Genetically divergent cytotypes of *Vaccinium uliginosum* co-occurring in the central Eastern Alps can be distinguished based on the morphology of their flowers

Geneticky divergentní cytotypy *Vaccinium uliginosum* ze střední části Východních Alp se liší morfologií květů

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The cloudberries (*Vaccinium uliginosum* s. lat.) are among the most frequent constituents of dwarf shrub communities in the Alps. Recently, we have shown that (i) both diploid and tetraploid cytotypes are widespread in the Eastern Alps and sometimes occur in mixed "populations" and that (ii) these two cytotypes correspond to two non-overlapping AFLP groups, which are in almost perfect congruence with two plastid lineages. Further (iii), we revealed that diploids and tetraploids show some degree of niche separation, but co-occur in low-alpine heath vegetation. Finally, (iv), in spite of the clear separation of diploids and tetraploids in the molecular data sets, we failed to detect consistent morphological differences. Here, we focused on patterns of genetic, ecological and morphological divergence between cytotypes at mixed-ploidy sites as well as on the underlying processes. Our study is based on a sampling of a hundred individuals each at three sites with previously determined co-occurrence of diploids and tetraploids in the Central Alps of Tirol, Austria. We employed an integrative approach combining flow cytometry, plastid DNA sequencing, AFLP fingerprinting, morphometry of leaf and flower characters as well as a characterization of ecological niches based on the vegetation surrounding the individual *Vaccinium* plants, in order to address the following questions. What is the small-scale distribution of diploid and tetraploid *V. uliginosum* s. lat. at the three sites investigated? Are the molecular genetic (AFLP and plastid DNA) relationships reflecting the previously detected divergence governed by differences in ploidy level or is there evidence of across-cytotype gene flow in areas of immediate co-occurrence of diploid and tetraploid *V. uliginosum* s. lat.? Is there evidence for ecological or morphological separation between cytotypes? More specifically, do flower characters allow for a better discrimination between cytotypes than the previously used leaf characters? We show that mixed-ploidy sites are clearly secondary contact zones as based on AFLP markers individuals cluster by cytotype and not by locality. We found no evidence for recent hybridization across the ploidy boundary but the intersection of biparentally inherited recombining AFLPs with maternally inherited plastid DNA sequences provided strong evidence for past inter-ploidy gene flow as one fifth of the tetraploids exhibited a haplotype otherwise restricted to diploids. Further, small-scale ecological segregation between cytotypes was weak, but statistically significant. Leaf characters indicated little differentiation between the cytotypes, whereas flower characters had relatively strong discriminative power. Overall, from a taxonomic point of view, we suggest that clear genome-wide differentiation combined with consistent morphological differences in flower size do not preclude the differentiation of two distinct species (diploid *V. gaultherioides* and tetraploid *V. uliginosum* s. str.).

K e y w o r d s: AFLP, cytotype coexistence, ecological differentiation, morphometrics, polyploidy

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Introduction

As in much of the arctic, boreal and temperate zones in the Northern Hemisphere (Hagerup 1933, Young 1970), the cloudberries (*Vaccinium uliginosum* s. lat.) are among the most frequent constituents of dwarf shrub communities in the Alps, ranging from lowland bogs through montane conifer forests to low-alpine heath communities. These plants form clones with an estimated maximal age ranging between 1390 and 1880 years (de Witte et al. 2012). Flowers are homogamous; self-compatibility but no apomixis is recorded (Jacquemart & Thompson 1996). In the Alps, *V. uliginosum* s. lat. forms a diploid-tetraploid complex; diploids are reported from the French Alps and tetraploids, with one record each, from the eastern Swiss and the Bavarian Alps (Lippert & Heubl 1989, Eidesen et al. 2007). Recently, we have shown that both cytotypes are actually widespread in the middle part of the Alpine arc; tetraploids are the only cytotype occurring in the Northern Limestone Alps, whereas both diploids and tetraploids range throughout the Central Alps and Southern Limestone Alps (Regele et al. 2017).

Previous research has shown that plastid DNA variation in *Vaccinium uliginosum* s. lat. is geographically structured into three major lineages (Alsos et al. 2005, Eidesen et al. 2007); two of them, the Amphi-Atlantic lineage and the Arctic-Alpine lineage, are reported also in the Alps in tetraploid and diploid individuals, respectively (Regele et al. 2017). Range-wide amplified fragment length polymorphism (AFLP) analysis reveals five geographic groups, which can be explained by them surviving in different Pleistocene refugia (Eidesen et al. 2007). Based on the same approach we detected two divergent groups within the Alps, which almost perfectly match the two cytotypes and the two plastid DNA lineages; the single exception is a tetraploid individual with a plastid haplotype of the Arctic-Alpine lineage (Regele et al. 2017).

In marked contrast to other polyploid complexes, there is little evidence for consistent niche differentiation between diploid and tetraploid *V. uliginosum* s. lat. in the Alps. Rather, diploids are restricted to high-altitude heath communities at or above the timberline, whereas tetraploids span a wide altitudinal range from pre-Alpine bogs over conifer forests to low-alpine heath communities (Regele et al. 2017). The chosen experimental design, however, revealed only three mixed-ploidy sites and thus prevented inferences about local niche differentiation. In other groups such as the autopolyploid complex of *Senecio carniolicus* detailed studies reveal strong niche differentiation among the cytotypes (now delineated as species; Flatscher et al. 2015) in spite of their co-occurrence within very short distances (a few decimetres; Hülber et al. 2009, Sonnleitner et al. 2010). Overall, niche differentiation is considered a prevalent mechanism in maintaining the stable coexistence of cytotypes, which is rarely experimentally demonstrated using reciprocal transplantations (Kolář et al. 2017).

As the intricate morphological variation in *V. uliginosum* s. lat. has a quantitative rather than qualitative nature, and much variability appears to be environmentally induced (Young 1970), the taxonomic status of these taxa in the southern European mountain ranges is controversial. Treatments range from recognizing a single morphologically plastic species (Polatschek et al. 1999) to discriminating *V. uliginosum* s. str. and *V. gaultherioides* as subspecies (Young 1970) or even species (Aeschimann et al. 2004, Fischer et al. 2008). In our previous study leaf characters failed to show consistent morphological differences. In particular, absolute size characters exhibit a strong inverse

correlation with altitude within the tetraploid cytotype, which prevented their use for reliable determination. It was concluded that future research should (i) focus on mixed-cytotype localities and thus eliminate the confounding effect of wide altitudinal gradients on morphological and ecological differentiation and (ii) include morphometric characters from reproductive organs in order to provide a definite answer to whether *V. uliginosum* s. str. and *V. gaultherioides* are two diagnosable evolutionary units that merit taxonomic recognition (Regele et al. 2017).

Here, we adopt an approach that is informed by the results of our previous study (Regele et al. 2017) and highly complementary. It is based on an in-depth sampling of a hundred individuals at each of three sites with previously determined co-occurrence of diploid and tetraploid *V. uliginosum* s. lat. in the Central Alps in Tirol, Austria. We employ an integrative approach combining flow cytometry, plastid DNA sequencing, AFLP fingerprinting, morphometry of leaf and flower characters as well as characterization of ecological niches via species coverage and Landolt indicator values of the vegetation surrounding the *Vaccinium* individuals in order to address the following questions. (i) What is the small-scale distribution of diploid and tetraploid *V. uliginosum* s. lat. at the three sites sampled? (ii) Are molecular genetic (AFLP and plastid DNA) relationships reflecting the previously detected divergence governed by differences in ploidy level or is there evidence of across-cytotype gene flow in areas where diploid and tetraploid *V. uliginosum* s. lat. co-occur? Is there evidence for (iii) ecological or (iv) morphological separation between cytotypes? More specifically, do flower characters allow for a better discrimination of diploids and tetraploids than the previously used leaf characters? Finally, we evaluate our results in terms of whether they support the taxonomic recognition of the two cytotypes.

Material and methods

Field survey and collection of plant material

We focused on three sites where diploids and tetraploids co-occur in the Central Alps of Tirol, Austria (Fig. 1), i.e. Zillertaler Alpen: Filzenkogel, NE slope, N 47°08'04" E 11°52'04", altitude 1913–1988 m a.s.l., hereafter P1; Zillertaler Alpen: near Mezuinalm, N 47°15'53" E 11°50'09", altitude 1777–1948 m a.s.l., hereafter P2; Stubaier Alpen: Kühtai, Mittertal, N 47°12'36" E 10°59'17", altitude 1977–2043 m a.s.l., hereafter P3. At each site, in order to avoid sampling clones, 100 individuals, each at least 1 metre apart from each other, were chosen at random. We aimed to sample one individual each from all discrete patches of *V. uliginosum* s. lat.; therefore, the area sampled varied across the three sites. During fieldwork, we were unable to distinguish the two cytotypes morphologically; therefore, any sampling bias can be safely excluded. In order to characterise the ecological niches of the two cytotypes within the three sites studied, for each individual sampled we recorded the accompanying vascular plants (except for *V. uliginosum* s. lat.), as well as the moss and lichen species growing in a 1 m^2 square centred on the target individual (henceforth termed relevé) and estimated their cover using the refined nine-degree Braun-Blanquet scale (Reichelt & Wilmanns 1973). In addition, we assessed the cover of the moss layer, the herbaceous plant layer and the total vegetation; the geographic position was determined using a handheld GPS. The coordinates of each individual were used

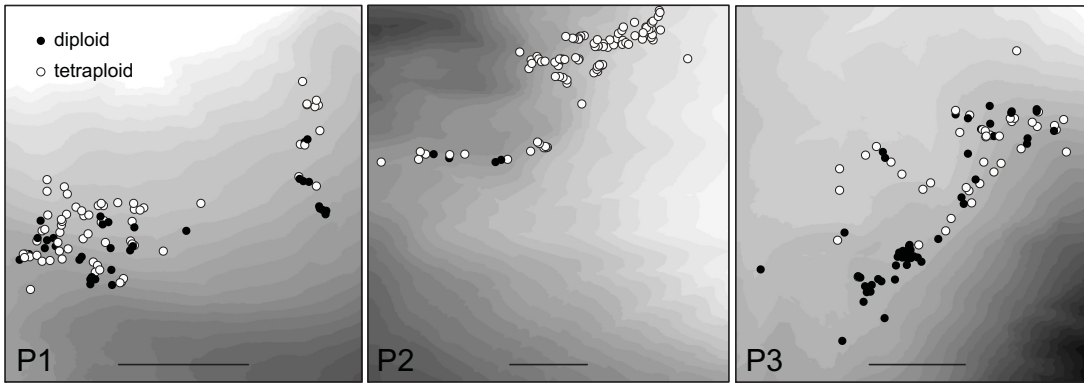

Fig. 1. – Spatial distribution of individuals sampled at the three sites of *Vaccinium uliginosum* s. lat. investigated in the central Eastern Alps. Increasing altitude is indicated by darker shading. The scale bar represents 200 m. Altitude is based on a digital elevation model with a spatial resolution (pixel size) of 1 m, provided by the Government of the Austrian Federal State Tyrol, Department of Geoinformation.

to calculate Moran's I, a measure of spatial autocorrelation, for P1 and P3 with the function Moran.I in R 3.4.3 (R Core Team 2017). Fresh leaf material was dried and stored in silica gel for AFLP and flow cytometric analyses. A vegetative shoot was sampled from all individuals as a herbarium voucher, and flowers collected from 101 individuals at P1 and P3 were stored in 80% ethanol for morphometric analyses; P2 could not be sampled at the appropriate phenological stage.

Estimation of relative genome size

Relative genome size (RGS) was estimated using flow cytometry (FCM) following the protocol described in Suda & Trávníček (2006). Nuclei of silica-gel-dried leaf tissue and reference standard (*Bellis perennis*, 2C = 3.38 pg; Schönswetter et al. 2007) were stained using 4',6-diamidino-2-phenylindole (DAPI). The relative fluorescence intensity of 3000 particles was recorded with a Partec CyFlow Space flow cytometer (Sysmex Partec GmbH, Münster, Germany) and Partec FloMax software was used to evaluate the histograms. The reliability of the measurements was assessed by calculating coefficients of variation (CVs) for the G1 peaks of both the analysed sample and the reference standard. Analyses yielding a CV threshold of > 5% were discarded.

DNA extraction, AFLP fingerprinting and analysis of AFLP data

We chose 180 individuals for DNA extraction in order to cover a similar number of individuals of each cytotype as well as all three of the sites investigated. The selected samples span both cytotypes' distributions within each site. P2 is under-represented as it contained only 4% diploids. Similar amounts of dried tissue (~10 mg) were used for DNA extraction following a CTAB protocol (Doyle & Doyle 1987) with some modifications (Schönswetter et al. 2002). AFLP profiles were generated following established protocols (Vos et al. 1995) with modifications either described in Schönswetter et al. (2009) or detailed below. One blank sample (DNA replaced by water) was included to test for systematic contamination, and reproducibility was tested by replicating 10 individuals

(Bonin et al. 2004). We used the same selective primers as in our previous study (Regele et al. 2017), i.e. *Eco*RI (6-FAM)ACA/*Mse*I-CAG, *Eco*RI (VIC)AGG/ *Mse*I-CAT and *Eco*RI (NED)AGC/*Mse*I-CAG (6-FAM-labelled primer: Sigma-Aldrich, St. Louis, Missouri, U.S.A.; NED- and VIC-labelled primers: Applied Biosystems, Foster City, California, U.S.A.) and employed the same protocol. The selective PCR product was purified using Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden), applied to a MultiScreen-HV plate (Millipore, Molsheim, France) in three steps of 200 µL each and packed at 600 *g* for 1, 1 and 5 min, respectively. Then 1.0 µL of the elution product was mixed with 10 µL formamide (Applied Biosystems) and 0.1 µL GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130 automated capillary sequencer at the Department of Botany and Biodiversity Research of the University of Vienna (Austria).

Electropherograms were analysed using Peak Scanner version 1.0 (Applied Biosystems) and default peak detection parameters, except for light peak smoothing. The minimum fluorescence threshold was set to 50 relative fluorescence units (rfu). Automated binning and scoring of the AFLP fragments were done using RawGeno 2.0–1 (Arrigo et al. 2009) in R 3.4.3 (R Core Team 2017) with the following settings: scoring range = 100–500 bp, minimum intensity = 50 rfu, minimum bin width = 1 bp and maximum bin width = 1.5 bp. Fragments with a reproducibility < 85% based on sample-replicate comparisons were eliminated. Fragments were coded as present (1) or absent (0) for all three primer combinations and combined in a binary data matrix. In order to explore the origin of the tetraploids, we determined the frequency of AFLP markers for each cytotype and each site separately. Non-hierarchical K-means clustering (Hartigan & Wong 1979) was done using the function find.clusters in adegenet (R 3.4.3; Jombart 2008). A total of 50 000 independent runs were carried out for each assumed value of K (i.e., the number of genetic groups, in our case ranging between 1 and 10) and the intergroup inertia recorded. The most likely number of groups was selected using intergroup inertia as a proxy of clustering accuracy (Evanno et al. 2005). A principal coordinate analysis (PCoA) based on a matrix of Jaccard distances among individuals was calculated using FAMD (Schlüter & Harris 2006).

Plastid DNA sequencing

We sequenced the intergenic plastid spacer separating *trn*S from *trn*G (Hamilton 1999), previously shown to be variable in *V. uliginosum* (Alsos et al. 2005, Regele et al. 2017). All 176 individuals, from which we obtained interpretable AFLP fingerprints, were sequenced. All reactions were carried out in a MasterCycler Gradient thermocycler (Eppendorf, Hamburg, Germany). For most individuals, the region was amplified in a 10 µl reaction containing 3.43 µl RedTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich), forward and reverse primer at 10 mM final concentration, 0.82 µl BSA (1 mg/ml; Promega, Madison, Wisconsin, U.S.A.) and 0.5 µl DNA of unknown concentration. PCR conditions were 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 45 seconds; final elongation was 72°C for 5 minutes. Twenty-three individuals failed to yield a PCR product; these were amplified with Phusion DNA Polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.) in a 20 µl reaction containing 0.2 µl polymerase, forward and reverse primers at 10 mM final concentration, 10 mM dNTPs, 4 µl 5× Phusion buffer and 1 µl DNA. PCR

conditions were 98°C for 30 seconds followed by 35 cycles of 98°C for 10 seconds, 63.4C° for 20 seconds, 72°C for 15 seconds; final elongation was 72°C for 10 minutes.

The quality of the PCR products was checked on 1.5% TBE agarose gels. Subsequently, the amplification products were purified enzymatically using Exonuclease I and Fast Alkaline Phosphatase (Thermo Fisher Scientific) according to the manufacturer's instructions. Sanger sequencing was done by a commercial sequencing facility (Eurofins MWG Operon, Ebersberg, Germany). Geneious 5.5.6 (Biomatters, Auckland, New Zealand) was used to align the sequences. Based on our previous study (Regele et al. 2017) we sequenced using only the *trn*S primer, even if discrimination of the common arctic-alpine haplotypes A and D, which is irrelevant for the present study, was not possible. As we were only interested in the extent of haplotype mixture between the cytotypes, no further analyses were conducted. Plastid haplotypes are named as in Regele et al. (2017).

Analysis of ecological data

The cover data recorded in the refined Braun-Blanquet scale (Reichelt & Wilmanns 1973) were transformed using the ordinal transformation of van der Maarel (1979). Plant and lichen species that occurred only in one relevé were excluded. Environmental conditions were characterized by mean Landolt indicator values (Landolt 2010), which describe the ecological requirements of species in terms of climate (temperature, T; continentality, K; light, L) and soil parameters (moisture, F; reaction, R; nutrients, N; humus content, H; aeration, D; moisture variability, W) ranging from 1 (low) to 5 (high). For species indifferent to a particular indicator, we used the median of the concerned indicator value in the relevé; *V. uliginosum* s. lat. was omitted from the calculation. The princomp function was used to carry out principal component analyses (PCAs) based on indicator values weighted by species cover in percent in three different data sets, P1, P3 and a set of the combined data from all three sites. Prior to this, the dataset was visually checked for normal distribution and Pearson correlation and non-parametric Spearman rank coefficients were computed to reveal the correlation structure. The cover values of accompanying vascular plants, lichens and bryophytes were visualized using non-metric multidimensional scaling (NMDS) and the function metaMDS in vegan (Oksanen et al. 2016). To evaluate the ecological differentiation of cytotypes we performed a redundancy analysis (RDA) using the function rda in vegan (Oksanen et al. 2016), based on Hellinger-transformed cover values. The transformation was done using the function decostand in vegan (Oksanen et al. 2016). The RDA was applied to the same three data sets as mentioned above.

Analysis of morphological data

Five mature leaves from the middle part of different leafy shoots of each herbarium specimen were glued on paper and scanned with a resolution of 600 dpi. Four leaf characters were measured using ImageJ and five flower characters were scored manually using a digital calliper. Flower characters were only available for 101 individuals, whereas leaf characters could be scored on all individuals. The latter included leaf area, leaf circularity, i.e. $4\pi(\text{area}/\text{perimeter}^2)$, a value of 1.0 for which indicates a perfect circle, the ratio leaf length / leaf width and the angle at the base of the lamina. The flower characters were length and maximal width of corolla, length of style, width of the constriction below the

corolla fringe and length of the pedicel. As preliminary analyses revealed that flower characters differentiate between diploids and tetraploids more strongly than leaf characters, we present analyses of 101 individuals based exclusively on flower characters as well as analyses of all individuals based exclusively on leaf characters.

Pearson correlation and non-parametric Spearman rank coefficients were computed to reveal correlation structure among the characters and to ensure that no strong correlations (> 0.95) between characters were present. Two outliers were removed from the dataset of flower characters. After standardization to zero mean and unit variance, a principal component analysis (zero-centred PCA based on a covariance matrix) was used to display the pattern in the overall variation along the first two components. To express the extent of morphological differentiation between the two ploidy-defined groups a linear discriminant analysis (LDA) and a classificatory discriminant analysis were carried out on the leaf, flower and combined data sets. Analyses were done using MorphoTools (Koutecký 2015) in R 3.4.3 (R Core Team 2017). ANOVAs for single morphometric variables were calculated using the aov function.

Results

Ploidy levels, AFLPs and plastid sequences

FCM analyses yielded high-resolution histograms and revealed two different ploidies. Mean ratios between sample and standard were 0.379±0.008 (mean±SD) for diploids and 0.785±0.013 for tetraploids, which are very similar to our previous results (Regele et al. 2017). Whereas at P1 and P3 diploids and tetraploids were sampled in comparable numbers (34% and 56% diploids, respectively), at P2 only 4% were diploid. No odd-ploidy cytotypes were detected. We were not able to obtain the RGS from four individuals, which were excluded from all analyses.

In total, 348 AFLP fragments were scored for 176 individuals; we failed to obtain reproducible fingerprints from four individuals, which were excluded. The error rate based on 10 sample-replicate comparisons was 2.5% (Bonin et al. 2004). Tetraploids harbour 28 private alleles of which seven have a frequency of between 13% and 33%, whereas diploids harbour only eight private alleles of which only two are frequent (both 14%). Six of the seven frequent private alleles of tetraploids occur at all three sites, the seventh occurs at two sites. Evaluation of the inter-cluster inertia in the K-means clustering indicates that K = 2 is the best number of clusters (intergroup inertia = 5.9% of the total inertia), separating diploids from tetraploids. We obtained *trn*S–*trn*G sequences from 171 individuals; five individuals repeatedly failed to amplify, probably due to a mutation at the priming site. Genbank accession numbers are presented in Electronic Appendix 1. We detected 71 diploids with haplotype A/D (haplotypes A and D from Regele et al. 2017 could not be separated due to the chosen sequencing approach, see above), 82 tetraploids with haplotype K and 18 tetraploids with haplotype A/D (Fig. 2).

Ecology

As Moran's I test for spatial autocorrelation yielded significant P-values and I(observed) is greater than I(expected), positive spatial correlation can be assumed for sites P1 and P3

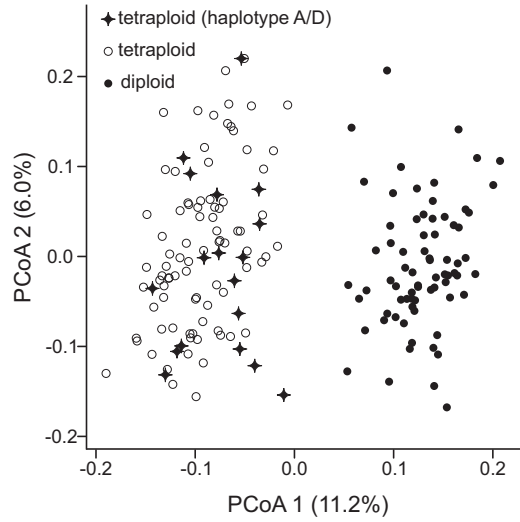

Fig. 2. – Principal coordinate analysis of Jaccard distances among AFLP multilocus phenotypes of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. Black circles represent diploid, white circles tetraploid and black stars tetraploid individuals with the plastid haplotype A/D, which is predominantly found in diploids.

Table 1. – Results of redundancy analyses (RDA) conducted to evaluate ecological differentiation between diploid and tetraploid cytotypes of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. Study site P2 was excluded due to the low number of diploid individuals sampled.

		df	Variance	F	P
combined data set	model	1	35.14	11.42	< 0.001
	residuals	296	910.69		
P1	model	1	0.0084	1.98	0.016
	residuals	98	0.4149		
P3	model	1	0.0215	3.94	< 0.001
	residuals	98	0.5342		

(P1: I(observed) = 0.091, I(expected) = –0.010, S.D. = 0.027, P < 0.001; P3: I(observed) = 0.186, I(expected) = –0.010, S.D. = 0.030, P < 0.001); at P2 too few diploids were found for a meaningful analysis. All Landolt indicator values were normally distributed and no correlation coefficient exceeded 0.95 for any pair of indicator values. The first two axes of the PCA of averaged Landolt indicator values of accompanying plant species explained 31.8% and 20.1% of the total variation (Fig. 3A); the narrower distribution of the diploids strongly overlaps the wider distribution of the tetraploids. The characters N (factor loading of the first component: 0.93), K (0.63), R (0.50) and T (0.47) influenced the structure most strongly. In the separate PCAs of P1 and P3 the separation is weaker than in the combined data set (Electronic Appendix 1). The NMDS of cover values of accompanying plant species showed a strong overlap between diploids and tetraploids (stress = 0.253; Fig. 3B). In contrast, RDAs, in spite of suggesting that there is more variation within species than between them, detected significant differences in the cover of accompanying plant species, at least for the combined data set and for P3; at P1 there were no significant differences (Table 1).

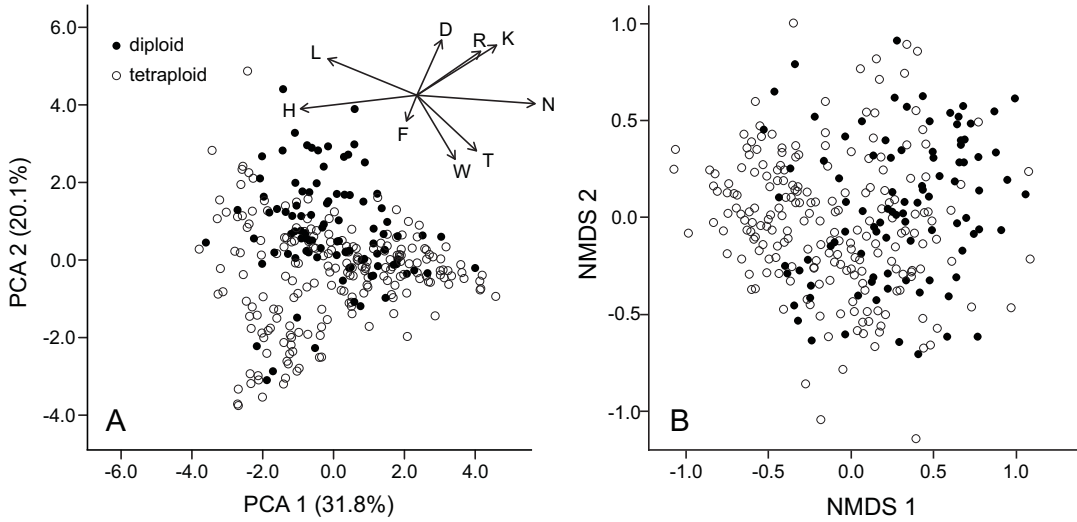

Fig. 3. – Ecological differentiation between diploid (black) and tetraploid (white) cytotypes of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. (A) principal component analysis based on average weighted Landolt indicator values of the surrounding vegetation. The inset shows relationships among the particular values projected in the same ordination space as the samples (D, aeration; F, moisture; H, humus content; K, continentality; L, light; N, nutrient; R, reaction; T, temperature; W, moisture variability). (B) non-metric multidimensional scaling of cover values of accompanying plant species. Stress value: 0.253.

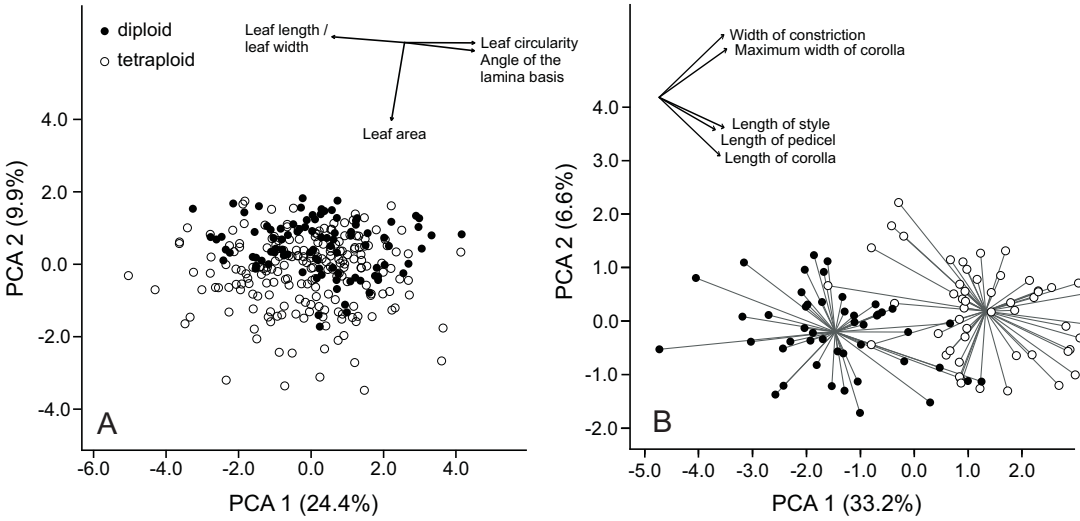

Fig. 4. – Principal component analyses illustrating morphological differentiation between diploid (black) and tetraploid (white) individuals of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. Arrows in the insets represent the contribution of the characters to the overall explained variation. For details see Table 2. (A) variation in leaf characters of all individuals; (B) variation in flower characters of 101 individuals. Lines connect to the centroid of each group.

Table 2. – Factor loadings for all morphometric characters (units are in squared brackets) used in the morphometric analyses of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. Principal component and linear discriminant analyses were done separately for leaf and flower characters. PC 1, factor loadings of the first component of the principal component analysis; PC 2, factor loadings of the second component of the principal component analysis; LDA, scores of discriminant function.

Leaf characters	PC 1	PC 2	LDA
Leaf area [mm^2]	–0.17	–1.00	–0.07
Leaf circularity	0.89	0.00	0.03
Leaf length / leaf width	–0.92	0.07	0.05
Angle at the base of the lamina [°]	0.88	–0.10	0.36
Flower characters	**PC 1**	**PC 2**	**LDA**
Length of pedicel [mm]	0.40	–0.30	0.16
Length of corolla [mm]	0.43	–0.54	–0.51
Maximum width of corolla [mm]	0.48	0.45	1.40
Width of the constriction below the corolla fringe [mm]	0.46	0.59	0.26
Length of style [mm]	0.46	–0.28	0.78

Table 3. – Absolute differences in flower characters between di- and tetraploid individuals of *Vaccinium uliginosum* s. lat. in the central Eastern Alps. The values represent 10% and 90% percentiles and the values in parentheses are minima and maxima.

Flower characters	Diploid	Tetraploid
Length of pedicel [mm]	(2.26–) 2.69–4.47 (–5.24)	(2.77–) 3.46–5.35 (–5.96)
Length of corolla [mm]	(3.23–) 3.51–4.33 (–4.74)	(3.63–) 3.85–4.71 (–5.27)
Maximum width of corolla [mm]	(2.27–) 2.54–3.19 (–3.54)	(2.90–) 3.28–4.25 (–4.46)
Width of the constriction below the corolla fringe [mm]	(1.63–) 1.93–2.35 (–2.49)	(2.10–) 2.27–2.92 (–5.34)
Length of style [mm]	(2.46–) 2.68–3.26 (–3.75)	(2.70–) 3.03–3.83 (–4.06)

Table 4. – Significance of linear discriminant analyses (LDA) based on different sets of morphometric characters measured for di- and tetraploid individuals of *Vaccinium uliginosum* s. lat. in the central Eastern Alps.

		df	Chi-square	F	P
Leaves and flowers	model	9	18.633	28.88	< 0.001
	residuals	89	54.127		
Leaves	model	4	0.251	24.31	< 0.001
	residuals	290	0.749		
Flowers	model	5	0.695	42.37	< 0.001
	residuals	93	0.301		

Morphology

Correlation coefficients did not exceed 0.95 for any character pair; therefore, all nine characters listed in Table 1 were retained. The PCA ordination diagram of the four leaf characters (296 individuals; Fig. 4A, Table 2) did not show any differentiation between diploids and tetraploids. In contrast, the PCA based on the flower characters suggested a relatively clear structure (Fig. 4B). Along the first axis diploid and tetraploid individuals were separated. The first two axes described 33.2% and 6.6% of the total variation. All characters contributed to the observed differentiation to a similar extent (Table 2). Likewise, the LDA based on flower characters showed a strong separation between diploids

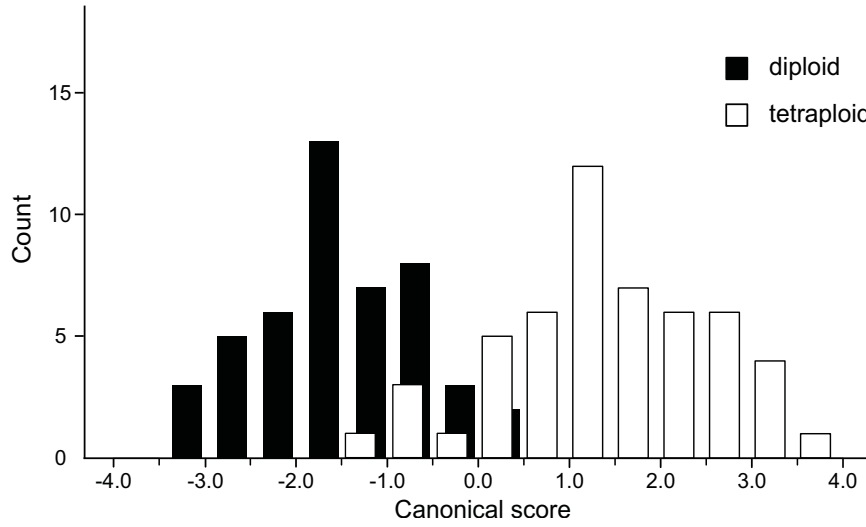

Fig. 5. – Histogram of the linear discriminant analysis of diploid (black) and tetraploid (white) individuals of *Vaccinium uliginosum* s. lat. in the central Eastern Alps based on flower characters of 101 individuals. For the contribution of the characters to the linear discriminant functions see Table 2.

and tetraploids (Fig. 5); the character maximum width of corolla contributed the most to the discrimination, followed by length of style (Table 2). A classificatory discriminant analysis of the combined data set of leaves and flowers classified 94% (diploids: 96%, tetraploids: 92%) of the individuals correctly. The same analysis conducted for flower characters separately correctly classified 93% (diploids: 96%, tetraploids: 90%) of the individuals in spite of a wide overlap of measured sizes (Table 3). LDAs were highly significant for all three data sets (Table 4). Independently conducted ANOVA models showed significant differences for all flower characters as well as for leaf area and angle at the base of the lamina (see Electronic Appendix 2 for details).

Discussion

Our previous study focussing on the large-scale distribution of diploid and tetraploid cytotypes of *Vaccinium uliginosum* s. lat. across the middle section of the Eastern Alps revealed the existence of mixed-ploidy "populations" (Regele et al. 2017). Here, we focus on patterns of genetic, ecological and morphological divergence between cytotypes in such populations as well as on the underlying processes. Importantly, in the area investigated, mixed-ploidy populations of *Vaccinium uliginosum* s. lat. are clearly forming secondary contact zones. This is supported by two lines of evidence. (i) Based on AFLP markers individuals clearly cluster by cytotype and not by locality (Fig. 2); the latter scenario would be expected in the case of local, recurrent origin of tetraploids. (ii) The seven frequent private AFLP fragments in tetraploids are not restricted to a single study site, but occur at all three sites with similar frequencies. This, again, rejects the hypothesis that tetraploids are locally evolved autopolyploids, but more likely indicates a long independent evolution, probably involving an origin well outside the Alpine region. Altogether,

we suggest that secondary contact zones formed in the course of the postglacial recolonization of the Alps from different glacial refugia, which were probably situated in the southern Alps for diploids and the forelands of the northern Alps for tetraploids (Regele et al. 2017).

Frequencies of diploids and tetraploids varied strongly across the three sites investigated. At P1 and P3, we sampled roughly comparable numbers of diploids and tetraploids whereas at P2 there were only 4% diploids probably because of the relatively low altitude. Although the small-scale distribution patterns (Fig. 1) revealed a relatively strong intermingling of cytotypes, there was significant spatial autocorrelation at P1 and P3 as revealed by Moran's I. This might indicate (weak) ecological differentiation or, alternatively, reflect limited dispersal or ability to reproduce vegetatively. The latter hypothesis, however, appears unlikely, as no clones were detected by the AFLP analysis. Longevity along with the ability to reproduce vegetatively and by selfing and, consequently, the lack of a need for regular sexual reproduction probably enabled *V. uliginosum* s. lat. to escape minority cytotype exclusion (see also Chrtek et al. 2017). In other polyploid complexes spatial segregation is identified as an important driver of successful cytotype co-occurrence, for example in *Chamerion angustifolium* (Sabara et al. 2013), *Gymnadenia conopsea* (Trávníček et al. 2011b), *Knautia arvensis* (Kolář et al. 2009), *Pilosella echioides* (Trávníček et al. 2011a) and *Senecio carniolicus* s. lat. (Sonnleitner et al. 2010).

In spite of sampling individuals of both cytotypes in close proximity we found no evidence for recent hybridization across the ploidy boundary. First, we did not detect any triploids in the c. 300 individuals investigated using flow cytometry. Second, the PCoA of AFLP data revealed no overlap of cytotypes along the first principal coordinate; the second principal coordinate, which reflected the intra-cytotype variation, explained only roughly half of the variation explained by the first coordinate (Fig. 2). The intersection of biparentally inherited, recombining AFLPs with maternally inherited plastid DNA sequences, however, provided strong evidence for past inter-ploidy gene flow. Roughly one fifth of the tetraploids exhibited haplotype A/D (Fig. 2), which is otherwise restricted to diploids. We suggest that co-occurrence of both cytotypes has fostered the occurrence of tetraploids with "diploid" plastid haplotypes because it enabled mating of unreduced egg cells of diploids with reduced male gametes of tetraploids in the past. Subsequent gene flow is then likely to have homogenized the nuclear genome towards that of tetraploids. Altogether, it is obvious that previous studies (Eidesen et al. 2007, Regele et al. 2017), which indicate a good congruence between AFLPs, plastid DNA haplotypes and ploidy level, have underestimated the number of tetraploids carrying plastid haplotypes captured from diploids. It is unclear if this points to the local emergence of tetraploids from diploid ancestors or – at the other extreme – to a single hybridization event in the past.

Frequency of across-cytotype gene flow and genetic differentiation among cytotypes differ strongly across the systems studied. In *Larrea tridentata* there is a stronger genetic differentiation between sympatric di- and tetraploids than between tetraploids and hexaploids. The occurrence of "diploid" haplotypes in 5% of tetraploid individuals is considered a strong indication for crosses between these two cytotypes (Laport et al. 2016). For diploid *Anthoxanthum alpinum* and tetraploid *A. odoratum* Pimentel & Sahuquillo (2008) show that individuals cluster genetically by locality and not by cytotype and that tetraploids from Scandinavia are more closely related to diploids from

Scandinavia than to tetraploids from the Iberian Peninsula. In the *Vaccinium oxycoccos* aggregate diploid *V. microcarpum* is genetically clearly distinguished from tetraploid *V. oxycoccos* with little evidence for gene flow. In comparison to *V. uliginosum* s. lat. it is likely that the last-mentioned system is further advanced along the speciation continuum, as the morphological differentiation is stronger and the tetraploids appear to outcompete the diploids (Smith et at. 2015).

Neither non-metric multidimensional scaling (Fig. 3A) of the cover values of accompanying plant species nor the PCA of the average weighted Landolt indicator values (Fig. 3B) revealed a clear small-scale ecological segregation between diploids and tetraploids, but the PCA showed that the distribution of the diploids is approximately a subset of that of the tetraploids, which is not only consistent with the general hypothesis that polyploids have a larger ecological range than their diploid relatives (Weiss-Schneeweiss et al. 2013), but also reflects the large-scale distribution pattern of the two cytotypes (Regele et al. 2017). We stress that the prevalence of tetraploids at humid microsites suggested by the PCA (lower left corner in Fig. 3A) is due to the fact that site P2, where tetraploids strongly predominate, is generally more humid than the two other sites, where no such segregation was obvious at the site level (Electronic Appendix 1). In contrast, an RDA revealed significant ecological separation of the cytotypes in the combined data set as well as at site P3 (Table 1). These results are surprising, as in the PCA the separation within P1 appears stronger than at P3. One reason for the somewhat ambiguous results may be that ecological separation, important to overcome minority cytotype exclusion, evolves slowly in *V. uliginosum* s. lat. due to the longevity of the individuals, the strong vegetative reproduction and the likely young age of the heteroploid populations. Clearer results may have been obtained in reciprocal transplant experiments, which are, however, not feasible as *V. uliginosum* s. lat. is a clonal, slow growing woody perennial. Other polyploid complexes, for instance the *V. oxycoccos* aggregate (Asada 2001) or the *Senecio carniolicus* aggregate (Hülber et al. 2009) do show a clear significant ecological segregation among cytotypes, which may even be greater in areas of cytotype co-occurrence ("niche displacement"; Sonnleitner et al. 2016). This may be due to their shorter life cycles (e.g. in *Senecio carniolicus* aggregate) or longer period of co-occurrence. It is important to stress that the sites sampled were explicitly selected to represent contact zones between the two entities, which have much greater ecological amplitudes when their Alpine range or even their entire ranges are taken into account (Alsos et al. 2005, Eidesen et al. 2007, Regele et al. 2017). In any event, our results are in line with those of Regele et al. (2017), which support the frequently criticized (e.g. Petit & Thompson 1999) view that polyploids have larger ecological amplitudes than diploids.

With the exception of the characters "leaf area" – big leaves are exclusively found on tetraploid plants, which are generally more variable in their leaf size than diploids – and "angle at the base of the lamina" we found no differentiation in leaf characters (Fig. 4A) between the cytotypes, which reflects the results of our previous study (Regele et al. 2017). In contrast, flower characters differentiate between diploids and tetraploids relatively well (Fig. 4B, Table 2) and allow for a correct classification of 96% and 90% of diploids and tetraploids, respectively (Fig. 5). Adding leaf characters increased the average correct assignment by only 1% and does not impact the results of the LDA (Table 4). Differences are, however, not qualitative but only quantitative as tetraploids bear significantly bigger flowers (Table 3, Electronic Appendix 1). These findings concur with the

situation in the *V. oxycoccos* aggregate, where tetraploids are more variable in morphology than diploids and flower characters differentiate better between cytotypes than leaf characters (Smith et al. 2015). It is unclear if the difference in flower size is driven by, or at least reflected in, different spectra of pollinators. Generally, pollinators of alpine plants are relatively unspecific (Olesen & Jordano 2002), which may support the hypothesis that the different flower size is a direct effect of the bigger cell sizes (Gregory 2001) of tetraploids. This is also supported by the fact that we did not observe any separation in phenology, which is often triggered by differences in pollinator spectra. In contrast, Smith et al. (2015) found different numbers of di- and tetraploids when sampling flowering individuals of the *Vaccinium oxycoccos* aggregate. As outlined above, leaf size shows no clear ploidy-correlated pattern but a strong overlap between di- and tetraploids, which may be explained by a stronger modifying effect of the environment on leaves than on flowers.

Conclusions

Our results from zones where diploid and tetraploid cytotypes of *V. uliginosum* s. lat. are in close contact with one another complicate the somewhat simplistic picture emerging from our previous study (Regele et al. 2017). Most significantly, we show that (i) tetraploid plants carrying a plastid haplotype characteristic of diploids are relatively frequent. Further, (ii) it is evident that diploids and tetraploids are not indistinguishable as previously suggested based on leaf characters, but can be relatively safely discriminated when flowering. In turn, it is obvious at least for plants from high altitudes, where both cytotypes co-occur, that determinations based on other characters, which are the overwhelming majority because of the short and early flowering season, have a high chance of being wrong.

The question remains how to deal with the two cytotypes taxonomically. As indicated before, treatments vary widely from recognizing two species to merging both cytotypes into a single species. In general, taxonomic treatments of polyploid complexes with co-occurring cytotypes differ strongly. Whereas in the autopolyploid complex of *Senecio carniolicus* s. lat. the cytotypes are delineated as species (Flatscher et al. 2015), in *Gymnadenia conopsea* s. lat. this is only partly possible because of a mismatch of genetic lineages and morphological groups (Trávníček et al. 2011b). In several other groups no taxonomic recognition is envisaged, usually due to the impossibility of morphologically characterizing the cytotypes, for instance in *Chamerion angustifolium* (Kennedy et al. 2006), *Galax urceolata* (Nesom 1983), *Pilosella echioides* (Trávníček et al. 2011a), *Ranunculus kuepferi* (Cosendai et al. 2013) and *Solidago altissima* (subspecies are described but are doubtful; Etterson et al. 2016). We strongly emphasise that it is premature to draw taxonomic conclusions about a species complex with a Holarctic distribution based on results from three sites in the Austrian Central Alps. However, in the absence of data from other areas we cautiously suggest that our results of a clear genome-wide differentiation combined with consistent morphological differences in flower size do at least not reject the differentiation of two distinct species (diploid *V. gaultherioides* and tetraploid *V. uliginosum* s. str.) as for instance proposed by Fischer et al. (2008).

See www.preslia.cz for Electronic Appendices 1–2

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Souhrn

Vlochyně (*Vaccinium uliginosum* s. lat.) jsou jednou z nejvýznamnějších složek vysokohorské keříčkové vegetace Alp. Nedávno jsme zjistili, že ve Východních Alpách se vyskytují jak diploidy, tak tetraploidy, a že oba cytotypy někdy vytvářejí smíšené populace. Tyto cytotypy odpovídají dvěma genetickým skupinám odhaleným metodou AFLP a z velké části se shodují také se dvěma haploidními skupinami. Diploidy a tetraploidy také mají částečně odlišné niky. I přes zřetelnou diferenciaci na molekulární úrovni nebyly nalezeny spolehlivé morfologické znaky. V této studii jsme se zaměřili na genetickou, ekologickou a morfologickou diferenciaci mezi cytotypy uvnitř smíšených populací v Tyrolsku. Využili jsme kombinaci metod zahrnující průtokovou cytometrii, sekvenování chloroplastové DNA, analýzu metodou AFLP, morfometriku vegetativních i generativních orgánů a zkoumání ekologických nik prostřednictvím analýzy složení okolní vegetace. Cílem bylo zjistit, jaké je rozšíření obou cytotypů na malé škále, zda molekulární metody potvrdí diferenciaci mezi oběma cytotypy, zda mezi nimi v těchto smíšených populacích dochází ke genovému toku a jestli existuje nějaká ekologická a morfologická diferenciace. Zjistili jsme, že tyto smíšené populace představují sekundární hybridní zónu, neboť genotypy jednotlivých rostlin odhalené metodou AFLP se klastrují podle cytotypů a nikoliv podle lokalit. Nenašli jsme žádný důkaz pro výskyt právě probíhající hybridizace mezi oběma cytotypy, ale kombinace molekulárních metod odhalila, že k hybridizaci došlo v minulosti, neboť 1/5 tetraploidů má haplotyp vyskytující se jinak jen u diploidů. Ekologická diferenciace na malé škále byla nízká, ale statisticky průkazná. Znaky na listech byly nevýznamné pro morfologickou identifikaci cytotypů, zatímco znaky na květech se ukázaly jako významné. Z taxonomického pohledu zřetelná genetická diferenciace spolu s rozdíly ve velikosti květů umožňují rozlišení dvou druhů, diploidní *V. gaultherioides* a tetraploidní *V. uliginosum* s. str.

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