

## Microsatellite variation in three calcium-tolerant species of peat moss detected specific genotypes of *Sphagnum warnstorffii* on magnesium-rich bedrock

Variabilita mikrosatelitů tří druhů kalcitolerantních rašeliničků odhalila specifické genotypy *Sphagnum warnstorffii* rostoucí na hořčíkem bohatých podkladech

Eva Mikulášková<sup>1</sup>, Adam Veleba<sup>1</sup>, Jakub Šmerda<sup>1</sup>, Aleš Knoll<sup>2</sup>  
& Michal Hájek<sup>1</sup>

<sup>1</sup>Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, CZ-611 37 Brno, Czech Republic, e-mail: evamikul@gmail.com; <sup>2</sup>CEITEC MENDELU, Mendel University in Brno, Zemědělská 1, CZ-613 00 Brno, Czech Republic

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Peat mosses are a key functional group in peatlands, driving biogeochemical cycles, habitat development and changes in species composition. They are generally intolerant of calcium and magnesium bicarbonate, but some species are adapted to mineral-rich fens. A previous study found a coincidence between genetic variation and the ability to tolerate high pH/calcium levels in *Sphagnum warnstorffii*. Here we compare its microsatellite variation with that of two rarer calcium-tolerant species (*Sphagnum subnitens*, *S. contortum*), using a novel data set from Eurasia. Because physiological experiments indicate that *S. warnstorffii* can tolerate high magnesium levels, we included also samples from dolomite and serpentinite. Genetic diversity of *S. warnstorffii* was higher than that of other species. The Bayesian analysis in program Structure resulted in two population groups of *S. warnstorffii*. One group coincided with dolomite (Italy, Austria, Estonia) and moderately magnesium-rich (but calcium-poor) rocks (serpentinite, metadolerite, cordierite-bearing migmatite on the Bohemian Massif), while the second one coincided with magnesium-poor bedrock across Eurasia. The principal coordinate analysis revealed a cline between populations from magnesium-rich and magnesium-poor bedrocks, with populations from dolomite and serpentinite forming one extreme. Populations from magnesium-poor bedrock located far from any dolomite or serpentinite formed the opposite extreme of the cline. We demonstrate for the first time that magnesium toxicity may drive bryophyte microevolution, as has repeatedly been shown for vascular plants, including ferns.

**Key words:** *Bryophyta*, calcium tolerance, genetic variation, magnesium toxicity, microsatellites, peatland, population structure, *Sphagnum contortum*, *Sphagnum subnitens*, *Sphagnum warnstorffii*

### Introduction

*Sphagnum* mosses are a dominant component of peatlands, a habitat covering more than three percent of the global land area, making up at least half of the world's wetlands, storing one third of the global carbon pool and harbouring many endangered species (Tahvanainen 2011, Loisel et al. 2012, Rydin & Jeglum 2013, Jiroušek et al. 2015). Two contrasting ecosystems occur within peatlands, dominated either by *Sphagnum* species or

by non-sphagnaceous, usually weft-forming calcium-tolerant bryophytes (Vitt & Wieder 2008, Hájek et al. 2014, Udd et al. 2015). *Sphagna* affect ecosystem processes differently from other mosses (Peterka et al. 2014); they acidify the environment, raise the peatland surface above the water table, take up most nutrients, hamper seed germination and seedling establishment of vascular plants, decrease nutrient mineralization by litter decomposition and generally act as a strong competitive filter for other plants. They thus regulate the vegetation structure to which vascular plants respond. Peatland mosses are generally physiologically intolerant of calcium and magnesium bicarbonate (Clymo 1973, Hájek et al. 2014, Vicherová et al. 2015), especially when either calcium or magnesium is supplied alone (Vicherová et al. 2015). However, some *Sphagnum* species are partially tolerant of a high calcium supply and high pH. They are called calcium-tolerant species in the ecological literature and when they become established, they may substantially change the ecosystem dominated by non-sphagnaceous mosses. Some of them have inconsistent pH-niche breadths in different regions (Mikulášková et al. 2015, Plesková et al. 2016), which is mirrored in regional fen ecosystem composition and functioning (Hájek et al. 2006). Peatland diversity and development, therefore, cannot be fully understood without a thorough understanding of calcium-tolerance in *Sphagnum* mosses.

The evolution of species within the genus *Sphagnum* is driven more by water table depth (i.e. the hummock-hollow gradient) than water chemistry (Johnson et al. 2015). However, there are several calcium-tolerant species in phylogenetically diverse sections (representing different clades), usually splitting in the terminal nodes of the phylogenetic tree (e.g. Shaw et al. 2003): sect. *Acutifolia* (*S. warnstorffii*, *S. subnitens*, *S. subfulvum*), sect. *Subsecunda* (*S. contortum*), sect. *Squarrosa* (*S. teres*) or sect. *Sphagnum* (*S. centrale*). This pattern fits the concept of alpha and beta niche (Ackerly et al. 2006) in which traits related to within-community niche differences (alpha niche) are supposed to evolve earlier, while traits differentiating species affinities for different macrohabitats (beta niche) evolve later. Mikulášková et al. (2015) report an adaptive role of pH for *S. warnstorffii*, which most frequently and most consistently grows in calcium-rich fens in different regions (Hájek et al. 2014, Plesková et al. 2016). Genetic diversity of its populations correlated partially with water pH, independent of geography.

In this study we follow the study of Mikulášková et al. (2015) by collecting new data for *S. warnstorffii* accompanied by samples of two other calcium-tolerant species. We also collected samples of *S. contortum*, a species in which a different pattern was expected due to its semi-aquatic life form. Peat mosses with a submerged habit are directly affected by water chemistry because they do not produce hummocks and so cannot raise their capitula above the level of the groundwater. In addition, we also sampled another hummock-forming species, *S. subnitens*. This species frequently occurs where the pH and calcium concentrations are high (Hájek et al. 2006, 2014, Johnson et al. 2015), but physiological experiments indicate it is calcium-intolerant (Kooijman 2012, Vicherová et al. 2015). Karlin et al. (2011) report its uniform genetic structure in North America and interpret this as spread from one haploid parent, and a somewhat more variable genetic structure in Europe. Nevertheless, the genetic structure in Europe has not been compared with that of other calcium-tolerant species nor interpreted ecologically. In this study, we did not aim to merely reproduce the results of the previous paper (Mikulášková et al. 2015) and did not sample along a steep gradient in pH. Instead, we focused on hitherto unexplored geological bedrock. Because physiological experiments (Vicherová et al.

2015) indicate that both calcium and magnesium are toxic for peatland mosses, but that *S. warnstorffii* is able to tolerate high levels of magnesium, we determined whether magnesium tolerance coincides with genetic structure at a broad geographical scale. Therefore, we sampled *S. warnstorffii* growing on dolomite and serpentinite. The effect of magnesium-rich bedrock, however, could not be directly determined for the other two species, *S. subnitens* and *S. contortum*. They rarely occur on serpentinite or dolomite bedrock and, indeed, only a few populations were found on these bedrocks. In addition, *S. subnitens*, unlike *S. warnstorffii*, does not survive cultivation in magnesium hydrogencarbonate (Vicherová et al. 2015). It is hypothesized that the rather broad niche of *S. warnstorffii*, including fens on serpentinite and dolomite, results from its specific genetic variation.

Magnesium-rich environments generally act as an important evolutionary trigger for vascular plants and ferns (Rajakaruna 2004, Kolář et al. 2012, 2014a, Vicić et al. 2014), but for bryophytes the evidence for serpentinite or dolomite ecotypes is poor (Shaw 1991). We aimed to advance the understanding of the genetic background of calcium-tolerance in bryophytes by answering the following questions: (i) Will the significant genetic variation in *S. warnstorffii* reported by Mikulášková et al. (2015) be confirmed using a new independent data set? (ii) If so, does it exceed that of other species incorporated in the current study, a semi-aquatic (*S. contortum*) and calcicolous yet physiologically calcium-intolerant (*S. subnitens*)? (iii) Is the genetic variation in *S. warnstorffii* and other species associated with specific geographical region or specific bedrocks?

## Material and methods

### Field sampling

Localities in the Dolomitic Alps (and nearby regions), on the Bohemian Massif (Czech Republic) and in Bulgaria, Estonia and Siberia served as a source of data for further exploring the genetic variability within *S. warnstorffii* (Fig. 1). In the Alps we selected three localities with abundant *S. warnstorffii*: one located in the central part of the Dolomites (Sciliar Mts: Palud din Ladins, see Gerdol & Tomaselli 1997), one on the western margin of the Dolomites (Passo Campo Magno, Bracco et al. 2004) and one near the northern margin of the Dolomites (Kartitscher Sattel, Austria; Steiner 1992). The question was whether this extensive area with a dolomite bedrock supports a specific ecotype and whether it extends beyond the core area in the Dolomites. For Siberia (the Altai, the Western Sayan) we used samples collected for another project on recent similarities with European glacial landscapes (e.g. Horsák et al. 2015). We included Siberia because of the possible effect of Pleistocene history on the genetic structure of *S. warnstorffii*, that is acknowledged by Mikulášková et al. (2015). Finally, we collected more data from the Vitosha Mts in Bulgaria, which were also partially included in Mikulášková et al. (2015). The populations of this species in this mountain range are abundant, yet isolated from other *S. warnstorffii* populations and occur in very calcium- and magnesium-poor fens. In addition, there are no fens on magnesium-rich bedrock, such as dolomite or serpentinite, in Bulgaria (Hájek et al. 2008). The field collections usually included 6–10 samples per locality (Electronic Appendix 1).

For the comparison of the three calcium-tolerant species (*S. warnstorffii*, *S. subnitens*, *S. contortum*; Electronic Appendix 1) we selected only regions where all three species

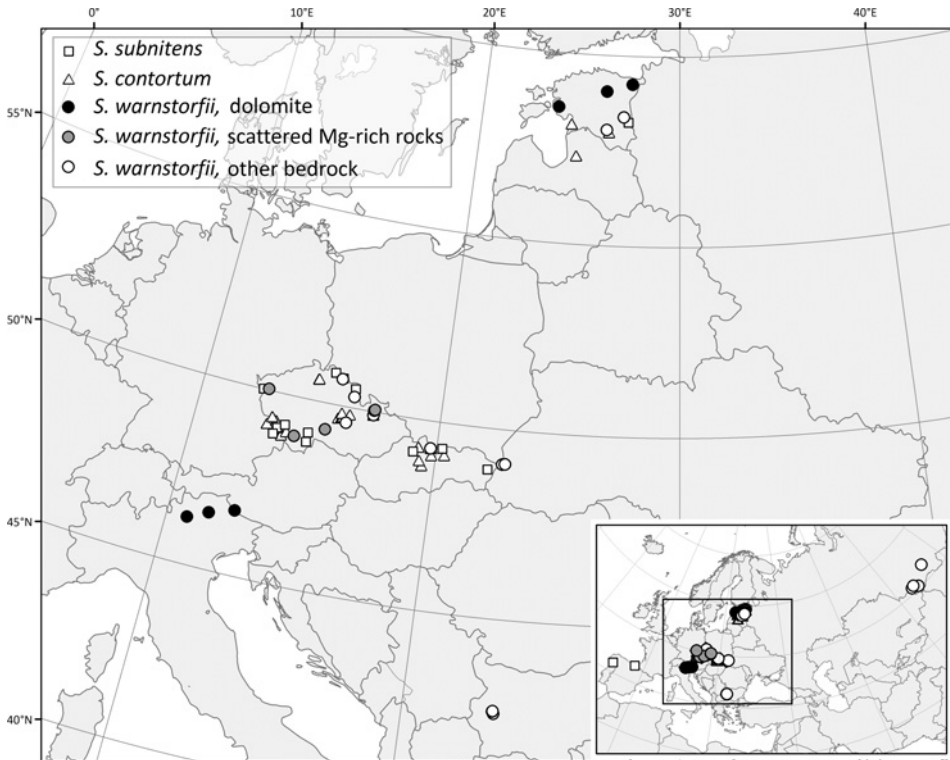


Fig. 1. – Distributions of the populations of *Sphagnum contortum*, *S. subnitens* and *S. warnstorffii* sampled in this study. For *S. warnstorffii*, different bedrock is indicated by the colour of the circle (grey colour for scattered Mg-rich rocks including Mg-mix and serpentinite; see Table 1).

occurred sympatrically at a regional scale: the Czech Republic plus Slovakia (localities explored by Hájek et al. 2014 and Mikulášková et al. 2015) and Estonia (localities visited during the 5th Meeting on the Biology of *Sphagnum*, 2012). Both regions are rather diverse in terms of geological bedrock (Table 1). In addition, several herbarium specimens were included in the analysis (Electronic Appendix 1), usually consisting of 1–3 samples per locality.

### Molecular methods

All genetic analyses were based on gametophytes. DNA was extracted from the capitulum using the CTAB protocol described by Doyle & Doyle (1987) and modified as described at <http://biology.duke.edu/bryology/protocols.html>. The microsatellite genotyping protocols are described by Ricca et al. (2008). The primer sequences for microsatellite amplification are those provided by Shaw et al. (2008). The following 18 markers were used in this study: 1, 3, 4, 4long, 7, 9, 10, 12, 14, 17, 18, 19, 20, 22, 26, 28, 29 and 30. Microsatellites were amplified using fluorescent labelled primers in 8 µl multiplexed polymerase chain reactions, each targeting a set of three markers. Standard

Table 1. Classification of the bedrocks that was used for the characterization of the localities studied and interpretation of the PCoA gradients. Data on rock chemistry are taken from Parker (1967) and cited regional sources.

I. Dolomitic regions (“Dolomite”)	Characterized by high concentration of both magnesium and calcium, but Mg:Ca ratio is greater than on pure limestone. Dolomite contains 16–21% MgO and 29–31% CaO. Dolomite bedrock occurs at a large scale and forms a landscape matrix, well supporting evolutionary processes. Geographically it corresponds to the Dolomites (the Alps) and dolomitic-limestone and dolomitic-marl regions in Estonia.
II. Scattered serpentinites on the Bohemian Massif (“Serpentine”)	Serpentine bedrock contains a high fraction of MgO (dozens of units of percentage), but only single percentage unit of CaO, causing an excess of magnesium over calcium. On the other hand, serpentinites do not form a landscape matrix on the Bohemian Massif and they are scattered within magnesium-poor crystalline rocks. They are nevertheless one of a few areas where species of high-pH fens may occur.
III. Crystalline bedrock with scattered magnesium-rich rocks on the Bohemian Massif (“Mg-mix”)	This bedrock is dominated by calcium-poor and magnesium-poor crystalline rocks, but rocks with extremely high Mg:Ca ratio are locally abundant and support high-pH fens. They are either metadolerite (the Jeseníky Mts) with up to 5–15% of MgO, or migmatitized association of cordierite-biotite-muscovite paragneisses containing up to ca 4% MgO but with a high Mg:Ca ratio (max. 4.3; Suk 1964). These bedrocks hence may support the occurrence magnesium-tolerating ecotypes that evolved on serpentinite or dolomite bedrock.
IV. Other crystalline bedrock on the Bohemian Massif, Bulgaria, Estonia and Siberia (“Other”)	(A) Crystalline or sandstone bedrock with single units of MgO and CaO content: granite, granitoid, phyllite, sandstone, siltstone, quartzite, ortho-gneiss and migmatite without cordierite. This group however consists of regions where magnesium-rich bedrock occurs nearby: Bohemian Massif (serpentinites, group II), Estonia (dolomites, group I) and Altai-Sayan (serpentine in ophiolite complexes; Glorie et al. 2011) (B) Crystalline bedrock with single units of MgO and CaO: syenite and monzonite (the poorest bedrock). This group include the Vitoshka Mts (Bulgaria), which is far from any magnesium-rich bedrock.

thermocycling conditions (Shaw et al. 2008) were used for all primer sets with no additional optimization. The PCR products were diluted in sterile water and 1.2 µl of the dilution was mixed with GS500 size standard and Hi-Di™ Formamide (Applied Biosystems) for fragment analysis on an ABI 3500 Genetic Analyzer (Life Technologies). Sizes and genotype assignments were measured and made using a GeneMarker, version 2.4.0 (Softgenetics).

### Statistical analyses

Samples with seven or more missing loci were excluded from the analyses. Total of 141 samples for *S. warnstorffii*, 23 for *S. subnitens* and 40 for *S. contortum* were included in the genetic analyses (full dataset).

Interpopulation and interspecies relationships were calculated and visualized using principal coordinates analysis (PCoA) in GenAlEx v. 6.5 (Peakall & Smouse 2006). Clones were excluded from this analysis, so the dataset consist of 68 samples of *S. warnstorffii*, 18 of *S. subnitens* and 30 of *S. contortum* (full dataset without clones). Genetic distances were calculated using a dissimilarity matrix (Jaccard’s distance). The

PCoA was calculated with default settings from the genetic distance matrices using covariance matrices with data standardization.

In order to compare genetic diversity among species, only samples from the Czech Republic, Slovakia and Estonia were included. The dataset contained only unique genotypes (max three unique genotypes per locality and species, samples with missing data were excluded) which resulted in 24 samples of *S. warnstorffii*, 16 of *S. subnitens* and 30 of *S. contortum* (“diversity dataset”). Unbiased diversity of locus (uh) was calculated in GenAlEx for each species and locus, and averaged for loci of each species.

The PCoA analysis was performed using the diversity dataset (GenAlEx) and variance for each species group was calculated using the PERMDISP method (Anderson 2006) with the function `betadisper` (package `vegan` v. 2.3.0, Oksanen et al. 2013) in R v. 2.15.3. The differences between variances were analysed using the functions `anova` and `TukeyHSD` in R.

The Bayesian clustering method implemented in program Structure 2.3.4 (Pritchard et al. 2000) was used to test the genetic admixture/integrity of the closely related species *S. contortum*, *S. subnitens* and *S. warnstorffii* (full dataset without clones). Admixture analyses of the dataset were run with  $K = 1$  through to  $K = 7$ , with 10 replicate runs of one million generations (following a burn-in of 100,000 generations) at each value of  $K$ .

For *S. warnstorffii* alone, an admixture analysis in Structure 2.3.4 (Pritchard et al. 2000) with LOCPRIOR model was applied. Affiliation to bedrock was used as a prior. LOCPRIOR model assists clustering if the signal of the structure in data sets is relatively weak (details for both analysis, with and without LOCPRIOR, are shown in Electronic Appendix 1). Ten replicate runs for each  $K$  ranging from 1 to 5 were carried out with a burn-in length of one million and followed by one million iterations for each value of  $K$ . The optimum number of clusters ( $K$ ) and similarity among results of different runs for the same  $K$  were calculated using the R script `Structure-sum-2009`. Population structure was graphically displayed using program `distruct` 1.1 (Rosenberg 2004).

The resulting pattern in *S. warnstorffii* was interpreted by an ordinal variable “bedrock” that represents the bedrock categories ordered according to decreasing absolute and relative (to calcium) contents of magnesium (scale 1–4; Table 1). Correlation of this variable with the first three PCoA axes was tested using Spearman’s test in Statistica 12 (Statsoft 2013). Because this variable was created as a posteriori interpretation of resulting PCoA axis and not as a priori basis for hypothesis testing, the test should be understood as a kind of description rather than the rigorous testing of the effect of magnesium.

The same bedrock categories were visualized in the results of the admixture analysis of *S. warnstorffii* in Structure 2.3.4 (Pritchard et al. 2000) using the LOCPRIOR model.

## Results

### *Microsatellite variation*

A total of 204 samples of individual gametophytes (141 *S. warnstorffii*, 40 *S. contortum*, 23 *S. subnitens*) were analysed and 116 unique multi-locus genotypes were identified. Seventeen microsatellite markers were used for the final evaluation of the data (marker no. 3 was omitted because of a high percentage of missing data), full dataset without clones contains 12.1% of missing data. Marker no. 26a was not used in the analyses of

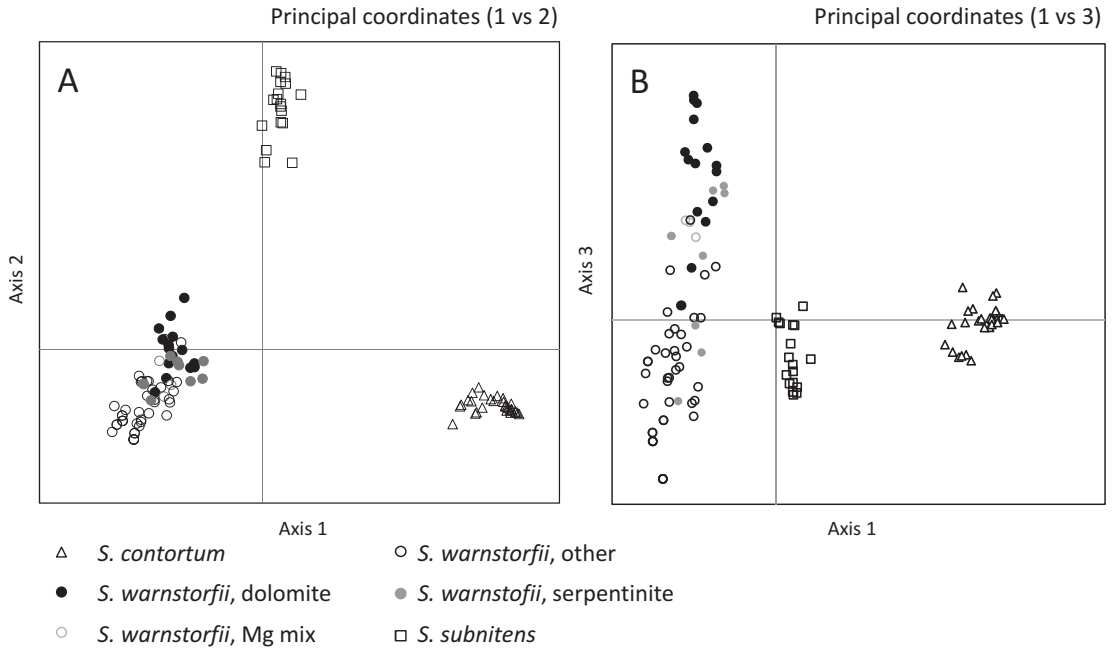


Fig. 2. – The first, second and third axes of the principal coordinates analysis (PCoA) comparing 116 multilocus haploid genotypes of *Sphagnum warnstorffii*, *S. contortum* and *S. subnitens* across 17 microsatellite markers for the dataset without clones. The first three principal coordinates account for 27.2%, 13.9% and 5.9% of the total genetic variation. *Sphagnum warnstorffii* samples are further classified according to bedrock (see Table 1 for details and explanation of bedrock codes).

*S. warnstorffii* because it was not amplified for this species (percentage of missing data was 4.4% in *S. warnstorffii* subset). All microsatellite markers in the whole dataset were polymorphic (markers 4long, 12, 17, 22, 26, and 30 had 2–5 alleles, markers 7, 18, 19, 20, and 28 had 6–11 alleles and markers 1, 34, 9, 10, and 14 were highly variable with 15–18 alleles (Electronic Appendix 1).

#### Principal coordinate analysis

The individual species are well distinguished by the PCoA analysis, the main gradient is formed by the differences among species in the analysis. The first axis explained 27.2% of the total variance, and the second and third 13.9% and 5.9% of genetic variability, respectively. The first axis sorted the individual species. Second axis sorted populations according to both the species level (however, it separated only *S. subnitens*) and bedrock (for *S. warnstorffii*). Third axis sorted populations in the *S. warnstorffii* cloud according to bedrock (Fig. 2). The ordinal variable “bedrock” correlated with the second ( $P < 0.003$ , Spearman  $R = 0.358$ ) and the third ( $P < 0.001$ , Spearman  $R = -0.440$ ) PCoA axes.

The average unbiased genetic diversity in the diversity dataset was highest for *S. warnstorffii* ( $n = 24$ ,  $\mu_{uh} = 0.471$ ), while for *S. subnitens* ( $n = 16$ ,  $\mu_{uh} = 0.410$ ) and

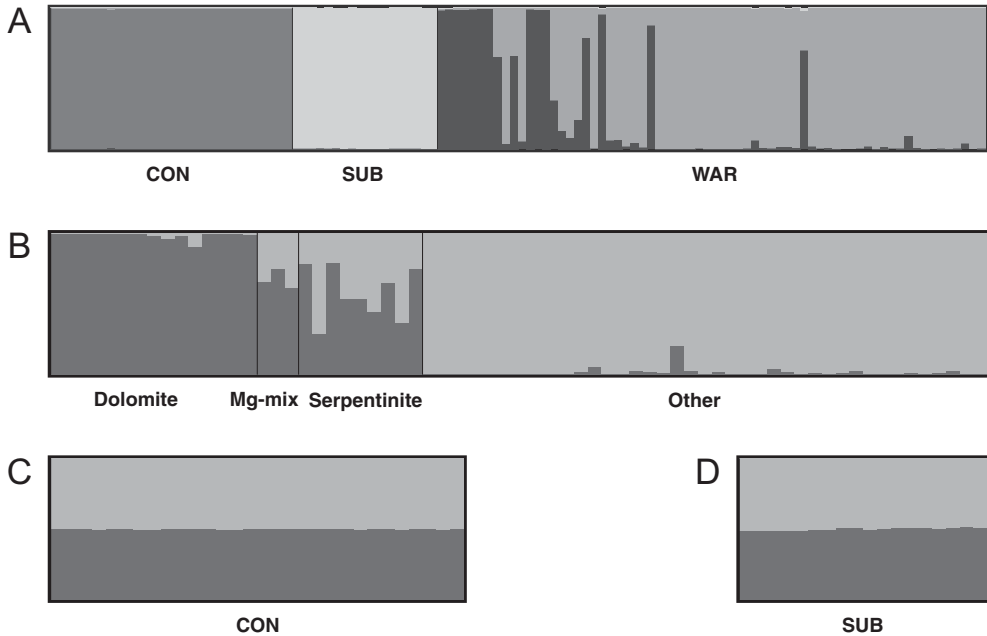


Fig. 3. – (A) Uniform genetic structure of *Sphagnum contortum* and *S. subnitens* contrasts with the two interpretable Structure groups recorded for *S. warnstorffii*. Each sample is represented by a vertical bar, and the colour indicates the relative percentage membership of each inferred genetic cluster ( $K = 4$ ). (B) Population structure of *S. warnstorffii*. Samples are classified according to bedrock for  $K = 2$  (see Table 1 for details and explanation of bedrock codes). (C) Population structure of *S. contortum* for  $K = 2$ . (D) Population structure of *S. subnitens* for  $K = 2$ .

*S. contortum* ( $n = 30$ ,  $\mu_{\text{H}} = 0.402$ ) it was almost equal. The comparison of the PCoA variances showed a significant difference between the three species analysed (ANOVA  $F = 6.059$ ,  $P = 0.004$ ). The variance recorded in *S. warnstorffii* was higher than that in both *S. contortum* ( $P < 0.01$ ) and *S. subnitens* ( $P < 0.05$ ).

### Genetic structure

The Bayesian analysis indicated that three clusters ( $K = 3$ ) best reflect the genetic structure present in the complete dataset of all three target species. Details for choosing the best  $K$  based on the results from structure.sum script are listed in Electronic Appendix 1. This result corresponds clearly with the three separate *Sphagnum* species. When the level  $K = 4$  was used, the cluster of *S. warnstorffii* was subdivided into two groups according to bedrock (Fig. 3A). This result corroborates that of the PCoA, whose third axis (Fig. 2) sorted samples along a gradient stretching from dolomitic and serpentinite bedrock to bedrock with low magnesium and calcium contents and/or a low Ca:Mg ratio. While *S. contortum* and *S. subnitens* appear genetically uniform (Fig. 3C, 3D), a detailed inspection of *S. warnstorffii* confirmed a subdivision into two different genetic groups (Fig. 3B). In Structure, samples from dolomite and “other” (i.e. magnesium-poor; Table 1) bedrocks



were assigned to their own genetic groups with more than 90% probability of membership (with one exception showing a 78% probability of membership). Samples from serpentinites and Mg-mix (Table 1) had intermediate genotypes with the lowest probability of being assigned to one of the two resulting clusters within *S. warnstorffii* (66–80%).

## Discussion

### *Higher genetic variation recorded for Sphagnum warnstorffii than the other species*

Using a geographical subset from the countries where all three species were recorded (Czech Republic, Slovakia, Estonia) we found a significantly higher genetic variation for *S. warnstorffii* than *S. subnitens* and *S. contortum*, which confirms previous speculations (Pakarinen 1979). The previous study of Mikulášková et al. (2015) concludes that this genetic variation is partly accounted for by differences in pH and partly unexplained, perhaps by a legacy of glacial refugia or introgression. The results of the present study, which uses an independent data set including samples from Siberia, neither supported the macroclimatic and refugia explanations, nor reproduced a transitional cluster of the populations towards another species because of introgression. Instead, the new results indicate a possible ecological-biogeographical explanation based on magnesium toxicity at a low Ca:Mg ratio that may operate on some bedrocks (serpentinite, dolomite). *Sphagnum warnstorffii* is one of few species of *Sphagnum*, which frequently grows in calcium- and especially magnesium-rich fens, while the physiological tolerance of calcium and magnesium by *S. subnitens* and *S. contortum* is low (Kooijman 2012, Vicherová et al. 2015). Despite this, *S. subnitens* is frequently found in calcium-rich fens (Hájek et al. 2014). We hypothesize that this discrepancy may be due to hummock formation that helps its meristems avoid coming into contact with toxic ions, as demonstrated for another calcifuge species of peat moss, *S. fuscum* (Granath et al. 2010). *Sphagnum contortum* does not occur in the most mineral rich fens and most acid mires. We assume that the broad niche of *S. warnstorffii* with respect to pH, calcium and magnesium might be connected with a higher level of genetic differentiation among the populations on different bedrocks, resulting in the high genetic variation recorded in this study.

The previous study of Mikulášková et al. (2015) reports finding a principal gradient in the genetic variation of *S. warnstorffii*, which is not correlated with environmental chemistry, and a secondary gradient correlated with pH. The latter pH gradient obviously does not correspond with the magnesium-conditioned differentiation found in this study, because there were very few samples from dolomite or serpentinite included in the previous study. One sample from serpentinite and one from metadolerite fell into the distinct group of samples that shaped the principal gradient and was interpreted either as a result of introgression or a legacy of glacial refugia in the study of Mikulášková et al. (2015). The new data indicates that the magnesium-tolerant genotype may also contribute to that unexplained variation.

### *Magnesium toxicity as an evolutionary trigger for wetland bryophytes?*

The two Structure groups and the major cline identified for *S. warnstorffii* can be explained by the character of the bedrock in the subregions studied (Table 1, Fig. 3B).

Geography also coincides partially with this cline, clumping samples from the Dolomites and from the Vitoshka Mts at opposite ends of the gradient. Nevertheless, bedrock (i.e. regional groundwater chemistry) seems to be more important. Both genetic groups can occur sympatrically in one country depending on the bedrock (Estonia, Czech Republic) or may even occur in the same fen (small serpentinite islands on the magnesium-poor Bohemian Massif). On the other hand, geographically distant samples from similar bedrocks can be genetically very close (e.g. the samples from dolomitic bedrocks in Estonia and the Alps).

Individual samples were easily classified into four groups characterized by bedrock, especially in terms of local occurrence of magnesium-rich substrates and their regional commonness (Table 1). This classification fits well with site scoring along the second and mainly third PCoA axes that sorts *S. warnstorffii* samples. Not only Mg content and Mg:Ca ratio, but also geological landscape setting affects the sorting of the samples and presence of particular genotypes. The large dolomite massif in the Alps probably does not support any other ecotype than the magnesium-tolerant one, while crystalline regions with scattered serpentinite islands may harbour both genotypes and transitional types between them (Table 1, Fig. 3B).

The coincidence between magnesium-rich geological bedrock and genetic structure is surprisingly close. We did not expect such a strong effect and hence the sampling of the data was not more focused on magnesium tolerance. In spite of this, the magnesium effect seems to be dominant in our data set. It is generally known that serpentinite or dolomite bedrock drives evolutionary processes such as natural selection and speciation in vascular plants and ferns (Rajakaruna 2004), but there is insufficient evidence of this for bryophytes. Shaw (1991) found no evidence that serpentinite plants of *Funaria flavicans* were more tolerant of high Mg:Ca ratios or high nickel combined with a high Mg:Ca ratio. We are not aware of any study reporting ecotypic adaptation for the Mg:Ca ratio in bryophytes. Also results for vascular plants are not always supportive. Quintela-Sabarís et al. (2010) and Kolář et al. (2014b) report no evidence of genetic differentiation between serpentinite and non-serpentinite populations of target vascular plants. Kolář et al. (2014a), by contrast, report Mg stress as an adaptive character of *Knautia arvensis*. The reasons for these differences may be the different scales at which the studies were carried out, different physiological effect of magnesium on individual species and the effect of other factors that mask the magnesium effect, such as geography or drought. Salmerón-Sánchez et al. (2014) report that geographical isolation and arid environments in general are more important factors for genetic differentiation in *Jurinea pinnata* than dolomite or gypsum bedrock. The genetic structure of *S. warnstorffii* populations is so clearly governed by magnesium-rich bedrock because (i) in (serpentinite or dolomite) wetlands it is not limited by drought, unlike in a dryland habitat, (ii) good dispersal ability of bryophytes (Sundberg 2013, Mikulášková et al. 2015) diminishes geographical effects and redistributes genotypes across entire regions where magnesium-rich substrates are present and (iii) magnesium toxicity causally affects the survival of peat mosses at least at the stage of protonemata, which are sensitive to metal ions (Kapur & Chopra 1989, Vicherová et al. 2015). Magnesium is an important intracellular element that is accumulated inside cells (Hájek et al. 2014). Vicherová et al. (2015) experimentally found that magnesium bicarbonate is more toxic and has a different effect on survival and growth of fen species of bryophytes than calcium bicarbonate, especially when supplied alone. In

their study, only *S. squarrosum*, *S. teres*, *S. flexuosum* and *S. warnstorffii* survived in 2.4 mM  $\text{Mg}(\text{HCO}_3)_2$  and when  $\text{Mg}^{2+}$  were combined with  $\text{Ca}^{2+}$ , each of the ions either ceased to be toxic or was generally less toxic. It is therefore possible that magnesium-tolerant genotypes also occur in *S. squarrosum*, *S. teres* and *S. flexuosum*. On the other hand, these species are quite rare on serpentinite or dolomite bedrock, perhaps because of a shortage of phosphorus and potassium (compare Hájek et al. 2015).

The adaptive role of magnesium for peat mosses, together with the previously reported adaptive role of pH (Mikulášková et al. 2015), represent a rather recent evolutionary mechanism, forming a beta niche in the sense of Ackerly et al. (2006), which is in marked contrast to the adaptation to water level that drives the macroevolution of peat mosses (Johnson et al. 2015). An analogous pattern is recorded for vascular plants, although the physiological background of calcicole behaviour may be different (Hájek et al. 2014). For vascular plants pH niche shifts are frequently recorded (Hájková et al. 2008), perhaps because a switch-over between adaptation to acidic and calcareous substrates may not require a complicated reorganization of the genome (Tyler 2003). Phylogenetic analyses of vascular plants show that variance in species niche position along a pH gradient can be better explained at the level of species than at the levels of genera or family (Prinzing et al. 2001).

#### *Future prospects*

A strong evolutionary role of magnesium toxicity for wetland bryophytes via contrasting bedrock properties (Mg content, Mg:Ca ratio, commonness of magnesium-rich substrate) is a novel result that opens up new directions in research on the genetic structure of bryophytes. Because the strength of this effect was unexpected, the sampling was not designed to test the a priori formulated hypothesis about magnesium-tolerant ecotypes. Further studies are therefore needed. They should include more samples from the magnesium-rich and magnesium-poor regions in the Alps or include Scandinavia or transects from dolomite or serpentinite regions to those with magnesium-poor bedrocks. Sampling could be accompanied by detailed local measurements of magnesium and calcium concentration, but the magnesium effect may be more apparent at regional than local scales. Even more useful could be the experimental cultivation of individual genotypes under different Mg:Ca ratios and the searching for specific genes and physiological traits that determine magnesium tolerance. Besides hummock formation, control over the balance of intracellular Ca/Mg uptake/efflux is a presumable factor in calcium and magnesium tolerance (Vicherová et al. 2015); resolving the genetic background of this adaptation is an exacting task for the future. Recently started sequencing of the *Sphagnum* genome, with complementary analyses of gene expression using transcriptomics (Johnson et al. 2015) may hence shed more light on the genetic background of calcium and magnesium tolerance in peat mosses.

See [www.preslia.cz](http://www.preslia.cz) for Electronic Appendix 1

## Souhrn

Rašeliničky (*Sphagnum* sp.) jsou určující funkční skupinou na rašeliništích. Ovlivňují biogeochemické cykly i vývoj ekosystému včetně druhového složení. Až na takzvané kalcitolerantní druhy netolerují velké koncentrace hydrogenuhličitanů vápníku a hořčíku ve vodě. V předchozí studii jsme zjistili souvislost mezi genetickou variabilitou druhu *Sphagnum warnstorffii* a pH prostředí. V této studii porovnáváme mikrosatelitní markery *S. warnstorffii* a dalších dvou kalcitolerantních druhů (*S. subnitens*, *S. contortum*). Protože fyziologické experimenty ukázaly, že *S. warnstorffii* může tolerovat vysoké hladiny hořčíku, zahrnuli jsme do studie i vzorky z mokřadů na dolomitech a hadcích. Genetická diverzita *S. warnstorffii*, zjištěná na základě 17 mikrosatelitních markerů, byla vyšší než genetická variabilita ostatních dvou analyzovaných druhů. Analýza pomocí programu Structure ukázala dvě výrazné genetické entity *S. warnstorffii*. Jedna skupina vzorků souvisela s dolomitovým podložím v italských a rakouských Alpách a v Estonsku a s hořčíkem bohatým, avšak vápníkem chudým, podložím Českého masívu (serpentinity, metadolerity, migmatizované pararuly s kordieritem). Druhá skupina vzorků souvisela s podložím s nízkým obsahem hořčíku z různých míst Eurasie. Analýza hlavních koordinát ukázala, že spíše než o geneticky oddělené entity se jedná o klinální variabilitu, kde populace z dolomitů (Alpy, Estonsko) a hadců (Český Masív) představují jeden extrém a populace z nehořečnatého podloží vzdáleného od hadců a dolomitů (Bulharsko) představují druhý extrém. Naše studie je první, byť neúplným, dokladem toho, že toxicita hořčíku může ovlivňovat mikroevoluci mechorostů, což bylo dosud doloženo jen pro cévnaté rostliny včetně kapradin.

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