plant ($F = 6.1$, $P = 0.002$). Environmental conditions significantly influenced leaf morphology (habitat effect: $F = 39.4$, $P < 0.001$; climate effect: $F = 10.9$, $P = 0.001$) and shoot morphology (climate effect: $F = 7.5$, $P = 0.007$), while the shape of generative organs was influenced only by ploidy level.

DISCUSSION

The current cytotype distribution

Central Europe is acknowledged as a potential region of origin of *Campanula rotundifolia* agg. (Sutherland *et al.*, 2018; Wilson *et al.*, 2020) because it may host the original diploids. From here, further spread of the complex was facilitated by polyploidization (Sutherland and Galloway, 2018). However, the longitudinal pattern of a ploidy increase from Central Europe to Western Europe and North America (Sutherland *et al.*, 2018) is not supported in this study. Available chromosome counts (Böcher, 1936; Podlech, 1965) limited knowledge of the full diploid distribution, and the presence of polyploids in Central Europe was omitted, partly due to the intricate taxonomy of this group. The presented data favour the hypothesis of the polytopic origin of polyploids on a small (e.g. tetraploids from the Western Carpathians, the Bohemian Massif and autotetraploids in mixed-ploidy populations, Fig. 1) as well as a large geographical scale [e.g. hexaploids in the Pannonian Basin; Fig. 1, in Northern Italy (Fenaroli *et al.*, 2013) and in Britain and Ireland (Wilson *et al.*, 2020), tetraploids in the Western Carpathians/the Bohemian Massif; Fig. 1, in Britain (Wilson *et al.*, 2020) and North America (Sutherland *et al.*, 2018)].

In contrast to previous cytotype distributions based on published chromosome counts, cytotypes are geographically clustered (Fig. 1, Supplementary Data Material S1). Diploids have a distribution centre in the Bohemian Massif and are otherwise rare or endemic in Europe and North America, except for North Scandinavia (Laane *et al.*, 1983). The largest discordance was found for the tetraploid cytotype detected in

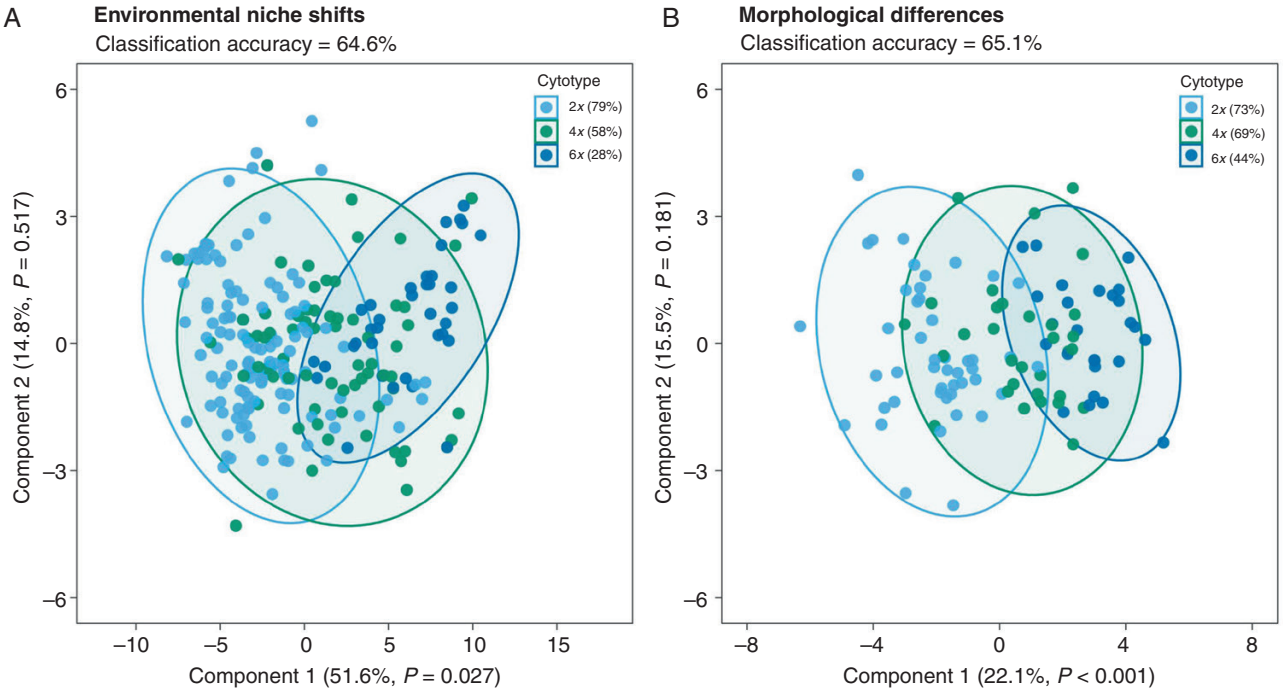

FIG. 2. OPLS-DA score plots of predictive components with 95 % confidence ellipses showing differences in environmental niches (A) and morphology (B) among three major cytotypes of *Campanula rotundifolia* agg. The relative proportion of variance explained by the components is displayed in parentheses along with probabilities (*P*). Cross-validated classification accuracies and sensitivities are given as percentages for each model and cytotype, respectively.

all regions (the Bohemian Massif, the Pannonian Basin and the Western Carpathians). Populations from the Pannonian Basin (populations with site IDs: 57, 66, 90, 97, 101) were not studied for morphological and environmental differentiation because of their low number and uncertain origin. These populations nested in the contact zone of the diploid and hexaploid cytotypes may represent autopolyploids that successfully established uniform ploidy populations. Alternatively, these populations may have remained from a formerly large tetraploid area that was now found to be hexaploid. However, none of these populations was included in studies involving chromosome counts (Supplementary Data Material S1). These tetraploids could also represent hybrids between diploids and hexaploids that may rarely be formed (Meeus *et al.*, 2020) despite the relatively high postzygotic reproductive barrier between 2*x* and 6*x* cytotypes (Scott *et al.*, 1998). Additional tetraploid populations were scattered within the diploid area, mostly on serpentinite outcrops (Fig. 1). The stressful nature of serpentinite environments (Brady *et al.*, 2005) may lead to higher production of unreduced gametes (Ramsey and Schemske, 2002; Wilson *et al.*, 2020) or may serve as a refugium for rare cytotypes, thus bypassing minority-cytotype exclusion (Kolář *et al.*, 2012). A range expansion was detected for the hexaploid cytotype. It is almost exclusive in the Pannonian Basin, where hexaploids were mostly reported either from mixed-ploidy or formerly tetraploid populations (Gadella, 1964; Kovanda, 1967, 1970*a*, 1977, 1983; Mráz, 2005; Supplementary Data Material S1). Contact zones similar to those between 2*x*–6*x* and 6*x*–4*x* that roughly follow the borders of the studied regional features or between the Czech Republic and Slovakia were also detected for other ploidy-variable taxa (Mráz *et al.*, 2008; Trávníček *et al.*, 2010; Kobrlová *et al.*, 2016; Macková *et al.*, 2020).

Mixed-ploidy population dynamics and potential DNA aneuploids

Flow cytometry detected a shift in the frequency and location of mixed-ploidy populations (Supplementary Data Material S1). This approach screened the actual ploidy variation in the population, while previous studies used chromosome counts on seedlings from seeds collected at the locality (Gadella, 1964; Kovanda, 1966, 1970*a*, *b*; Rauchová, 2007). The possible establishment and survival of such seedlings may differ in the field and experimental cultivation (Sutherland and Galloway, 2016; Meeus *et al.*, 2020). Experimental 2*x*–4*x* crosses yielded an almost equal frequency of 3*x* and 4*x* offspring (Sutherland and Galloway, 2016) However, in simulated 2*x*–4*x* open-pollinated contact zones, the ratio of homoploid and heteroploid crosses varied depending on cytotype frequency and pollinator preferences for the rare cytotype (Sutherland *et al.*, 2020). Neither 3*x* nor 4*x* hybrids are likely to contribute to the interploidy gene flow in mixed-ploidy populations (Sutherland and Galloway, 2021). In simulated 2*x*–4*x* contact zones, the persistence of minority cytotypes was facilitated by pollinator preferences for the rare ploidy level and near-complete postzygotic reproductive isolation. The lack of intermediate cytotypes in mixed-ploidy populations (2*x*–4*x*, 2*x*–6*x*) or the 2*x*–4*x* contact zone supports the hypotheses of a strong triploid block and no secondary contact zone (Scott *et al.*, 1998; Sutherland and Galloway, 2016; Lafon-Placette

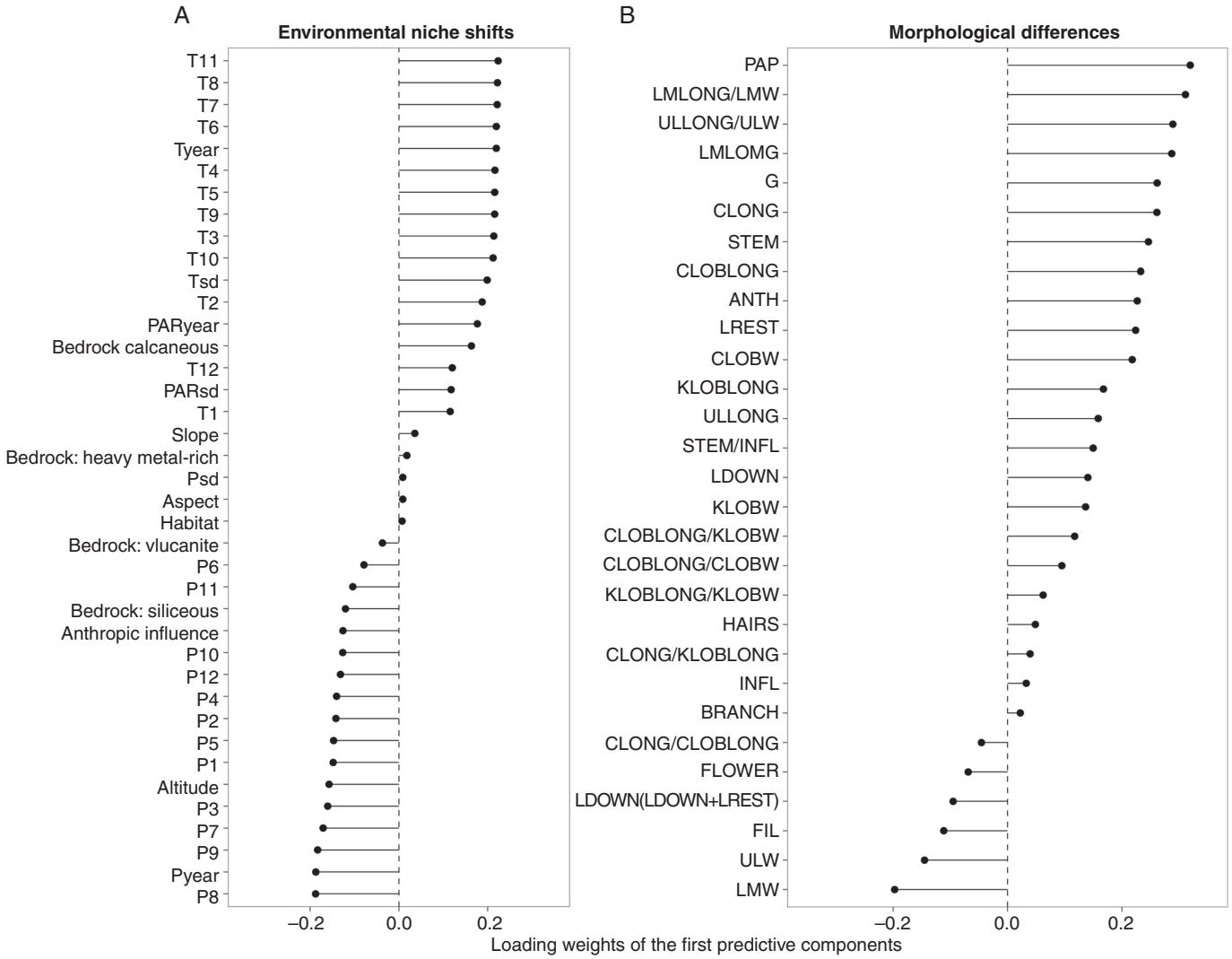

FIG. 3. Variable weights for the first (significant) predictive components of OPLS-DAs (Fig. 2) performed on environmental conditions (A) and morphological characteristics (B) of *Campanula rotundifolia* agg. cytotypes. For abbreviations of variable names, see Supplementary Data Material S2 and Table S3.

et al., 2017; Castro *et al.*, 2020) where cytotype spatial separation may be enhanced via reinforcement (Sutherland and Galloway, 2021). Mixed-ploidy (2*x*–4*x*) localities within the diploid range thus probably represent a primary contact zone of 2*x* and auto-4*x*, mediated by a high production of diploid unreduced gametes (Castro *et al.*, 2018) and low viability of possibly formed triploids. The lack of tetraploids, pentaploids or other cytotypes in the 2*x*–6*x* mixed-ploidy population suggests strong endosperm barriers between these two cytotypes (Scott *et al.*, 1998; Meeus *et al.*, 2020), supported by altitudinal cytotype separation (Schönswetter *et al.*, 2007*a*). An almost similar proportion of diploids and hexaploids suggests it is a secondary contact zone. Unlike 2*x*–4*x* experimental crosses of *Campanula rotundifolia* agg. that yielded relatively balanced frequencies of 3*x* and 4*x* offspring, the 4*x*–6*x* crosses yielded almost exclusively pentaploids (Sutherland and Galloway, 2016). However, in simulated 4*x*–6*x* open-pollinated contact zones, the pollinator preferences for the rare cytotype altered the ratio of homoploid and heteroploid crosses with regard to cytotype frequency (Sutherland *et al.*, 2020). In contrast to simulated 2*x*–4*x* contact zones, in simulated 4*x*–6*x* contact zones, the lack of postzygotic reproductive barriers and frequent formation of viable and fertile 5*x* may lead to asymmetric gene flow depending on cytotype frequency and introgression (Sutherland *et al.*, 2020; Sutherland and Galloway, 2021). In contrast to the results of Sutherland *et al.* (2020), Wilson *et al.* (2020) detected a variable one-sided barrier favouring the persistence of tetraploids in 4*x*–6*x* mixed-ploidy populations in Britain. We found pentaploids and hexaploids within a predominantly tetraploid population (Fig. S3 and Supplementary Data Material S3), similar to the pattern in Britain (Wilson *et al.*, 2020), Germany (Nierbauer *et al.*, 2017) and North America (Sutherland and Galloway, 2018). These hexaploids are probably autopolyploids and form pentaploids via a 4*x*–6*x* backcross, and pentaploids may further participate in the heteroploid hybridization (Laport *et al.*, 2016; Wilson *et al.*, 2020; Peskoller *et al.*, 2021; Sutherland and Galloway, 2021). At this locality, higher ploidies were found near tourist paths,

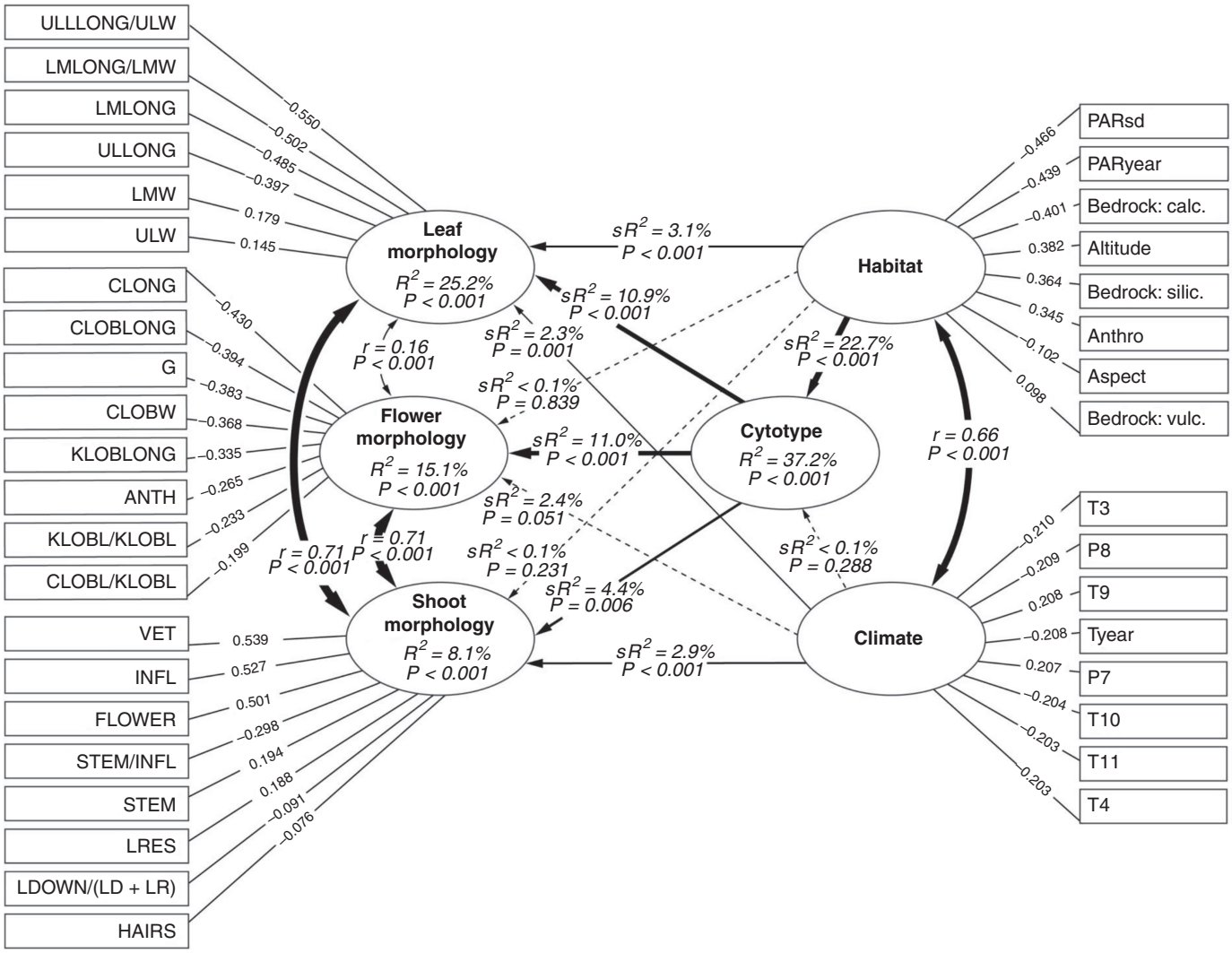

FIG. 4. Structural equation model linking environmental characteristics (habitat and climate) with the distribution of cytotypes, leaf morphology, flower morphology and shoot morphology. Arrows represent significant (solid lines) and non-significant (dashed lines) relationships between variables. Unidirectional arrows denote direct effects of one variable on another, and their size is proportional to semi-partial marginal determination coefficients (sR^2). Bidirectional arrows denote parwise correlations between variables, and their size is proportional to Pearson correlation coefficients (r). For each response variable, variance explained by predictors (marginal determination coefficients – R^2) and statistical significance of whole models (P) are displayed. Morphological and environmental characteristics are latent variables derived as first components from principal component analyses on the original data. The most important original variables (rectangles) are shown along with their loadings on the latent morphological and environmental variables. For abbreviations of variable names, see Supplementary Data Material S2 and Table S3.

which implies a role of human-induced stress in the higher production of unreduced gametes (Wilson *et al.*, 2020).

Potential DNA aneuploids were found in all cytotypes, including pentaploids (Supplementary Data Material S3). Chromosome counts for 4*x C. gentilis* with 68 euploid chromosomes detected a variation of 67, 70, 73 and 75 chromosomes for aneuploids with a respective deviation of the DAPI ratio in flow cytometry analyses (Rauchová, 2007). Similar deviations were observed in Britain at sites with heavy metal soil for tetraploids and autohexaploids (Wilson *et al.*, 2020). This finding further supports the role of environmental stress in the production of unreduced gametes (Ramsey and Schemske, 1998). However, Rauchová (2007) also counted an individual with a DAPI ratio corresponding to a potential DNA aneuploid as a tetraploid with 68 euploid chromosomes, which suggests intraspecific genome size variation. A mix of potential DNA aneuploids and individuals with different genome sizes was also observed for the studied individuals (Supplementary Data Material S3).

A shift in cytotype distribution

The shift between published and observed ploidy distribution favouring higher ploidy levels in mixed- and uniform-ploidy populations (Supplementary Data Material S1) suggests that polyploids are well established and have higher colonization success. However, the time needed to successfully replace the lower ploidy detected by earlier chromosome counting would be relatively short (M. Kovanda performed his studies on

Campanula rotundifolia agg. 50 years ago), and in simulated contact zones of *Campanula rotundifolia* agg., the cytotypes produced progeny of the same ploidy level more frequently than expected under random mating (Sutherland *et al.*, 2020; Wilson *et al.*, 2020). Thus, the likelihood of counting a seedling of a different ploidy than that of the mother plant is relatively low, especially in 4*x* or 4*x*–6*x* populations. Moreover, Wilson *et al.* (2020) showed that under open pollination, tetraploid mothers produced almost exclusively tetraploid offspring, while hexaploid progeny was more variable, including many pentaploids and aneuploids.

Although our sampling for the ploidy level estimates by flow cytometry did not cover the entire population, two cytotypes were often detected in populations from which only 11 individuals were sampled (Supplementary Data Table S1). Localities with published chromosome counts were sampled more thoroughly (30 individuals at a minimum; Supplementary Data Material S1). Nevertheless, the previously published cytotypes still may have been undetected in some undersampled populations.

Several studies on other plant species also show large discrepancies between published and observed ploidy variation in a similar pattern (exclusive occurrence of higher ploidy levels) for populations counted by M. Kovanda, and they assigned these differences to errors or difficulties in chromosome counting or to sampling artefacts (Laane *et al.*, 1983; Weiss *et al.*, 2002; Rauchová, 2007; Vít *et al.*, 2012; Macková *et al.*, 2020; Wilson *et al.*, 2020). This raises awareness that the original data do not reflect the actual cytogeography. However, these data are still kept in online karyological databases (e.g. Marhold *et al.*, 2007: database available at http://www.chromosomes.sav.sk; Rice *et al.*, 2015: database available at http://ccdb.tau.ac.il), which may bias further metanalyses using these sources (Porturas *et al.*, 2019; Rice *et al.*, 2019). Therefore, the use of published data without verifying the cytotype distribution in the field with high-throughput methods, such as flow cytometry, is highly questionable. More cytogeographical studies are thus needed because they very often discover unexpected variability, either at the local (Chumová *et al.*, 2015; Trávníček *et al.*, 2012; Caperta *et al.*, 2017; Nierbauer *et al.*, 2017; Wilson *et al.*, 2020) or at broad geographical scales (Krejčíková *et al.*, 2013; Čertner *et al.*, 2017; Nierbauer *et al.*, 2017; Paule *et al.*, 2017; Hanušová *et al.*, 2019; Rejlová *et al.*, 2019). New data on ploidy variation provide valuable insights into the evolutionary mechanisms shaping the gene flow dynamics of polyploids and, consequently, speciation. Minority cytotypes can help to detect interploidy hybridization through a triploid or a pentaploid bridge (Mandáková *et al.*, 2013; Peskoller *et al.*, 2021) with consequences for conservation genetics (Nierbauer *et al.*, 2017; Macková *et al.*, 2018), to detect the dynamic formation of unreduced gametes and neopolyploids (Wilson *et al.*, 2020; Sutherland and Galloway, 2021) and reveal cryptic variation that may result in speciation events (Flatscher *et al.*, 2015). Describing cytotypes as different species has its pitfalls such as in the case of *Campanula gentilis* and *C. moravica*. These two taxa were delimited from *C. rotundifolia* agg. mainly because of their different chromosome numbers (*C. moravica*) or the presence of mixed-ploidy populations (*C. gentilis*), and minute morphological differences related to WGD. The morphological differentiation was later doubted (Kovanda, 2002; Rauchová, 2007; Šemberová, 2013), leaving geographical distribution and chromosome number the only reliable identifiers of *C. gentilis* and *C. moravica,* respectively. A shift in cytotype distribution is also of relevance to the *loci classici* of *C. gentilis* and *C. moravica*, which implies a need for a detailed taxonomical revision. Taxonomic complexity, unclear geographical distribution and low morphological differentiation of individual species led us to omit the species names in this study.

Cytotype morphological and environmental differentiation as prezygotic reproductive barriers

Higher morphological variation, often created by polyploidy, was not confirmed in higher polyploids (6*x*) despite the trend toward larger size observed in some traits, especially leaf length and width. Similarly, morphological differentiation was more pronounced between diploids and polyploids than between higher ploidies (Table 1, Fig. 2B; Supplementary Data Table S4; Porturas *et al.*, 2019). Only cytotype pairs involving hexaploids and those that were spatially isolated differed morphologically. Morphological changes induced by WGD may vary within the same cytotype (Laport and Ramsey, 2015; López-Jurado *et al.*, 2019), suggesting that allopatric tetraploids probably represent lineages independently formed via recurrent formation. The lack of an environmental niche shift suggests that adaptations to local microclimatic or microhabitat conditions mirrored by the different geomorphological histories and flora of the two regions (Kaplan, 2012; Mráz and Ronikier, 2016) played a role in the tetraploid morphological differentiation (Castro *et al.*, 2018; López-Jurado *et al.*, 2019; Wilson *et al.*, 2020). The lower postzygotic reproductive barrier and higher gene flow between higher ploidy levels (Sutherland and Galloway, 2021) would need to be compensated for by a strong prezygotic reproductive isolation (e.g. by altering phenology or pollinator preferences). However, in simulated contact zones of *C. rotundifolia* agg., pollinators did not prefer a specific cytotype but they overvisited the rare one (Sutherland *et al.*, 2020). This contrasts with studies comparing plant–pollinator interactions along an elevational gradient where differences in plant morphology correlated with altitude and a related shift in pollinator size (Maad *et al.*, 2013). In addition, differences in phenology were detected between plants from different European countries (Preite *et al.*, 2015) but not on a smaller regional scale, among the plants from Britain and Ireland (Wilson *et al.*, 2020). While diploids from across Europe flowered later than some European tetraploids (Gadella, 1964), diploids and tetraploids in the Bohemian Massif started flowering earlier than hexaploids and tetraploids in the Pannonian Basin and Western Carpathians, suggesting a longitudinal shift in phenology.

The narrowest environmental niche breadth and the niche shift from all other cytotypes suggest niche specialization of the hexaploid cytotype and support the niche-filling hypothesis (López-Jurado *et al.*, 2019). In contrast, tetraploids had the widest niche, similar to the pattern of niche expansion observed in other polyploid complexes (Karunarathne *et al.*, 2018; López-Jurado *et al.*, 2019; Molina-Henao and Hopkins, 2019). However, this was true only when tetraploids from the Bohemian

Massif and the Western Carpathians were merged in the analysis. The environmental niche breadth of tetraploids from each area (either Bohemian Massif or Western Carpathians) was still broader than that of hexaploids but narrower than that of diploids (Table 1). Diploids had the broadest niche. While polyploids may benefit from gene neofunctionalization serving as pre-adaptation to a broader distribution, diploids, as the original and first cytotype, had the longest time to spread and adapt to the local environment.

The lack of niche shift (Table 1) between diploids and tetraploids, either in parapatry or in allopatry, may indicate a partly sufficient postzygotic reproductive barrier because no triploids were detected in the field. The triploid block may be bypassed by the tetraploid progeny (Sutherland and Galloway, 2016), and ongoing gene flow could explain the lack of morphological differentiation. However, no gene flow (Sutherland and Galloway, 2021) and no mixed-ploidy populations were found in the contact zone between diploids and tetraploids in the Bohemian Massif (Fig. 1), suggesting low viability of triploid and tetraploid hybrids. In contrast, the lack of pentaploid hybrids between parapatric tetraploids and hexaploids despite the lack of morphological differentiation may imply that the environmental niche shift is an efficient prezygotic reproductive barrier promoting assortative mating (Husband and Schemske, 2000; Sonnleitner *et al.*, 2010; Castro *et al.*, 2020). However, the environmental niche shift turned out not to be affected by the level of cytotype spatial isolation or strength of the postzygotic reproductive isolation because it was detected only for cytotype pairs, including hexaploids, irrespective of parapatry or allopatry. Thus, it is probably not due to reinforcement but rather reflects the niche specialization of the hexaploid cytotype according to the niche filling hypothesis (López-Jurado *et al.*, 2019).

The strong postzygotic reproductive isolation in 2*x*–4*x* contact zones results in neotetraploids and unfit triploids not participating in heteroploid gene flow. Isolated cytotypes thus may, via reinforcement or minority cytotype exclusion, undergo independent evolution (Sutherland and Galloway, 2021). In contrast, lower postzygotic isolation of polyploids allows the formation of viable and fertile pentaploids participating in 4*x*–6*x* gene flow (Wilson *et al.*, 2020; Sutherland and Galloway, 2021). Even if slowing the divergence (Sutherland and Galloway, 2021), pentaploids may represent an important bridge between the two cytotypes, allowing polyploids to benefit from a larger shared gene pool. We did not detect morphological or phenological differentiation between parapatric polyploids (4*x* Western Carpathians –6*x*) that could imply reinforcement. Despite the lack of selection for assortative mating, no mixed-ploidy populations or pentaploids were detected in the contact zone. Environmental niche shifts driven by the specialization of the hexaploids may thus serve as an efficient prezygotic reproductive barrier.

Consequences of climate, environment and ploidy on plant morphology

WGD immediately manifests in plant phenotype (Laport and Ramsey, 2015), phenology (Simón-Porcar *et al.*, 2017) and reproductive mode (Pannell *et al.*, 2004; Meeus *et al.*, 2020). However, reproductive isolation is more pronounced in established polyploids than in synthetic neopolyploids (Husband *et al.*, 2016; Porturas *et al.*, 2019), which suggests that local adaptation, environmental niche shifts (López-Jurado *et al.*, 2019) and reinforcement enhancing assortative mating (Kirkpatrick, 2000; Hopkins, 2013) could act as prezygotic reproductive barriers. The interactions between climate, environment and ploidy level on cytotype morphology and distribution are complex (Fig. 4). Despite niche specialization of hexaploids, the cytotype distribution was more influenced by local habitat conditions (e.g. bedrock, PAR and altitude) than climate. Leaf and shoot morphology were influenced by ploidy and environmental conditions, which mirrors the intricate taxonomic complexity and endemism of *Campanula rotundifolia* agg. (Podlech, 1965; Kovanda, 2002; Kovačić, 2004; Mansion *et al.*, 2012). Concerning local adaptations, the morphology of leaves and the overall plant may provide the best adaptive potential (Laport and Ramsey, 2015) without affecting pollinators or thus plant reproduction. Therefore, despite the tendency towards larger size in some traits, the longer and narrower leaves of hexaploids may be a xeromorphic adaptation to the drier and warmer Pannonian Basin, while diploids with wider and shorter leaves prefer the humid and colder Bohemian Massif (Figs 1 and 3). In contrast, generative traits were influenced by ploidy level only and differed among the higher polyploids (Fig. 3). Through floral morphology, a shift in pollinator preferences or phenology may imply reinforcement and serve as one of the prezygotic reproductive barriers (Hopkins, 2013; Laport and Ramsey, 2015), although this is not immediately apparent (Castro *et al.*, 2020).

CONCLUSION

The discrepancies revealed between published and revised ploidy variation imply a need for high-throughput methods complementing chromosome counting. Environmental niche specialization of hexaploids of *Campanula rotundifolia* agg. may ensure their reproductive isolation from parapatric tetraploids under the lack of a strong postzygotic reproductive barrier (Sutherland and Galloway, 2016). In contrast, morphological differentiation was detected almost exclusively for spatially isolated cytotypes, including allopatric tetraploids, which implies their independent origin (Wei *et al.*, 2017; López-Jurado *et al.*, 2019).

The absence of hybrids despite the lack of morphological and environmental differentiation between parapatric diploids and tetraploids emphasizes the strength of the triploid block. However, ongoing gene flow towards tetraploids potentially occurs due to the formation of tetraploids via unreduced gametes (Sutherland and Galloway, 2016), which could explain the lack of morphological differentiation between parapatric diploids and tetraploids.

Cytotype distribution was influenced by local habitat conditions, and the level of cytotype spatial isolation had no impact on reproductive isolation. The triploid block and environmental niche shifts are the main mechanisms of reproductive isolation, driven both by the ploidy level and environment. Polyploidy is the only variable influencing flower shape, which emphasizes the role of WGD as a prezygotic reproductive barrier (Kennedy *et al.*, 2006). The lack of

pollinator fidelity between newly *in silico* created polyploids and their diploid ancestors (Porturas *et al.*, 2019) and between evolutionarily young cytotypes of *Campanula rotundifolia* agg. (Roquet *et al.*, 2009; Sutherland *et al.*, 2020) implies a need for longer adaptive coevolution for pollinators to effectively distinguish between cytotypes. The synergy between polyploidy, environment and morphology is complex and varies between different ploidy levels and their level of spatial isolation.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Material S1: FCM estimated ploidy level in localities with known chromosome counts. Material S2: Morphological variables. Material S3: Relative genome size and potential DNA aneuploids. Figure S1: Photos of *Campanula rotundifolia* agg. Figure S2: Validation plots for the OPLS-DA. Figure S3: A mixed-ploidy population. Table S1: Localities. Table S2: Environmental variables. Table S3: Summary of environmental variables. Table S4: Summary of morphological variables.

ACKNOWLEDGEMENTS

The authors are grateful to Filip Kolář, Martin Čertner, Jana Nosková, Kristýna Hanušová, Kateřina Hanušová, Kristýna Hlavatá, Monika Pavlíková, Ludmila Rejlová, Romana Urfusová, Jana Rauchová, Jana Mořkovská, Adam Knotek (Faculty of Science, Charles University), Radek Štencl (Nature Conservation Agency of the Czech Republic) and Petr Petřík (Institute of Botany, CAS) for additional sampling and Dušan Senko (Slovak Academy of Sciences) for help with assessing the environmental variables. Pavel Trávníček, Zuzana Chumová (Institute of Botany, CAS) and two anonymous reviewers provided constructive comments that improved the manuscript. The English text was edited by the American Journal Experts (www.aje.com). JS, KŠ and RS designed the study; KŠ conducted sampling, flow cytometry analyses and morphological measurements; KŠ and MS analysed the data, and KŠ and MS wrote the initial draft of the manuscript. KŠ, MS, KM and RS contributed to the final version of the manuscript. The paper is dedicated to the memory of Jan Suda.

FUNDING

The study was supported by Charles University [project GA UK No. 670213] to KŠ. KŠ and RS were supported by the long-term research project of the Czech Academy of Sciences, Institute of Botany (RVO 67985939). MS was supported by the Operational Programme Integrated Infrastructure (OPII), funded by the ERDF [ITMS 313011T721].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

LITERATURE CITED

Anderson MJM. 2006. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics* **62**: 245–253.

Balao F, Herrera J, Talavera S. 2011. Phenotypic consequences at the micro-evolutionary scale of polyploidy and genome size dynamics. *Journal of Evolutionary Biology* **192**: 256–265.

Barker M, Rayens W. 2003. Partial least squares for discrimination. *Journal of Chemometrics* **17**: 166–173.

te Beest M, Le Roux JJ, Richardson DM, *et al.* 2012. The more the better? The role of polyploidy in facilitating plant invasions. *Annals of Botany* **109**: 19–45.

Bjørnstad ON. 2019. *ncf: spatial covariance functions*. R package version 1.2-8. https://ento.psu.edu/directory/onb1.

Bjørnstad ON, Falck W. 2001. Nonparametric spatial covariance functions: estimation and testing. *Environmental and Ecological Statistics* **8**: 53–70.

Blackith RE, Reyment RA. 1971. *Multivariate morphometrics*. London: Academic Press.

Böcher TW. 1936. Cytological studies on *Campanula rotundifolia*. *Hereditas* **22**: 269–277.

Brady KU, Kruckeberg AR, Bradshaw HD. 2005. Evolutionary ecology of plant adaptation to serpentine soils. *Annual Review of Ecology, Evolution, and Systematics* **36**: 243–266.

Brochmann C, Brysting AK, Alsos IG, *et al.* 2004. Polyploidy in arctic plants. *Biological Journal of the Linnean Society* **82**: 521–536.

Broennimann O, Fitzpatrick MC, Pearman PB, *et al.* 2012. Measuring ecological niche overlap from occurrence and spatial environmental data. *Global Ecology and Biogeography* **21**: 481–497.

Caperta AD, Castro S, Loureiro J, *et al.* 2017. Biogeographical, ecological and ploidy variation in related asexual and sexual *Limonium* taxa (Plumbaginaceae). *Botanical Journal of the Linnean Society* **183**: 75–93.

Castro M, Castro S, Figueiredo A, Husband BC, Loureiro J. 2018. Complex cytogeographical patterns reveal a dynamic tetraploid–octoploid contact zone. *AoB Plants* **10**: 1–18.

Castro M, Loureiro J, Figueiredo A, Serrano M, Husband BC, Castro S. 2020. Different patterns of ecological divergence between two tetraploids and their diploid counterpart in a parapatric linear coastal distribution polyploid complex. *Frontiers in Plant Science* **11**: 315.

Castro M, Loureiro J, Husband BC, Castro S. 2020. The role of multiple reproductive barriers: strong post-pollination interactions govern cytotype isolation in a tetraploid–octoploid contact zone. *Annals of Botany* **126**: 991–1003.

Čertner M, Fenclová E, Kúr P, *et al.* 2017. Evolutionary dynamics of mixed-ploidy populations in an annual herb: dispersal, local persistence and recurrent origins of polyploids. *Annals of Botany* **120**: 303–315.

Christensen RHB. 2019. *ordinal - regression models for ordinal data*. R package version 2019.4-25. https://github.com/runehaubo/ordinal

Chumová Z, Krejčíková J, Mandáková T, Sud J, Trávníček P. 2015. Evolutionary and taxonomic implications of variation in nuclear genome size: lesson from the grass genus Anthoxanthum (Poaceae). *Plos One* **10**: e0133748. doi:10.1371/journal.pone.0133748.

Claude J. 2008. *Morphometrics with R*. New York: Springer Science & Business Media.

De Marco P, Nóbrega CC. 2018. Evaluating collinearity effects on species distribution models: an approach based on virtual species simulation. *PLoS One* **13**: e0202403.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* **2**: 2233–2244.

Doyle JJ, Sherman-Broyles S. 2017. Double trouble: taxonomy and definition of polyploidy. *New Phytologist* **213**: 487–493.

Eriksson L, Johansson E, Kettaneh-Wold N, Trygg J, Wikström C, Wold S. 2006. *Multi- and megavariate data analysis. Part II. Advanced applications and method extensions*. Umea: Umetrics Academy.

Fenaroli F, Pistarino A, Peruzzi L, Cellinese N. 2013. *Campanula martinii* (Campanulaceae), a new species from northern Italy. *Phytotaxa* **111**: 27–38.

Flagel LE, Wendel JF. 2009. Gene duplication and evolutionary novelty in plants. *New Phytologist* **183**: 557–564.

Flatscher R, García PE, Hülber K, *et al.* 2015. Underestimated diversity in one of the world's best studied mountain ranges: the polyploid complex of *Senecio carniolicus* (Asteraceae) contains four species in the European Alps. *Phytotaxa* **213**: 1–21.

Gadella TWJ. 1964. Cytotaxonomic studies in the genus *Campanula*. *Wentia* **11**: 1–104.

Glennon KL, Ritchie ME, Segraves KA. 2014. Evidence for shared broad-scale climatic niches of diploid and polyploid plants. *Ecology Letters* **17**: 574–582.

Hanušová K, Čertner M, Urfus T, *et al.* 2019. Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) probably stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. *Annals of Botany* **123**: 845–855.

Hopkins R. 2013. Reinforcement in plants. *New Phytologist* **197**: 1095–1103.

Hülber K, Sonnleitner M, Suda J, *et al.* 2015. Ecological differentiation, lack of hybrids involving diploids, and asymmetric gene flow between polyploids in narrow contact zones of *Senecio carniolicus* (syn. *Jacobaea carniolica*, Asteraceae). *Ecology and Evolution* **5**: 1224–1234.

Husband BC, Baldwin SJ, Sabara HA. 2016. Direct vs. indirect effects of whole-genome duplication on prezygotic isolation in chamerion angustifolium: Implications for rapid speciation. *American Journal of Botany* **103**: 1259–1271.

Husband BC, Schemske DW. 2000. Ecological mechanisms of reproductive isolation between diploid and tetraploid *Chamerion angustifolium*. *Journal of Ecology* **88**: 689–701.

Jiao Y, Wickett NJ, Ayyampalayam S, *et al.* 2011. Ancestral polyploidy in seed plants and angiosperms. *Nature* **473**: 97–100.

Jiménez-Valverde A, Nakazawa YJ, Lira-Noriega A, Peterson Townsend A. 2009. Environmental correlation structure and ecological niche model projections. *Biodiversity Informatics* **6**: 28–35.

Kaplan Z. 2012. Flora and phytogeography of the Czech Republic. *Preslia* **84**: 505–573.

Karunarathne P, Schedler M, Martínez EJ, Honfi AI, Novichkova A, Hojsgaard D. 2018. Intraspecific ecological niche divergence and reproductive shifts foster cytotype displacement and provide ecological opportunity to polyploids. *Annals of Botany* **121**: 1183–1196.

Kennedy BF, Sabara HA, Haydon D, Husband BC. 2006. Pollinator-mediated assortative mating in mixed ploidy populations of *Chamerion angustifolium* (Onagraceae). *Oecologia* **150**: 398–408.

Kirkpatrick M. 2000. Reinforcement and divergence under assortative mating. *Proceedings of the Royal Society B: Biological Sciences* **267**: 1649–1655.

Kobrlová L, Hroneš M, Koutecký P, Štech M, Trávníček B. 2016. *Symphytum tuberosum* complex in central Europe: cytogeography, morphology, ecology and taxonomy. *Preslia* **88**: 77–112.

Köhler C, Mittelsten Scheid O, Erilova A. 2010. The impact of the triploid block on the origin and evolution of polyploid plants. *Trends in Genetics* **26**: 142–148.

Kolář F, Čertner M, Suda J, Schönswetter P, Husband BC. 2017. Mixed-ploidy species: progress and opportunities in polyploid research. *Trends in Plant Science* **22**: 1041–1055.

Kolář F, Fér T, Štech M, *et al.* 2012. Bringing together evolution on serpentine and polyploidy: spatiotemporal history of the diploid-tetraploid complex of *Knautia arvensis* (Dipsacaceae). *PLoS One* **7**: e39988.

Kovačić S. 2004. The genus *Campanula* L. (Campanulaceae) in Croatia, circum-Adriatic and west Balkan region. *Acta Botanica Croatica* **63**: 171–202.

Kovanda M. 1966. Some chromosome counts in the *Campanula rotundifolia* Complex II. *Folia Geobotanica & Phytotaxonomica* **1**: 268–273.

Kovanda M. 1967. Polyploidie a variabilita v komplexu Campanula rotundifolia L. PhD Thesis, Charles University, Czech Republic.

Kovanda M. 1970*a*. Polyploidy and variation in the *Campanula rotundifolia* complex. Part I. (general). *Rozpravy Československé Akademie Věd* **80**: 5–95.

Kovanda M. 1970*b*. Polyploidy and variation in the *Campanula rotundifolia* complex. Part II. (Taxonomic) 1. Revision of the groups Saxicolae, Lanceolatae and Alpicolae in Czechoslovakia and adjacent regions. *Folia Geobotanica et Phytotaxonomica* **5**: 171–208.

Kovanda M. 1977. Polyploidy and variation in the *Campanula rotundifolia* complex. Part II. (Taxonomic) 2. Revision of the groups Vulgares and Scheuchzerianae in Czechoslovakia and adjacent regions. *Folia Geobotanica et Phytotaxonomica* **12**: 23–89.

Kovanda M. 1983. Chromosome numbers in selected Angiosperms. *Preslia* **55**: 193–205.

Kovanda M. 2002. A range extension for *Campanula moravica*. *Thaiszia* **12**: 179–181.

Krejčíková J, Sudová R, Lučanová M, *et al.* 2013. High ploidy diversity and distinct patterns of cytotype distribution in a widespread species of *Oxalis* in the Greater Cape Floristic Region. *Annals of Botany* **111**: 641–649.

Laane MM, Croff BE, Wahlstrøm R. 1983. Cytotype distribution in the *Campanula rotundifolia* complex in Norway, and cyto-morphological characteristics of diploid and tetraploid groups. *Hereditas* **99**: 21–48.

Lafon-Placette C, Johannessen IM, Hornslien KS, *et al.* 2017. Endosperm-based hybridization barriers explain the pattern of gene flow between *Arabidopsis lyrata* and *Arabidopsis arenosa* in Central Europe. *Proceedings of the National Academy of Sciences* **114**: E1027–E1035.

Landis JB, Soltis DE, Li Z, *et al.* 2018. Impact of whole-genome duplication events on diversification rates in angiosperms. *American Journal of Botany* **105**: 348–363.

Laport RG, Hatem L, Minckley RL, Ramsey J. 2013. Ecological niche modeling implicates climatic adaptation, competitive exclusion, and niche conservatism among *Larrea tridentata* cytotypes in North American deserts. *The Journal of the Torrey Botanical Society* **140**: 349–363.

Laport RG, Minckley RL, Ramsey J. 2016. Ecological distributions, phenological isolation, and genetic structure in sympatric and parapatric populations of the *Larrea tridentata* polyploid complex. *American Journal of Botany* **103**: 1358–1374.

Laport RG, Muhia P, Kennell C, Hasan H. 2017. Assessing water use variation among the cytotypes of the autopolyploid southwestern desert Creosotebush (*Larrea tridentata* [DC.] Coville: Zygophyllaceae). *Madroño* **64**: 32–42.

Laport RG, Ramsey J. 2015. Morphometric analysis of the North American creosote bush (*Larrea tridentata*, Zygophyllaceae) and the microspatial distribution of its chromosome races. *Plant Systematics and Evolution* **301**: 1581–1599.

Levin DA. 1975. Minority cytotype exclusion in local plant populations. *Taxon* **24**: 35–43.

Levin DA. 1983. Polyploidy and novelty in flowering plants. *The American Naturalist* **122**: 1–25.

Levin DA. 2019. Why polyploid exceptionalism is not accompanied by reduced extinction rates. *Plant Systematics and Evolution* **305**: 1–11.

López-Jurado J, Mateos-Naranjo E, Balao F. 2019. Niche divergence and limits to expansion in the high polyploid *Dianthus broteri* complex. *New Phytologist* **222**: 1076–1087.

Lüdecke D, Makowski D, Waggoner P. 2020. *Performance: assessment of regression models performance*. R package version 0.4.4. https://easystats.github.io/performance/.

Maad J, Armbruster WS, Fenster CB. 2013. Floral size variation in *Campanula rotundifolia* (Campanulaceae) along altitudinal gradients: patterns and possible selective mechanisms. *Nordic Journal of Botany* **31**: 361–371.

Macková L, Nosková J, Ďurišová Ľ, Urfus T. 2020. Insights into the cytotype and reproductive puzzle of *Cotoneaster integerrimus* in the Western Carpathians. *Plant Systematics and Evolution* **306**: 1–14.

Macková L, Vít P, Urfus T. 2018. Crop-to-wild hybridization in cherries—empirical evidence from *Prunus fruticosa*. *Evolutionary Applications* **11**: 1748–1759.

Mandáková T, Kovařík A, Zozomová-Lihová J, *et al.* 2013. The more the merrier: recent hybridization and polyploidy in *Cardamine*. *The Plant Cell* **25**: 3280–3295.

Mandáková T, Pouch M, Harmanová K, Zhan SH, Mayrose I, Lysak MA. 2017. Multispeed genome diploidization and diversification after an ancient allopolyploidization. *Molecular Ecology* **26**: 6445–6462.

Manly BFJ. 2006. *Randomization, bootstrap and Monte Carlo methods in biology*. Boca Raton: CRC Press.

Mansion G, Parolly G, Crowl AA, *et al.* 2012. How to handle speciose clades? Mass taxon-sampling as a strategy towards illuminating the natural history of *Campanula* (Campanuloideae). *PLoS One* **7**: e50076. doi:10.1371/journal.pone.0050076.

Mao Y, Gabel A, Nakel T, *et al.* 2020. Selective egg cell polyspermy bypasses the triploid block. *eLife* **9**: 1–15.

Marhold K, Mártonfi P, Mereďa P, Mráz P, **eds. 2007.** *Chromosome number survey of the ferns and flowering plants of Slovakia*. Bratislava: VEDA.

Mayrose I, Zhan SH, Rothfels CJ, *et al.* 2015. Methods for studying polyploid diversification and the dead end hypothesis: a reply to Soltis *et al.* (2014). *New Phytologist* **206**: 27–35.

Meeus S, Šemberová K, De Storme N, Geelen D, Vallejo-Marín M. 2020. Effect of whole-genome duplication on the evolutionary rescue of sterile hybrid monkeyflowers. *Plant Communications* **1**: 100093.

Mevik B-H, Wehrens R, Liland KH. 2019. *pls: partial least squares and principal component regression*. R package version 2.7-1. https://github.com/khliland/pls.

Molina-Henao YF, Hopkins R. 2019. Autopolyploid lineage shows climatic niche expansion but not divergence in *Arabidopsis arenosa*. *American Journal of Botany* **106**: 61–70.

Moore RC, Purugganan MD. 2005. The evolutionary dynamics of plant duplicate genes. *Current Opinion in Plant Biology* **8**: 122–128.

Mráz P. 2005. Chromosome number and DNA ploidy level reports from Central Europe - 1. *Biologia, Bratislava* **60**: 99–103.

Mráz P, Ronikier M. 2016. Biogeography of the Carpathians: evolutionary and spatial facets of biodiversity. *Biological Journal of the Linnean Society* **119**: 528–559.

Mráz P, Šingliarová B, Urfus T, Krahulec F. 2008. Cytogeography of *Pilosella officinarum* (Compositae): altitudinal and longitudinal differences in ploidy level distribution in the Czech Republic and Slovakia and the general pattern in Europe. *Annals of Botany* **101**: 59–71.

Muñoz-Pajares AJ, Perfectti F, Loureiro J, *et al.* 2017. Niche differences may explain the geographic distribution of cytotypes in *Erysimum mediohispanicum*. *Plant Biology* **20**: 139–147.

Murdoch D, Chow ED. 2018. *ellipse: functions for drawing ellipses and ellipse-like confidence regions*. R package version 0.4.1. https://CRAN.R-project.org/package=ellipse.

Nakagawa S, Johnson PCD, Schielzeth H. 2017. The coefficient of determination R^2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *Journal of the Royal Society Interface* **14**: 20170213.

Nierbauer KU, Paule J, Zizka G. 2017. Heteroploid reticulate evolution and taxonomic status of an endemic species with bicentric geographical distribution. *AoB Plants* **9**: plx002.

Oksanen J, Blanchet FG, Friendly M, *et al.* 2019. *vegan: community ecology package*. R package version 2.5-4. https://github.com/vegandevs/vegan.

Pannell JR, Obbard DJ, Buggs RJA. 2004. Polyploidy and the sexual system: what can we learn from *Mercurialis annua*? *Biological Journal of the Linnean Society* **82**: 547–560.

Parisod C, Besnard G. 2007. Glacial *in situ* survival in the Western Alps and polytopic autopolyploidy in *Biscutella laevigata* L. (Brassicaceae). *Molecular Ecology* **16**: 2755–2767.

Paule J, Wagner ND, Weising K, Zizka G. 2017. Ecological range shift in the polyploid members of the South American genus *Fosterella* (Bromeliaceae). *Annals of Botany* **120**: 233–243.

Peskoller A, Silbernagl L, Hülber K, Sonnleitner M, Schönswetter P. 2021. Do pentaploid hybrids mediate gene flow between tetraploid *Senecio disjunctus* and hexaploid *S. carniolicus* s. str. (*S. carniolicus* aggregate, Asteraceae)? *Alpine Botany*131, 151–160. https://doi.org/10.1007/s00035-021-00254-x.

Pinheiro J, Bates D. 2000. *Mixed-effects models in S and S-PLUS*. New York: Springer Science & Business Media.

Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC. 2019. *nlme: linear and nonlinear mixed effects models*. R package version 3.1-139. https://svn.r-project.org/R-packages/trunk/nlme/.

Podlech D. 1965. Revision der europäischen und nordafrikanischen Vertreter der Subsect. Heterophylla (Wit.) Fed. der Gattung *Campanula* L. *Feddes Repertorium* **71**: 50–187.

Porturas LD, Anneberg TJ, Curé AE, Wang S, Althoff DM, Segraves KA. 2019. A meta-analysis of whole genome duplication and the effects on flowering traits in plants. *American Journal of Botany* **106**: 469–476.

Preite V, Stöcklin J, Armbruster GFJ, Scheepens JF. 2015. Adaptation of flowering phenology and fitness-related traits across environmental gradients in the widespread *Campanula rotundifolia*. *Evolutionary Ecology* **29**: 249–267.

R Core Team. 2019. *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Ramsey J, Schemske DW. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. *Annual Review of Ecology and Systematics* **29**: 467–501.

Ramsey J, Schemske DW. 2002. Neopolyploidy in flowering plants. *Annual Review of Ecology and Systematics* **33**: 589–639.

Rauchová J. 2007. *Karyologická, fenetická a genetická diferenciace českého subendemického taxonu Campanula gentilis Kovanda*. MSc Thesis, Charles University, Czech Republic.

Ray-Mukherjee J, Nimon K, Mukherjee S, *et al.* 2014. Using commonality analysis in multiple regressions: a tool to decompose regression effects in the face of multicollinearity. *Methods in Ecology and Evolution* **5**: 320–328.

Rejlová L, Chrtek J, Trávníček P, Lučanová M, Vít P, Urfus T. 2019. Polyploid evolution: the ultimate way to grasp the nettle. *PLoS One* **14**: 1–24.

Rice A, Glick L, Abadi S, *et al.* 2015. The Chromosome Counts Database (CCDB) - a community resource of plant chromosome numbers. *New Phytologist* **206**: 19–26.

Rice A, Šmarda P, Novosolov M, *et al.* 2019. The global biogeography of polyploid plants. *Nature Ecology and Evolution* **3**: 265–273.

Roquet C, Sanmartín I, Garcia-Jacas N, *et al.* 2009. Reconstructing the history of Campanulaceae with a Bayesian approach to molecular dating and dispersal–vicariance analyses. *Molecular Phylogenetics and Evolution* **52**: 575–587.

Schneider U, Becker A, Finger P, Meyer-Christoffer A, Ziese M, Rudolf B. 2014. GPCC's new land surface precipitation climatology based on quality-controlled *in situ* data and its role in quantifying the global water cycle. *Theoretical and Applied Climatology* **115**: 15–40.

Schönswetter P, Lachmayer M, Lettner C, *et al.* 2007*a*. Sympatric diploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps are separated along an altitudinal gradient. *Journal of Plant Research* **120**: 721–725.

Schönswetter P, Suda J, Popp M, Weiss-Schneeweiss H, Brochmann C. 2007*b*. Circumpolar phylogeography of *Juncus biglumis* (Juncaceae) inferred from AFLP fingerprints, cpDNA sequences, nuclear DNA content and chromosome numbers. *Molecular Phylogenetics and Evolution* **42**: 92–103.

Scott RJ, Spielman M, Bailey H, Dickinson HG. 1998. Parent-of-origin effects on seed development in *Arabidopsis thaliana*. *Development* **125**: 3329–3341.

Segraves KA, Thompson JN, Soltis PS, Soltis DE. 1999. Multiple origins of polyploidy and the geographic structure of *Heuchera grossulariifolia*. *Molecular Ecology* **8**: 253–262.

Šemberová K. 2013. *Population cytotype structure and phenotypic variation in Campanula moravica*. MSc Thesis, Charles University, Czech Republic.

Shipley B. 2009. Confirmatory path analysis in a generalized multilevel context. *Ecology* **90**: 363–368.

Sliwinska E, Loureiro J, Leitch IJ, Šmarda P, Bainard J, Bureš P, *et al*. 2021. Application-based guidelines for best practices in plant flow cytometry. *Cytometry A*. **doi: 10.1002/cyto.a.24499**.

Simón-Porcar VI, Silva JL, Meeus S, Higgins JD, Vallejo-Marín M. 2017. Recent autopolyploidization in a naturalized population of *Mimulus guttatus* (Phrymaceae). *Botanical Journal of the Linnean Society* **20**: 1–19.

Soltis DE, Albert VA, Leebens-Mack J, *et al.* 2009. Polyploidy and angiosperm diversification. *American Journal of Botany* **96**: 336–348.

Soltis DE, Burleigh GJ. 2009. Surviving the K-T mass extinction: new perspectives of polyploidization in angiosperms. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 5455–5456.

Sonnleitner M, Flatscher R, Escobar García P, *et al.* 2010. Distribution and habitat segregation on different spatial scales among diploid, tetraploid and hexaploid cytotypes of *Senecio carniolicus* (Asteraceae) in the Eastern Alps. *Annals of Botany* **106**: 967–977.

Sonnleitner M, Hülber K, Flatscher R, *et al.* 2016. Ecological differentiation of diploid and polyploid cytotypes of *Senecio carniolicus sensu lato* (Asteraceae) is stronger in areas of sympatry. *Annals of Botany* **117**: 269–276.

Stevens CJ, Wilson J, McAllister HA. 2012. Biological Flora of the British Isles: *Campanula rotundifolia*. *Journal of Ecology* **100**: 821–839.

Suda J, Herben T. 2013. Ploidy frequencies in plants with ploidy heterogeneity: fitting a general gametic model to empirical population data. *Proceedings of the Royal Society B: Biological Sciences* **280**: 20122387.

Sutherland BL, Galloway LF. 2016. Postzygotic isolation varies by ploidy level within a polyploid complex. *New Phytologist* **213**: 404–412.

Sutherland BL, Galloway LF. 2018. Effects of glaciation and whole genome duplication on the distribution of the *Campanula rotundifolia* polyploid complex. *American Journal of Botany* **105**: 1760–1770.

Sutherland BL, Galloway LF. 2021. Variation in heteroploid reproduction and gene flow across a polyploid complex: one size does not fit all. *Ecology and Evolution* **11**: 9676–9688.

Sutherland BL, Miranda-Katz T, Galloway LF. 2020. Strength in numbers? Cytotype frequency mediates effect of reproductive barriers in mixed-ploidy arrays. *Evolution* **74**: 2281–2292.

Sutherland BL, Quarles BM, Galloway LF. 2018. Intercontinental dispersal and whole-genome duplication contribute to loss of self-incompatibility in a polyploid complex. *American Journal of Botany* **105**: 1–8.

Trávníček P, Eliášová A, Suda J. 2010. The distribution of cytotypes of *Vicia cracca* in Central Europe: the changes that have occurred over the last four decades. *Preslia* **82**: 149–163.

Trávníček P, Jersáková J, Kubátová B, *et al.* 2012. Minority cytotypes in European populations of the *Gymnadenia conopsea* complex (Orchidaceae) greatly increase intraspecific and intrapopulation diversity. *Annals of Botany* **110**: 977–986.

Trygg J, Wold S. 2002. Orthogonal projections to latent structures (O-PLS). *Journal of Chemometrics* **16**: 119–128.

Tutz G, Hennevogl W. 1996. Random effects in ordinal regression models. *Computational Statistics & Data Analysis* **22**: 537–557.

Vít P, Lepší M, Lepší P. 2012. There is no diploid apomict among Czech *Sorbus* species: a biosystematic revision of *S. eximia* and discovery of *S. barrandienica*. *Preslia* **84**: 71–96.

Wehrens R. 2011. *Chemometrics with R*. Berlin: Springer-Verlag.

Wei N, Tennessen JA, Liston A, Ashman TL. 2017. Present-day sympatry belies the evolutionary origin of a high-order polyploid. *New Phytologist* **216**: 279–290.

Weiss H, Dobeš C, Schneeweiss GM, Greimler J. 2002. Occurrence of tetraploid and hexaploid cytotypes between and within populations in *Dianthus* sect. *Plumaria* (Caryophyllaceae). *New Phytologist* **156**: 85–94.

Weiss-Schneeweiss H, Emadzade K, Jang TS, Schneeweiss GM. 2013. Evolutionary consequences, constraints and potential of polyploidy in plants. *Cytogenetic and Genome Research* **140**: 137–150.

Wendel JF. 2015. The wondrous cycles of polyploidy in plants. *American Journal of Botany* **102**: 1753–1756.

Westerhuis JA, Hoefsloot HC, Smit S, *et al.* 2008. Assessment of PLSDA cross validation. *Metabolomics* **4**: 81–89.

Wickham H. 2016. *ggplot2: elegant graphics for data analysis*. New York: Springer.

Wilson J, Perry A, Shepherd JR, Durán-Castillo M, Jeffree CE, Cavers S. 2020. Invasion, isolation and evolution shape population genetic structure in *Campanula rotundifolia*. *AoB Plants* **12**: 1–14.

Wold S, Sjöström M, Eriksson L. 2001. PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **58**: 109–130.

Wos G, Mořkovská J, Bohutínská M, *et al.* 2019. Role of ploidy in colonization of alpine habitats in natural populations of *Arabidopsis arenosa*. *Annals of Botany* **124**: 255–268.