Do ploidy level, morphology, habitat and genetic relationships in Alpine *Vaccinium uliginosum* allow for the discrimination of two entities?

Lze na základě ploidie, morfologie, stanovištních nároků a genetických vztahů mezi alpskými populacemi *Vaccinium uliginosum* odlišit dva taxony?

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Evolutionary processes such as recurrent origin of polyploids and enhanced gene flow among polyploids make polyploid complexes taxonomically highly intricate. One such complex is the mostly diploid and tetraploid cloudberries (*Vaccinium uliginosum* L. s.l.), which are not only one of the most frequently recorded dwarf shrubs in the Arctic, but also in mountain ranges such as the European Alps. Surprisingly, in spite of its ubiquity there is very little information on variation in ploidy level, habitat and morphology of *V. uliginosum* s.l. across the Alps and in adjacent areas; also the taxonomic status of *V. uliginosum* s. str. and *V. gaultherioides* is controversial. Based on five latitudinal transects sampled across the middle Alpine arc and an integrative approach combining flow cytometry, plastid DNA sequencing, amplified fragment length polymorphism fingerprinting, morphometrics and characterization of ecological niches via phytosociology and Landolt indicator values the following questions were addressed: (i) What is the large-scale distribution of diploid and tetraploid *V. uliginosum* s.l. in the area sampled? (ii) Are molecular genetic relationships governed by ploidy level differentiation or is there evidence for across-cytotype gene flow causing geography-correlated clustering? Is there evidence for (iii) ecological or (iv) morphological separation between cytotypes? We revealed that tetraploids occur throughout the area investigated in the Alps and in their northern forelands, whereas diploids appear to be restricted to the Central Alps and the Southern Limestone Alps. The two cytotypes corresponded to two non-overlapping groups in the AFLP data set, and were also in almost perfect congruence with two plastid lineages. The single evidence for gene flow between diploids and tetraploids was a tetraploid individual carrying a haplotype from an otherwise exclusively diploid lineage. Diploids and tetraploids show some degree of niche separation, which is mostly due to the exclusive occurrence of tetraploids at low altitudes; above 1500 m a.s.l. both cytotypes co-occur. Accordingly, tetraploids occur in a broader range of plant communities than diploids. In spite of the clear separation of diploids and tetraploids in the molecular data sets we failed to detect consistent morphological differences. In particular, absolute size characters exhibited a strong inverse correlation with altitude within the tetraploid cytotype, which prevents their use for reliable determination. Consequently, based on the characters employed here, it is currently impossible to morphologically discriminate diploid *V. gaultherioides* and tetraploid *V. uliginosum* s. str. in areas where their ranges overlap, i.e. in subalpine and alpine dwarf shrub communities.

K e y w o r d s: AFLP, cytotypes, flow cytometry, polyploidy, niche differentiation

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Introduction

Biota of mountain areas and the Arctic underwent pronounced reshuffling of distribution areas during the Pleistocene. In contrast to temperate biota (Hewitt 2004), it is likely that cold-adapted species expanded their ranges during glacials and experienced range restriction to refugia during interglacials (Stewart et al. 2010). This alternation of phases of allopatric differentiation and secondary contact is likely to have triggered the formation of polyploid complexes (Otto & Whitton 2000, Guggisberg et al. 2009). As a result of evolutionary processes such as recurrent origin of polyploids and enhanced gene flow among polyploids (Soltis & Soltis 2009) there are several polyploid complexes among the taxonomically most intricate non-apomictic groups (Abbott & Brochmann 2003). Whereas some polyploid complexes have been subjects of systematic evolutionary research for several decades (e.g. *Achillea*, Clausen et al. 1948), others, such as the *Senecio carniolicus* aggregate, were considered ploidy uniform until recently (Suda et al. 2007, Flatscher et al. 2015).

Throughout much of the arctic, boreal and temperate zones in the Northern Hemisphere, the cloudberries (*Vaccinium uliginosum* L. s.l.) are among the most frequent constituents of dwarf shrub communities ranging from lowland bogs through montane conifer forests to low-alpine heath communities (Hagerup 1933, Young 1970). They form mostly a diploid-tetraploid complex, with hexaploids only reported from Beringia and Japan (Young 1970, Eidesen et al. 2007). Previous research has shown that the variation in plastid DNA is geographically structured into three major lineages (Alsos et al. 2005, Eidesen et al. 2007), i.e. the Amphi-Atlantic lineage (tetraploid), Beringian lineage (tetraploid, hexaploid) and Arctic-Alpine lineage (diploid). Amplified Fragment Length Polymorphism (AFLP) analysis revealed five geographic groups, which could be explained by them surviving in different Pleistocene refugia. Last, nuclear ribosomal Internal Transcribed Spacer (ITS) sequences, in contrast, mostly differentiate between diploids and polyploids, but cloning reveals that most individuals actually contain sequences of both major ITS types. Incongruences among the three data sets may be due to evolutionary processes taking place on different time horizons, for instance major episodes of unidirectional nuclear gene flow between early diverged cytotypes in shared refugia (Eidesen et al. 2007).

In spite of the frequent occurrence of *V. uliginosum* s.l. in the Alps and adjacent areas there is little information on variation in ploidy level, habitat and morphology across populations. Diploids are reported from the French Alps and tetraploids, with one record each, from the eastern Swiss and Bavarian Alps (Lippert & Heubl 1989, Eidesen et al. 2007), Schwarzwald (Germany) and Vosges (France; Gregor & Hand 2006). As the intricate morphological variation has a quantitative rather than qualitative nature, and much variability appears to be environmentally induced (Young 1970), the taxonomic status of the taxa constituting *V. uliginosum* s.l. in the southern European mountain ranges is controversial. Treatments range from recognising 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).

Here, based on sampling five latitudinal transects across the middle part of the Alpine arc and an integrative approach combining flow cytometry, plastid DNA sequencing, amplified fragment length polymorphism (AFLP) fingerprinting, morphometrics and

characterization of ecological niches via phytosociology and Landolt indicator values of the vegetation surrounding the *Vaccinium* individuals we addressed the following questions: (i) What is the large-scale distribution of diploid and tetraploid cytotypes of *V. uliginosum* s.l. in the area sampled? (ii) Are molecular genetic (AFLP and plastid DNA) relationships governed by differences in ploidy level or is there evidence of across-cytotype gene flow causing geography-correlated clustering? Is there evidence for (iii) ecological or (iv) morphological separation between cytotypes? Finally, we evaluate our results in terms of whether they can be used to discriminate between taxa.

Material and methods

Sampling

In summer 2013 we sampled 135 individual plants from 45 populations of *V. uliginosum* s.l. along five north-south orientated transects through the middle part of the Alpine arc (Switzerland, Germany, Austria, Italy; Fig. 1, Table 1). Leaf material was dried and stored in silica gel for flow cytometry and DNA extraction. Representative parts of all the individuals sampled were preserved as herbarium specimens for morphometric analyses.

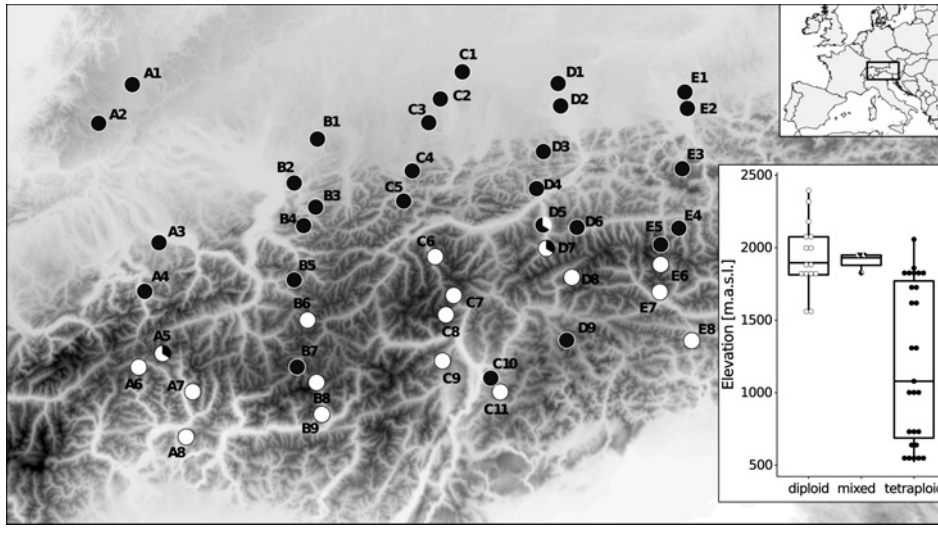

Fig. 1. – Populations sampled and ploidy level of the individuals of *Vaccinium uliginosum* s.l. studied in the middle part of the Alpine arc. Population identifiers correspond to Table 1 and pie charts indicate the ploidy level of the typically three investigated individuals per population (white, diploid; black, tetraploid). The upper insert indicates the position of the area sampled in Europe; the lower insert shows the altitudinal distribution of the populations studied.

Table 1. Sampled populations of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc. Except for population D3 with two investigated individuals three individuals were sampled and subjected to flow cytometry. For populations showing variation in cytotype or plastid haplotype the number of individuals per cytotype or haplotype is indicated in brackets. Genbank accession numbers include the associated haplotype in brackets for mixed populations.

ID	Sampling locality	Latitude N	Longitude E	Elevation [m a.s.l.]	n_{AFLP}	RGS ± SD	Ploidy	Plastid haplotype	trnS-trnG accession	trnL-trnF accession
A1	DE, Schwenninger Moos	48.040	8.524	710	3	0.778±0.015	4	K	MF427727	MF427785
A2	DE, Glaser Moos near Kesselberg, Bonndorf	47.822	8.252	1014	3	0.788±0.020	4	K	MF427735	MF427793
A3	CH, Turbenmoos near Etzel	47.176	8.758	981	3	0.787±0.003	4	K	MF427762	MF427821
A4	CH, Eggenberge below Hüenderegg	46.904	8.656	1628	3	0.781±0.005	4	K	MF427761	MF427820
A5	CH, Passo del Lucomagno	46.563	8.800	1931	3	0.362±0.004; 0.782	2(2), 4(1)	A(1), D(1), K(1)	MF427760 (K), MF427759 (A), MF427758 (D)	MF427819 (K), MF427818 (A), MF427817 (D)
A6	CH, Maggia valley, above lake Sambuco	46.488	8.615	1818	3	0.366±0.004	2	D	MF427754	MF427813
A7	CH, Alpe di Sceng	46.360	9.055	1560	3	0.359±0.004	2	A	MF427757	MF427816
A8	CH, western ridge of Monte Bar	46.104	8.999	1562	3	0.358±0.002	2	D(2)	MF427755-MF427756	MF427814-MF427815
B1	DE, Fetzachmoos	47.751	10.030	710	2	0.774±0.012	4	K	MF427743	MF427802
B2	AT, bog near Langen bei Bregenz	47.509	9.848	566	3	0.798±0.002	4	K	MF427749	MF427808
B3	CH, bog Bizau-Schönenbach	47.373	10.022	1023	3	0.799±0.012	4	K	MF427748	MF427807
B4	AT, Faschina, Zafernhorn, Gumpner Grätle	47.275	9.927	1854	3	0.791±0.009	4	K(2)	MF427750-MF427751	MF427809-MF427810
B5	CH, Schollberg, St. Antönien	46.975	9.853	2058	3	0.773±0.003	4	A, K(2)	MF427734 (A), MG252854-MG252855 (K)	MF427792 (A), MG252856-MG252857 (K)
B6	CH, Flüela pass	46.753	9.946	2394	3	0.367±0.003	2	D	MF427733	MF427791
B7	CH, Lej da Staz, St Moritz	46.499	9.870	1818	3	0.768±0.007	4	K(2)	MF427736-MF427737	MF427794-MF427795
B8	CH, Bernina pass	46.413	10.023	2318	3	0.364±0.004	2	D	MF427738	MF427796
B9	CH, Pescia Alta, Brusio	46.230	10.073	2099	2	0.354±0.002	2	D	MF427739	MF427797
C1	DE, Wildmoos near Gilching	48.112	11.224	576	3	0.808±0.004	4	K	MF427782	MF427841
C2	DE, Dettenhofer Filz	47.970	11.038	665	3	0.795±0.005	4	K	MF427725	MF427783
C3	DE, Oberoblander Filz	47.836	10.946	752	3	0.800±0.010	4	K	MF427726	MF427784
C4	DE, Schönleitenschrofen	47.571	10.808	1708	3	0.798±0.007	4	K	MF427780	MF427839
C5	AT, bog west of Berwang	47.409	10.736	1325	3	0.796±0.011	4	K	MF427779	MF427838
C6	AT, Niederthai, Hemerachalmen	47.103	10.971	1978	3	0.366±0.007	2	A	MF427747	MF427806
C7	IT, Oberglaueggalm, Timmelsjoch	46.882	11.111	2052	3	0.366±0.007	2	A	MF427746	MF427805
C8	IT, Lazins, Pfelders	46.782	11.064	1796	3	0.366±0.004	2	A(2)	MF427741-MF427742	MF427799-MF427800
C9	IT, Spitzn Sümpfl, Ulten	46.528	11.025	1870	3	0.368±0.003	2	A	MF427740	MF427798

ID	Sampling locality	Latitude N	Longitude E	Elevation [m a.s.l.]	n_{AFLP}	RGS ± SD	Ploidy	Plastid haplotype	trnS-trnG accession	trnL-trnF accession
C10	IT, Wölflmoor, Deutschnofen	46.430	11.410	1292	3	0.783±0.005	4	K	MF427744	MF427803
C11	IT, Lavaze Joch, Malga Varena	46.352	11.484	1846	3	0.366±0.007	2	A	MF427745	MF427804
D1	DE, Katzenreuter Filze	48.032	12.006	560	3	0.778±0.010	4	K	MF427781	MF427840
D2	DE, Filzenklaas S of Tuntenhausen	47.916	12.029	521	3	0.786±0.009	4	K	MF427752	MF427811
D3	DE, Spitzingsee moor	47.830	11.883	1079	2	0.795±0.010	4	K	MF427753	MF427812
D4	AT, S of lake Zirein, Rofan	47.464	11.812	1803	3	0.788±0.006	4	K	MF427763	MF427822
D5	AT, near Mezuinalm	47.266	11.844	1827	3	0.387; 0.792±0.003	2(1), 4(2)	A(1), K(2)	MF427765 (A), MF427764, MF427766 (K)	MF427824 (A), MF427823, MF427825 (K)
D6	AT, Gerlosplatte	47.242	12.141	1609	2	0.779±0.016	4	K	MF427732	MF427790
D7	AT, Filzenkogel, NE slope	47.135	11.877	1967	3	0.377±0.004; 0.768	2(2), 4(1)	A(2), K(1)	MF427767-MF427768 (A), MF427769 (K)	MF427826-MF427827 (A), MF427828 (K)
D8	IT, path towards Knuttenalm	46.969	12.083	1809	2	0.384±0.004	2	D	MF427776	MF427835
D9	IT, path towards Lavarellahütte	46.619	12.026	1846	3	0.784±0.013	4	K(2)	MF427777-MF427778	MF427836-MF427837
E1	DE, Röhrmoos near Innerwill	47.953	13.052	613	2	0.788±0.002	4	K	MF427729	MF427787
E2	DE, Ursprunger Moos	47.885	13.061	560	3	0.801±0.010	4	K	MF427730	MF427788
E3	DE, Gotzenalm, Feuerpalven	47.540	12.994	1745	3	0.794±0.007	4	K	MF427728	MF427786
E4	AT, surroundings of Jack-Hochalm, Rauris	47.221	12.956	1797	3	0.800±0.007	4	K	MF427731	MF427789
E5	AT, along Großglockner-Hochalpenstraße	47.131	12.808	1860	3	0.790±0.010	4	K	MF427770	MF427829
E6	AT, below Retschitzalpe	47.028	12.800	2019	3	0.400±0.001	2	A	MF427771	MF427830
E7	AT, surroundings of Zettersfeld	46.869	12.783	1896	3	0.399±0.006	2	A(2)	MF427772-MF427773	MF427831-MF427832
E8	IT, summit of Creta di Timau	46.596	13.009	2180	3	0.393±0.010	2	A(2)	MF427774-MF427775	MF427833-MF427834

Flow cytometry

Flow cytometry (FCM) of 4',6-diamidino-2-phenylindole (DAPI; final concentration 0.036 M) stained nuclei was used to estimate relative genome size (RGS) of silica gel-dried samples. The internal standard used to determine DNA amounts was *Bellis perennis* (2C = 3.38 pg; Schönswetter et al. 2007). Desiccated green leaf tissue (~ 0.5 cm^2) was chopped together with an appropriate amount of fresh reference standard and processed as described in Suda et al. (2007). The relative fluorescence intensity of 3,000 particles was recorded using a Partec CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Partec

FloMax software was used to evaluate the histograms, which were manually gated. RGS was calculated as the ratio of the relative fluorescence of sample and standard. The reliability of the measurements was assessed by calculating coefficients of variation (CV) for the G1 peaks of both the analysed sample and the reference standard. Analyses yielding a CV threshold of > 5% were discarded and the samples measured again.

DNA extraction, AFLP fingerprinting and analysis of AFLP data

Total genomic DNA was extracted from similar amounts of dried tissue (~10 mg) using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. 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 (DNA replaced by water) was included on each plate to test for contamination and reproducibility was tested by replicating 15 individuals from the same DNA extractions (Bonin et al. 2004). Out of the twelve initially screened selective primer combinations, the following three were chosen for the selective PCR (fluorescent dye in brackets): MseI-CAG and EcoRI-AAC(NED), Mse-CAT and EcoRI-ACA(6-FAM), Mse-CTT and EcoRI-AAG(VIC) (6-FAM labelled primers: Sigma-Aldrich, St. Louis, Missouri, USA; NED and VIC labelled primers: Applied Biosystems, Foster City, California, USA).

The selective PCR mix for the VIC and 6-FAM labelled primers contained 1 µl 10× RedTaq PCR reaction buffer (Sigma-Aldrich), 0.25 U RedTaq (Sigma-Aldrich), 0.22 µl dNTPs (10 mM; Applied Biosystems), 0.54 µl of each selective primer (MseI-primer: 5 µM; EcoRI-primer: 1 µM, Sigma-Aldrich) and 2 µl of the diluted preselective amplification product. The reaction mix for the NED labelled primer contained 0.4 U RedTaq. 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 minutes, respectively. Then 1 µl of the elution product was mixed with 10 µl formamide (Applied Biosystems) and 0.15 µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130 automated capillary sequencer.

Electropherograms were analysed using Peak Scanner version 1.0 (Applied Biosystems) and default peak detection parameters, except light peak smoothing. The minimum fluorescent threshold was set to 50 relative fluorescence units. Automated binning and scoring of the AFLP fragments were performed using RawGeno 2.0-1 (Arrigo et al. 2009) in R 3.3.0 (R Core Team 2016) with the following settings: scoring range = 50–500 bp, minimum intensity = 100 relative fluorescence units (rfu), minimum bin width = 1 bp, and maximum bin width = 1.5 bp. Fragments with a reproducibility lower than 80% based on sample-replicate comparisons were eliminated. Markers present or absent in only one individual were excluded. A Jaccard distance matrix among individuals was calculated using the vegdist() function in the R package vegan (Oksanen et al. 2016). Principal coordinate analysis was performed using the R function cmdscale(). In order to evaluate the correlation between Jaccard distances among AFLP multilocus phenotypes on the one hand and matrices of geographic as well as altitudinal distances on the other, we performed separate Mantel tests for di- and tetraploids using the function mantel() in the package vegan (correlation method: Spearman's rho, 10000 permutations).

Plastid DNA sequencing

We sequenced two plastid DNA regions, i.e. the intergenic spacer regions separating trnL from trnF (primers e–f; Taberlet et al. 1991) and trnS from trnG (Hamilton 1999), which were previously shown to be variable in *V. uliginosum* (Alsos et al. 2005). Sequences from *V. myrtillus* and *V. vitis-idaea* were included as outgroups (Genbank accessions DQ073325.1, DQ073326.1 and DQ073200.1, DQ073201.1; Alsos et al. 2005). Typically, in ploidy-uniform populations a single individual was sequenced, whereas in heteroploid populations sequences were obtained for all the individuals sampled. In addition, in eight populations one or two additional individuals were sequenced to test for intrapopulation variation. All reactions were carried out using a MasterCycler Gradient thermocycler (Eppendorf, Hamburg, Germany). Both regions were amplified in a 20 µl reaction containing 7.5 µl RedTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich), forward and reverse primer at 250 nM final concentration, 0.9 µl BSA (1 mg/ml; Promega, Madison, Wisconsin, U.S.A.) and 1 µl DNA. 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.

The quality of the PCR products was checked on 1% TBE-agarose gels. Subsequently, the amplification products were purified enzymatically using Exonuclease I and Fast Alkaline Phosphatase (Thermo Scientific) according to the manufacturer's instructions. Cycle sequencing reactions were performed separately for each primer using BigDye Terminator chemistry (Applied Biosystems) according to the manufacturer's protocol, followed by electrophoresis on an ABI 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Geneious 5. 5. 6 (Biomatters, Auckland, New Zealand) was used to assemble and edit the contigs and to align the sequences. The alignment of the concatenated plastid markers was analysed using statistical parsimony as implemented in TCS 1.21 (Clement et al. 2000) with the connection limit set to 95%; gaps were treated as a fifth character state. Indels longer than 1 bp were reduced to a single base pair column allowing these structural mutations to be counted as a single base pair mutation.

Morphometrics

As only a few individuals had flowers and/or fruits at the time of collection we focused on vegetative characters; comprehensive analyses of reproductive structures of plants from mixed-ploidy populations are underway (L. Silbernagl, unpublished).

Plant height, leaf size and leaf shape are suggested for discriminating between *V. gaultherioides* and *V. uliginosum* (Fischer et al. 2008). Plant height was recorded in the field. For morphometric analyses, five mature leaves from the middle part of different branches of each herbarium specimen were glued on paper and scanned at a 600 dpi resolution. Size measurements were performed using Lamina (Bylesjö et al. 2008) and the following characters were used: leaf length, leaf width, the ratio of leaf width at 25% and 75% of leaf length, and leaf circularity, i.e. $4\pi(\text{area}/\text{perimeter}^2)$ – a value of 1.0 indicates a perfect circle. To characterize leaf shape, we employed SHAPE (Iwata & Ukai 2002) to obtain a size and rotation independent mathematical description of the leaf outline based on elliptic Fourier transformation. For the combined analysis of size and shape characters, the individual mean of the aforementioned measurements and of the principal components 1 and 2 from the SHAPE analysis were used. The pairwise correlation between

measured characters was tested to avoid including strongly correlated (Pearson's $R^2 > 0.9$) characters. Measurements were transformed to achieve a normal distribution (assessed by Shapiro-Wilks tests). All characters were scaled and centred using the R function scale() and PCA was performed on the scaled characters using the princomp() function. To evaluate the correlation of characters with altitude, a linear regression was fitted using the lm() function. For a graphical representation, characters were plotted against altitude. Significance of differences was tested using a multivariate analysis of variance [MANOVA, R function manova()]. Homogeneity of variance was confirmed using Levene's test. ANOVAs for single morphometric variables were calculated using the summary.aov() function.

Linear discriminant analysis (LDA) of morphological characters was performed using the function lda(). For the LDA we aimed to mitigate the effect of the wide altitudinal gradient by separating the dataset into three groups: tetraploids collected below 1500 m a.s.l., tetraploids from above 1500 m a.s.l. and diploids (sampled only above 1500 m a.s.l.). This also discriminates tetraploids associated with bog vegetation and those from alpine dwarf shrub communities, where both cytotypes co-occur.

Ecological differentiation of cytotypes

Vegetation was sampled in 1 m^2 plots centred on each target individual, recording the percentage cover of vascular plants, lichens and bryophytes. The relevés were classified using TWINSPAN 2.3 (Hill & Šmilauer 2005) and omitting *V. uliginosum* s.l. from the indicator species list. Further, non-metric multidimensional scaling (NMDS) was performed using the function metaMDS(). Prior to analysis, square-root transformed cover values of accompanying species of plants (*V. uliginosum* s.l. was omitted) were converted to Hellinger distances using the function decostand() in vegan (Oksanen et al. 2016).

Environmental conditions were characterized by mean Landolt indicator values of accompanying species of vascular plants (Landolt et al. 2010), weighted by their relative cover. Landolt indicator values describe ecological requirements of species in terms of climatic parameters (temperature, T; continentality, K; light, L) and soil parameters (moisture, F; reaction, R; nutrients, N; humus content, H; aeration, D; moisture variability, W), all ranging from 1 (low) to 5 (high). For species indifferent to particular indicator values, we used the median of the concerned indicator values at the respective sites; *V. uliginosum* s.l. was omitted from the calculation. Principal component analysis was performed in R using the princomp() function and average indicator values of accompanying species weighted by the percentage they make up of the total cover.

Results

Flow cytometry, AFLP fingerprinting and plastid DNA sequencing

FCM analyses yielded high-resolution histograms and revealed the presence of two RGS classes (0.372±0.002 and 0.789±0.001, respectively), which translate into 2C-values of 1.26±0.01 pg and 2.67±0.00 pg (mean±SE), respectively. These values closely fit the estimates for chromosomally verified diploid and tetraploid *V. uliginosum* s.l. (1.22±0.01 pg and 2.67±0.02 pg) from Scandinavia (Eidesen et al. 2007). Ploidy within populations

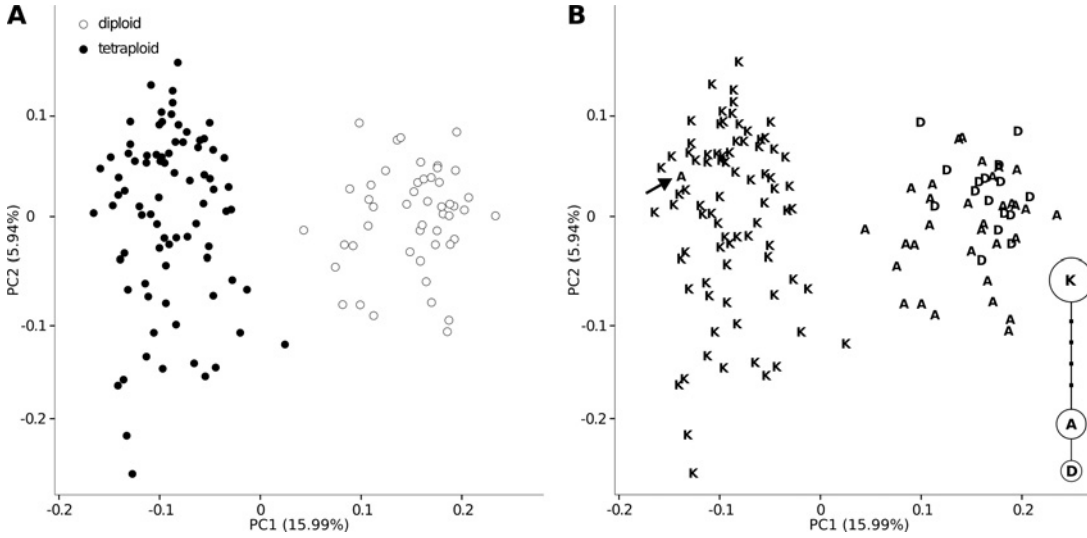

Fig. 2. – Principal coordinate analysis of Jaccard distances among AFLP multilocus phenotypes of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc. (A) White circles represent diploid and black circles tetraploid individuals. Letters in (B) reflect three different plastid DNA haplotypes, whose relationships are shown by a parsimony network; non-sampled haplotypes are indicated by black dots. The letters correspond to the haplotypes identified by Alsos et al. (2005). The arrow in Fig. 2B highlights one tetraploid individual with a haplotype predominantly found in diploids.

was uniform except for populations A5, D6 and D8; no triploids were detected. Populations in the northern forelands of the Alps are exclusively tetraploid, whereas diploids occupy the central and southern Alps (Fig. 1). A principal coordinate analysis of AFLP markers based on 248 polymorphic fragments (Fig. 2A) revealed two clearly separated clusters corresponding to diploids and tetraploids. Mantel tests revealed no correlation between genetic and geographic distances for tetraploids ($r = 0.05$, $P = 0.09$), whereas there was a significant correlation in diploids ($r = 0.35$, $P < 0.001$). No correlation between altitudinal and genetic distances was recorded for diploids ($r = 0.04$, $P = 0.285$), whereas tetraploids showed a weak correlation ($r = 0.06$, $P = 0.004$).

The concatenated matrix of trnS-trnG and trnL-trnF intergenic spacer sequences (GenBank accession numbers MF427725–MF427782, MG252854–MG252855 and MF427783–MF427841, MG252856–MG252857, respectively; Table 1) consisted of 1020 aligned positions. In total, three plastid haplotypes separated by a maximum of six mutational steps were found, which correspond to the haplotypes A, D and K identified by Alsos et al. (2005; Fig. 2B). Haplotypes segregated in terms of their ploidy levels and AFLP groups (Fig. 2B). Haplotypes A and D were present in diploid individuals, whereas in tetraploids haplotype K was predominant; only a single individual from the tetraploid population B5 had haplotype A. This was also the only case of occurrence of more than one haplotype in a ploidy-uniform population.

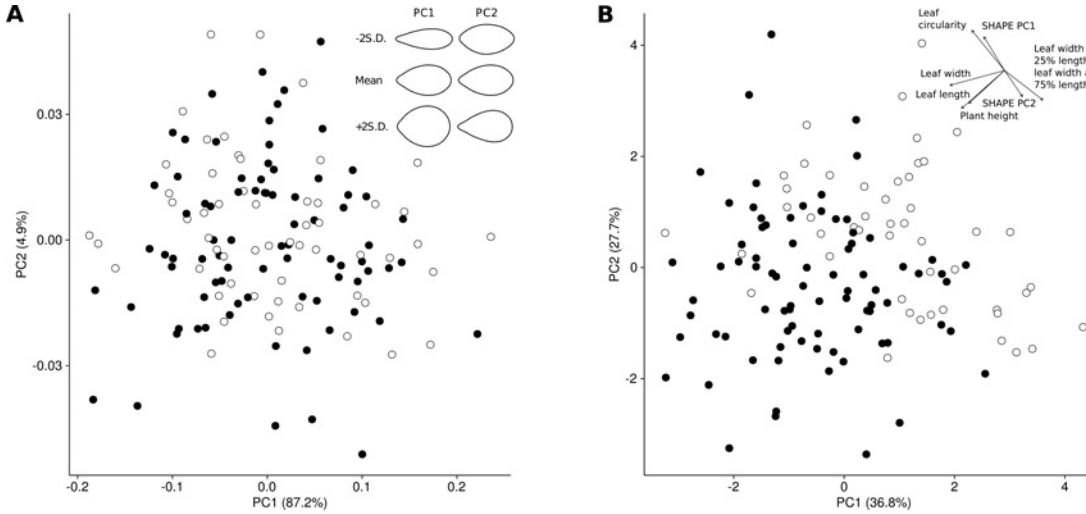

Fig. 3. – A, Variation in leaf shape of diploid (white) and tetraploid (black) individuals of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc based on reconstructed leaf outlines produced by the SHAPE analysis. The inset shows the variation of reconstructed leaf shapes along the first two principal components. B, Principal component analysis of diploid (white) and tetraploid (black) individuals of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc based on seven morphological characters. Arrows in the inset represent the contribution of the characters to the overall explained variation. A description of the morphological characters is provided in the main text; SHAPE PC1 and SHAPE PC2 are the first two principal components extracted by elliptic Fourier analysis of the leaf outlines shown in A.

Table 2. – ANOVAs comparing morphological characters between diploid and tetraploid *Vaccinium uliginosum* s.l.

Character		df	SS	MS	F	p
Leaf circularity	ploidy	1	5.757	5.757	0.272	0.603
	residuals	126	2665.53	21.155		
Leaf width	ploidy	1	0.329	0.329	32.062	<0.001
	residuals	126	1.291	0.010		
Leaf length	ploidy	1	0.364	0.364	46.779	<0.001
	residuals	126	0.980	0.008		
Width 25%/75%	ploidy	1	0.040	0.040	2.047	0.155
	residuals	126	2.450	0.019		
SHAPE PC1	ploidy	1	0.003	0.003	0.315	0.576
	residuals	126	1.052	0.008		
SHAPE PC2	ploidy	1	0.000032	0.000032	0.094	0.760
	residuals	126	0.044	0.00035		
Plant height	ploidy	1	33.201	33.201	55.659	<0.001
	residuals	126	75.161	0.597		

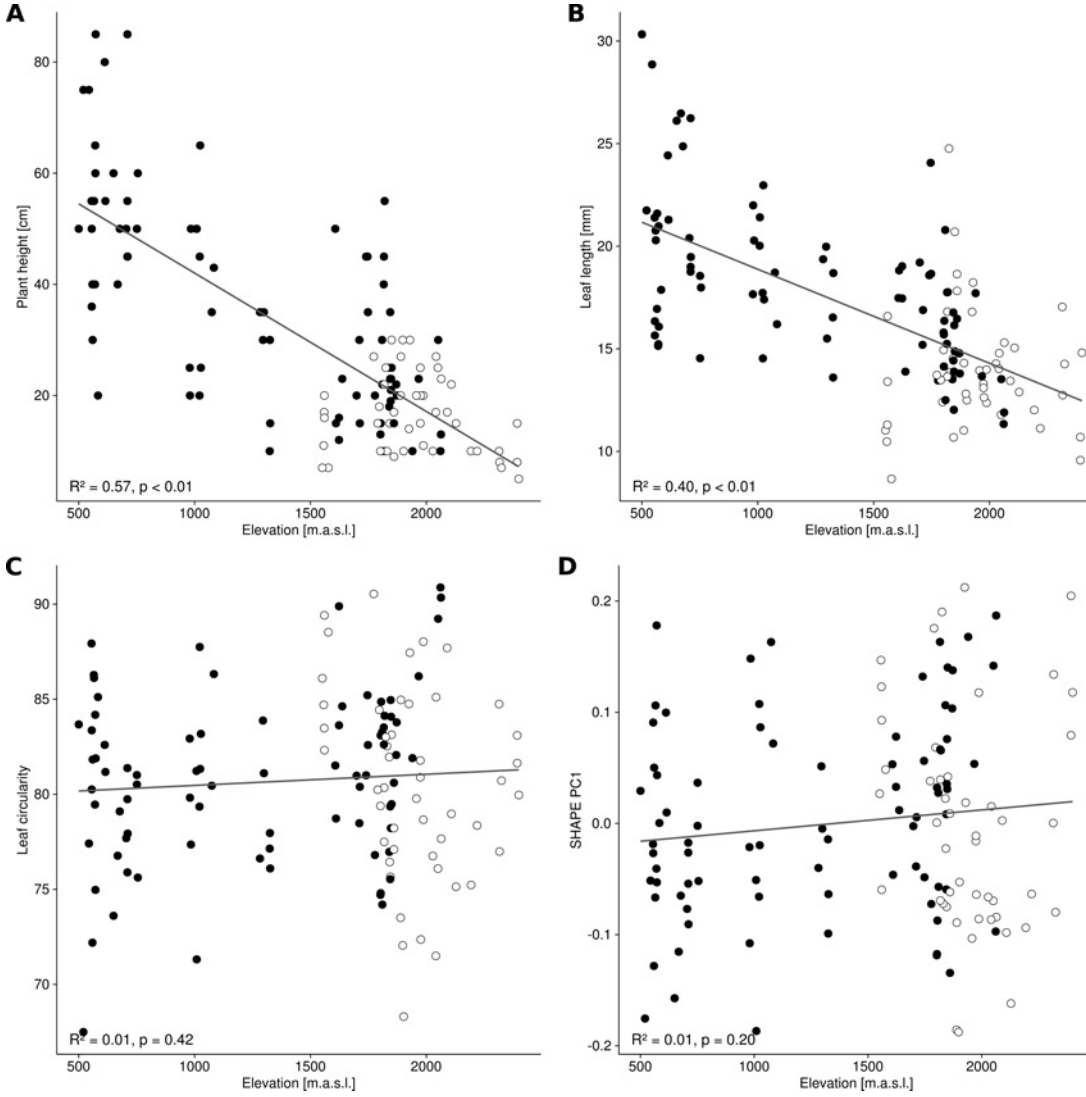

Fig. 4. – Scatterplots of morphologic characters scored in diploid (white) and tetraploid (black) individuals of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc and plotted against altitude. Plant height (A) and leaf length (B) show an inverse correlation with altitude, whereas leaf circularity (C) and the principal component 1 retrieved by SHAPE analysis (D) do not show such a trend.

Morphometrics

No distinct clusters were revealed by the SHAPE analysis (Fig. 3A), although the variation in leaf shape observed in the plants sampled was well represented in the reconstruction of leaf outlines. The recorded measurements did not show strong pairwise correlations; the strongest correlation was between leaf length and leaf width ($R^2 = 0.85$).

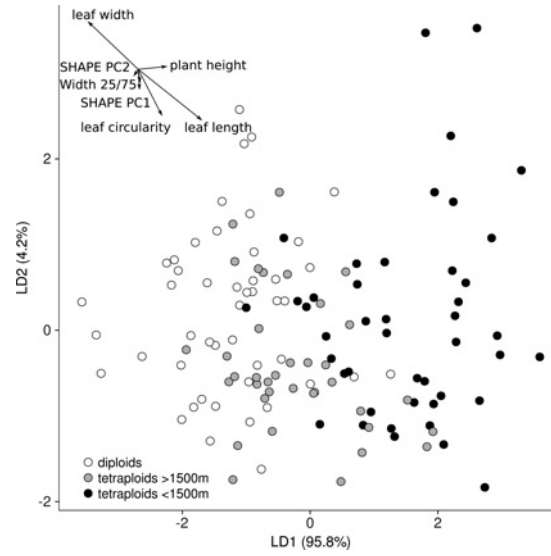

Fig. 5. – Linear discriminant analysis of morphological characters from diploid (white), low-altitude (<1500 m a.s.l.) tetraploid (black) and high-altitude (>1500 m a.s.l.) tetraploid (grey) cytotypes of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc. The inset shows the contribution of the characters to the linear discriminant functions. Morphological characters are described in the main text. Width 25/75 is the ratio of the leaf width at 25% and 75% of the length.

The MANOVA of the morphological characters revealed significant (Pillai's Trace = 0.4, F = 11.4, P < 0.001) differences between the two ploidy levels. ANOVAs for the single variables showed significant differences in leaf width, leaf length and plant height, whereas no such difference was observed in leaf shape characters. (Table 2). However, a principal component analysis of a combination of leaf shape characteristics and quantitative measurements of leaf size (Fig. 3B) showed no clear separation between diploids and tetraploids. Both analyses indicated similar variability in leaf shape; plant height, leaf length and leaf width contributed most to the separation of diploids from tetraploids, but the clusters overlap and the leaf size of tetraploid plants was highly variable. In addition, plotting absolute size characters such as leaf length and plant height against altitude revealed an inverse correlation of these characters with altitude (Fig. 4AB). Size-independent morphological characters such as leaf circularity or the principal component 1 from the SHAPE analysis (PC1) did not show this trend (Fig. 4CD). This observation was consistent across all the variables recorded (not shown: leaf width vs. altitude: $R^2 = 0.23$, $P < 0.01$; SHAPE PC2-altitude: $R^2 = 0.02$, $P = 0.11$; leaf width at 25% to leaf width at 75% of leaf length vs. altitude: $R^2 = 0.04$, $P = 0.03$).

The LDA of the morphological characters revealed that the diploids and low-altitude tetraploids differ whereas the high-altitude tetraploids overlap with the two other groups (Fig. 5). The characters contributing most to the separation were leaf size, plant height and, to a lesser extent, leaf circularity. Using the fitted model and the original data to classify the observations, diploids and low-altitude tetraploids were classified correctly in 81% and 77% of cases, respectively, whereas high-altitude tetraploids were classified correctly in only 44% of cases.

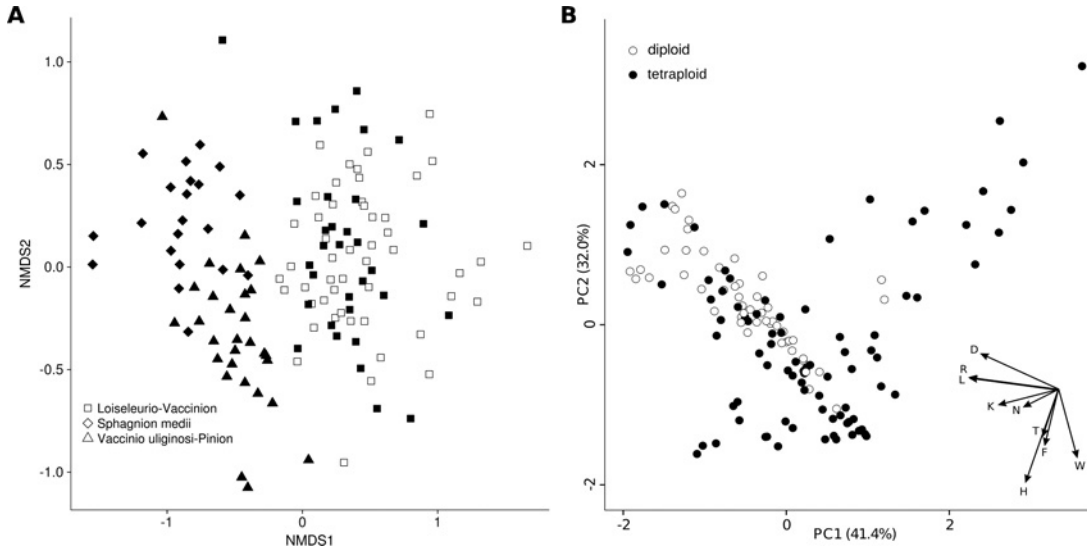

Fig. 6. – Phytosociological and ecological differentiation between diploid (white) and tetraploid (black) cytotypes of *Vaccinium uliginosum* s.l. from the middle part of the Alpine arc. A, non-metric multidimensional scaling; affiliation of relevés to three phytosociological alliances is indicated. Stress value, 0.205. B, Principal component analysis based on mean 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, nutrients; R, reaction; T, temperature; W, moisture variability.

Ecological differentiation between cytotypes

Diploids were confined to altitudes above 1500 m a.s.l. while tetraploids had a much broader altitudinal distribution (Fig. 1). The TWINSPAN analysis resulted in a differentiation of five units, which could be assigned to three phytosociological alliances. Diploids were restricted to *Loiseleurio-Vaccinion* communities (three TWINSPAN groups) whereas tetraploids were also found in *Sphagnion medii* and *Vaccinio uliginosi-Pinion* communities (two TWINSPAN groups). The NMDS corroborated the tripartite separation and showed little overlap between the three alliances (Fig. 6A). Environmental conditions assessed by mean weighted Landolt indicator values of the accompanying vascular plants showed no clear separation of diploid and tetraploid individuals (Fig. 6B). Diploids are confined to a comparatively narrow niche whereas the ecological conditions in areas where tetraploids occur vary considerably.

Discussion

Employing an integrative approach combining flow cytometry, plastid DNA sequencing, AFLP fingerprinting, morphometrics and characterization of ecological niches via phytosociology and Landolt indicator values of the surrounding vegetation we reveal that in the middle part of the Alpine arc *V. uliginosum* s.l. comprises two geographically weakly segregated cytotypes that are genetically differentiated, but overlap strongly in both morphology and habitat preferences.

Based on our sampling, tetraploids occur throughout the three major tectonic units of the middle part of the Alpine arc, Northern Limestone Alps, Central Alps and Southern Limestone Alps, and in the northern forelands of the Alps, whereas diploids are restricted to the Central Alps and the Southern Limestone Alps (Fig. 1). Interestingly, tetraploids predominate north of the Alpine main chain and are the only cytotype encountered in the Northern Limestone Alps and the northern forelands. In contrast, diploids prevail south of the main divide. This large-scale pattern may at least partly be explained by the more frequent occurrence of low-altitude wetlands such as raised bogs, which are exclusively inhabited by tetraploids, in the Northern Limestone Alps and their forelands.

Why diploids did not colonise the Northern Limestone Alps when they became ice-free after the last glaciation is unclear. A possible explanation may be sought in the scarcity of suitable habitats; on calcareous bedrock acidophilic dwarf shrub communities develop only on a thick, often water-logged humus layer (Grabherr 1993), which may be better suited for the tetraploid cytotype than for diploids. Alternatively, diploids probably survived the glaciations in refugia at the southern periphery of the Alps (Schönswetter et al. 2005) and have not yet recolonized the Northern Limestone Alps (i.e. incomplete range filling; Svenning & Skov 2007, Dullinger et al. 2012). It is likely that this mountain range, in contrast, was colonized by tetraploids surviving in the northern forelands. The latter hypothesis is supported by the fact that in Scandinavia, an area that is likely to have been recolonized from the tundra vegetation between the Alpine and Scandinavian ice sheets (Lang 1994, Burga et al. 1998), tetraploids clearly prevail (Eidesen et al. 2007). Divergent evolution of diploids in separate refugia followed by absence or scarcity of gene flow over wide distances, indicating restricted migration, is also supported by the Mantel test that revealed a significant correlation of genetic and geographic distances. No such correlation was found in tetraploids, probably suggesting existence of a single refugium or extensive postglacial gene flow. In contrast to diploids, tetraploids exhibited a weak correlation between genetic and altitudinal distances likely reflecting the distribution gap between lowland occurrences in bogs and alpine occurrences in dwarf shrub communities.

The two cytotypes strictly corresponded to two non-overlapping groups in the mostly nuclear derived, biparentally inherited (Bussell et al. 2005) AFLPs, and were also in almost perfect congruence with the two major lineages retrieved from non-recombining, maternally inherited (in Ericaceae: Szczecińska et al. 2014) plastid sequences (Fig. 2). We retrieved three haplotypes corresponding to the haplotypes A, D and K identified by Alsos et al. (2005), which fall into their circumpolar Arctic-Alpine lineage (haplotypes A, D) and the Amphi-Atlantic lineage (haplotype K). Divergence between the main plastid lineages is suggested to have happened before the Pleistocene, and the Arctic-Alpine lineage probably survived the last glaciation in southern mountain ranges (Alsos et al. 2005).

The observed congruence between ploidy level and the two plastid DNA lineages corroborates the hypothesis that the initial plastid DNA divergence followed early polyploidizations (Eidesen et al. 2007). The fact that the AFLP data show the same pattern of divergence, however, is in marked contrast with the assumption that recent gene flow via unreduced pollen from diploids to tetraploids is frequent (Eidesen et al. 2007). The single evidence for gene flow between diploids and tetraploids was a tetraploid individual from population B5 carrying haplotype A from the otherwise exclusively diploid Arctic-Alpine lineage (Alsos et al. 2005, Eidesen et al. 2007). This incongruence may be

explained by meiotic failure in megasporogenesis leading to an unreduced egg cell, which was then fertilized by an unreduced sperm cell. If a "triploid bridge" (Yamauchi et al. 2004) was involved, two possibilities exist. First, an unreduced egg cell was fertilized by a regular male gamete or, alternatively, a reduced egg cell was fertilized by an unreduced sperm cell, ultimately resulting in a tetraploid individual carrying a "diploid" haplotype. Subsequent gene flow may then have swamped the nuclear genome rendering it indistinguishable from that of the resident tetraploids.

Diploids and tetraploids show some degree of niche separation (Fig. 6), which is mostly due to the lack of diploids at low altitudes (Fig. 1B). In high-altitude habitats above 1500 m a.s.l. both cytotypes may co-occur as illustrated by populations A5, D5 and D7 (Fig. 1), which are ploidy-heterogeneous in spite of the small sample size of three individuals per population, which certainly resulted in an underestimate of ploidy mixtures. The cytotypes' altitudinal distribution is reflected in the plant communities inhabited. Whereas diploids exclusively grow in (sub)alpine dwarf shrub communities of the phytosociological alliance *Loiseleurio-Vaccinion*, tetraploids occupy different types of habitat from lowland swamps (*Sphagnion medii*) to swampy forests (*Vaccinio uliginosi-Pinion*) and subalpine-lower alpine dwarf shrub communities (*Loiseleurio-Vaccinion*, Grünebach 2013), thus spanning three vegetation classes (*Vaccinio uliginosi-Pinetea sylvestris*, *Oxycocco-Sphagnetea*, *Loiseleurio-Vaccinietea*; Grabherr 1993, Steiner 1993, Willner & Grabherr 2007). However, we acknowledge that fine-scale investigations in areas where the two cytotypes come into contact are needed in order to safely reject the hypothesis of a lack of ecological segregation; such investigations are underway (L. Silbernagl, unpublished). Previous studies in polyploid complexes have usually revealed niche differences among sympatric cytotypes (Lumaret et al. 1987, Felber-Girard et al. 1996, Sonnleitner et al. 2016), whereas the opposite is considered rare (Hanzl et al. 2014).

In spite of the clear separation of diploids and tetraploids in the molecular data sets we failed to detect consistent morphological differences (Fig. 3). Although absolute size characters used to discriminate *V. uliginosum* s. str. and *V. gaultherioides* such as plant height, leaf length or leaf width (Fischer et al. 2008) differed significantly between the two cytotypes (ANOVA analyses; Table 2), they were strongly inversely correlated with altitude within tetraploids, preventing their use for reliable determination (Fig. 4). Consequently, based on the characters employed here, it is currently impossible to morphologically distinguish the two cytotypes where their ranges overlap, i.e. in subalpine and alpine dwarf shrub communities. The LDA (Fig. 5) of morphological characters corroborates this result. The separation along the first linear discriminant reflects the altitudinally segregated habitats, bogs and alpine dwarf shrub communities, rather than the two cytotypes, which is consistent with field observations (D. Regele & P. Schönswetter, unpublished). We cannot exclude, however, that we have missed important traits; due to a lack of flowering material we could not assess generative characters such as the number of flowers per inflorescence (Fischer et al. 2008).

In conclusion, within *V. uliginosum* s.l. two major groups are congruently defined by ploidy level as well as by variation in AFLP and plastid DNA. These two groups, however, do not correlate with patterns of ecological and morphological divergence, currently preventing discrimination of diagnosable taxa. A similar situation was previously detected in eastern North American *V. oxycoccos* L. (Smith et al. 2015). Ongoing research (L. Silbernagl et al. in prep.) focussing on mixed-cytotype localities and thus eliminating

the disturbing effect of wide altitudinal gradients on morphological and ecological differentiation, which also includes morphometric characters from reproductive organs will hopefully provide a definite answer to whether *V. uliginosum* s. str. and *V. gaultherioides* are two diagnosable evolutionary units that merit taxonomic recognition.

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Souhrn

Evoluční procesy, jako je zvýšený genový tok mezi polyploidy a jejich opakovaný vznik, činí z polyploidních komplexů taxonomicky složité skupiny. *Vaccinium uliginosum* s.l. je příkladem takového komplexu, který zahrnuje převážně diploidy a tetraploidy a patří k nejčastěji se vyskytujícím zakrslým keříkům nejen v Arktidě, ale také v jižněji položených pohořích včetně Alp. Přes jejich častý výskyt existuje z Alp a přilehlých oblastí překvapivě málo informací o ploidní, stanovištní a morfologické variabilitě, a také skutečná taxonomická hodnota rostlin označovaných jako *V. gaultherioides* je nejasná. Podél pěti výškových transektů ve středních Alpách jsme studovali rostliny tohoto okruhu pomocí průtokové cytometrie, sekvenování chloroplastové DNA, metody AFLP a morfometrické analýzy, a popsali jsme jejich ekologické niky prostřednictvím fytocenologických snímků a Landoltových indikačních hodnot. Studovali jsme, (i) jaké je rozšíření diploidů a tetraploidů *V. uliginosum* s.l. na velké prostorové škále, (ii) zda jsou molekulárně genetické vztahy řízeny diferenciací ploidních úrovní a jestli jsou nějaké (iii) ekologické nebo (iv) morfologické rozdíly mezi oběma cytotypy. Zjistili jsme, že tetraploidi se vyskytují v celém zájmovém území v Alpách a jejich severním předhůří, zatímco výskyt diploidů je omezen na Centrální Alpy a Jižní vápencové Alpy. Analýza metodou ALFP odlišila oba cytotypy jako samostatné skupiny, které navíc téměř úplně odpovídaly dvěma chloroplastovým liniím. Jediným důkazem toho, že dochází k toku genů mezi diploidy a tetraploidy, byl tetraploidní jedinec s haplotypem jinak se vyskytujícím výlučně u diploidů. Niky diploidů a tetraploidů jsou do určité míry oddělené, což je způsobeno tím, že v nižších polohách se vyskytují pouze tetraploidi; nad 1500 n n.m. se však oba cytotypy vyskytují společně. Tetraploidi se také vyskytují v širším spektru rostlinných společenstev. Přestože molekulární metody oba cytotypy jasně odlišily, nezjistili jsme mezi nimi morfologické rozdíly, které by bylo možno využít k jejich spolehlivému určování. V současné době proto nelze na základě morfologických znaků rozlišit diploidní *V. gaultherioides* a tetraploidní *V. uliginosum* s. str.

References

Abbott R. J. & Brochmann C. (2003): History and evolution of the Arctic flora: in the footsteps of Eric Hultén. – Mol. Ecol. 12: 299–313.

Aeschimann D., Lauber K., Moser D. M. & Theurillat J. P. (2004): Flora alpina – Ein Atlas sämtlicher 4500 Gefässpflanzen der Alpen. – Haupt, Bern.

Alsos I. G., Engelskjøn T., Gielly L., Taberlet P. & Brochmann C. (2005): Impact of ice ages on circumpolar molecular diversity: insights from an ecological key species. – Mol. Ecol. 14: 2739–2753.

Arrigo N., Tuszynski J. W., Ehrich D., Gerdes T. & Alvarez N. (2009): Evaluating the impact of scoring parameters on the structure of intra-specific genetic variation using RawGeno, an R package for automating AFLP scoring. – BMC Bioinformatics 10: 33.

Bonin A., Bellemain E., Eidesen P. B., Pompanon F., Brochmann C. & Taberlet P. (2004): How to track and assess genotyping errors in population genetics studies. – Mol. Ecol. 13: 3261–3273.

Burga C. A., Perret R. & Vonarburg C. (1998): Vegetation und Klima der Schweiz seit dem jüngeren Eiszeitalter. – Ott, Thun.

Bussell J. D., Waycott M. & Chappill J. A. (2005): Arbitrarily amplified DNA markers as characters for phylogenetic inference. – Persp. Plant Ecol. Evol. Syst. 7: 3–26.

Bylesjö M., Segura V., Soolanayakanahally R. Y., Rae A. M., Trygg J., Gustafsson P., Jansson S. & Street N. R. (2008): LAMINA: a tool for rapid quantification of leaf size and shape parameters. – BMC Plant Biol. 8: 82.

Clausen J., Keck D. & Hiesey W. (1948): Experimental studies on the nature of species. III. Environmental responses of climatic races of *Achillea*. – Carnegie Inst. Washington Publ. 581: 1–129.

Clement M., Posada D. & Crandall K. A. (2000): TCS: A computer program to estimate gene genealogies. – Mol. Ecol. 9: 1657–1659.

Dullinger S., Willner W., Plutzar C., Englisch T., Schratt-Ehrendorfer L., Moser D., Ertl S., Essl F. & Niklfeld H. (2012): Post-glacial migration lag restricts range filling of plants in the European Alps. – Glob. Ecol. Biogeogr. 21: 829–840.

Eidesen P. B., Alsos I. G., Popp M., Stensrud Ø., Suda J. & Brochmann C. (2007): Nuclear vs. plastid data: complex Pleistocene history of a circumpolar key species. – Mol. Ecol. 16: 3902–3925.

Felber-Girard M., Felber F. & Buttler A. (1996): Habitat differentiation in a narrow hybrid zone between diploid and tetraploid *Anthoxanthum alpinum*. – New Phytol. 133: 531–540.

Fischer M. A., Oswald K. & Adler W. (2008): Exkursionsflora für Österreich, Liechtenstein und Südtirol. – Biologiezentrum der Oberösterreichischen Landesmuseen.

Flatscher R., García P. E., Hülber K., Sonnleitner M., Winkler M., Saukel J., Schneeweiss G. M. & Schönswetter P. (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.

Grabherr G. (1993): *Loiseleurio-Vaccinietea*. – In: Grabherr G. & Mucina L. (eds), Die Pflanzengesellschaften Österreichs. Teil II. Natürliche waldfreie Vegetation, p. 447–467, Fischer, Jena.

Gregor T. & Hand R. (2006): Chromosomenzahlen von Farn-und Samenpflanzen aus Deutschland 2. – Kochia 1: 135–140.

Grünebach M. (2013): Verbreitung und Abgrenzung zweier nahe verwandter *Vaccinium*-Arten in den Alpen und Gestaltung eines Unterrichtsprogramms für die Umsetzung der Pflanzensoziologie im gymnasialen Biologieunterricht. – Diploma thesis, Leopold-Franzens-Universität Innsbruck.

Guggisberg A., Mansion G. & Conti E. (2009): Disentangling reticulate evolution in an arctic-alpine polyploid complex. – Syst. Biol. 58: 35–73.

Hagerup O. (1933): Studies on polyploid ecotypes in *Vaccinium uliginosum* L. – Hereditas 18: 122–128.

Hamilton M. B. (1999): Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. – Mol. Ecol. 8: 513–525.

Hanzl M., Kolář F., Nováková D. & Suda J. (2014): Nonadaptive processes governing early stages of polyploid evolution: insights from a primary contact zone of relict serpentine *Knautia arvensis* (*Caprifoliaceae*). – Am. J. Bot. 101: 935–945.

Hewitt G. M. (2004): Genetic consequences of climatic oscillations in the Quaternary. – Phil. Trans. R. Soc. B. Biol. Sci. 359: 183–195.

Hill M. O. & Šmilauer P. (2005): TWINSPAN for Windows version 2.3. – Centre for Ecology & Hydrology, Huntingdon & University of South Bohemia, České Budějovice.

Iwata H. & Ukai Y. (2002): SHAPE: a computer program package for quantitative evaluation of biological shapes based on elliptic Fourier descriptors. – J. Hered. 93: 384–385.

Landolt E., Bäumler B., Erhardt A., Hegg O., Klötzli F., Lämmler W., Nobis M., Rudmann-Maurer K., Schweingruber F. & Theurillat J. (2010): Flora indicativa: Ökologische Zeigerwerte und biologische Kennzeichen zur Flora der Schweiz und Alpen. – Haupt, Bern.

Lang G. (1994): Quartäre Vegetationsgeschichte Europas. – G. Fischer, Jena.

Lippert W. & Heubl G. R. (1989): Chromosomenzahlen von Pflanzen aus Bayern und angrenzenden Gebieten. Teil 2. – Ber. Bayer. Bot. Ges. 60: 73–83.

Lumaret R., Guillerm J. L., Delay J., Ait Lhaj Loutfi A., Izco J. & Jay M. (1987): Polyploidy and habitat differentiation in *Dactylis glomerata* L. from Galicia (Spain). – Oecologia 73: 436–446.

Oksanen J., Blanchet F. G., Friendly M., Kindt R., Legendre P., McGlinn D., Minchin P. R., O'Hara R. B., Simpson G. L., Solymos P., Stevens M. H. H., Szoecs E. & Wagner H. (2016): vegan: Community Ecology Package. R package version 2.4-1 September 2016. – https://CRAN.R-project.org/package=vegan

Otto S. P. & Whitton J. (2000): Polyploid incidence and evolution. – Annu. Rev. Genet. 34: 401–437.

Polatschek A., Maier M. & Neuner W. (1999): Flora von Nordtirol, Osttirol und Vorarlberg. Band 2. Samenpflanzen: *Brassicaceae* bis *Euphorbiaceae*. – Tiroler Landesmuseum Ferdinandeum, Innsbruck.

R Core Team (2016): R: A language and environment for statistical computing. – The R Foundation for Statistical Computing, Vienna.

Schönswetter P., Solstad H., Escobar García P. & Elven R. (2009): A combined molecular and morphological approach to the taxonomically intricate European mountain plant *Papaver alpinum* s.l. (*Papaveraceae*) — taxa or informal phylogeographical groups? – Taxon 58: 1326–1348.

Schönswetter P., Stehlik I., Holderegger R. & Tribsch A. (2005): Molecular evidence for glacial refugia of mountain plants in the European Alps. – Mol. Ecol. 14: 3547–3555.

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

Smith T. W., Walinga C., Wang S., Kron P., Suda J. & Zalapa J. (2015) Evaluating the relationship between diploid and tetraploid *Vaccinium oxycoccos* (*Ericaceae*) in eastern Canada. – Botany 93: 623–636.

Soltis P. S. & Soltis D. E. (2009): The role of hybridization in plant speciation. – Annu. Rev. Plant Biol. 60: 561–588.

Sonnleitner M., Hülber K., Flatscher R., Escobar García P., Winkler M., Suda J., Schönswetter P. & Schneeweiss G. M. (2016): Ecological differentiation of diploid and polyploid cytotypes of *Senecio carniolicus* sensu lato (*Asteraceae*) is stronger in areas of sympatry. – Ann. Bot. 117: 269–276.

Steiner G. M. (1993): *Oxycocco-Sphagnetea*. – In: Grabherr G. & Mucina L. (eds), Die Pflanzengesellschaften Österreichs. Teil II. Natürliche waldfreie Vegetation, p. 166–181, Fischer, Jena.

Stewart J. R., Lister A. M., Barnes I. & Dalén L. (2010): Refugia revisited: individualistic responses of species in space and time. – Proc. Biol. Sci. 277: 661–671.

Suda J., Weiss-Schneeweiss H., Tribsch A., Schneeweiss G. M., Travníček P. & Schönswetter P. (2007): Complex distribution patterns of di-, tetra-, and hexaploid cytotypes in the European high mountain plant *Senecio carniolicus* (*Asteraceae*). – Am. J. Bot. 94: 1391–1401.

Svenning J. C. & Skov F. (2007): Could the tree diversity pattern in Europe be generated by postglacial dispersal limitation? – Ecol. Lett. 10: 453–460.

Szczecińska M., Gomolińska A., Szkudlarz P. & Sawicki J. (2014): Plastid and nuclear genomic resources of a relict and endangered plant species: *Chamaedaphne calyculata* (L.) Moench (*Ericaceae*). – Turk. J. Bot. 38: 1229–1238.

Taberlet P., Gielly L., Pautou G. & Bouvet J. (1991): Universal primers for amplification of three non-coding regions of chloroplast DNA. – Plant Mol. Biol. 17: 1105–1109.

Vos P., Hogers R., Bleeker M., Reijans M., van de Lee T., Hornes M., Frijters A., Pot J., Peleman J. & Kuiper M. (1995): AFLP: a new technique for DNA fingerprinting. – Nucleic Acids Res. 23: 4407–4414.

Willner W. & Grabherr G. (2007): Die Wälder und Gebüsche Österreichs. – Spektrum, München.

Yamauchi A., Hosokawa A., Nagata H. & Shimoda M. (2004): Triploid bridge and role of parthenogenesis in the evolution of autopolyploidy. – Am. Nat. 164: 101–112.

Young S. B. (1970): On the taxonomy and distribution of *Vaccinium uliginosum*. – Rhodora 72: 439–459.

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