Genetic variation in two cryptic species of the rare fen moss *Hamatocaulis vernicosus* in the Czech Republic

Genetická variabilita dvou kryptických druhů vzácného slatiništního mechu *Hamatocaulis vernicosus* v České republice

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Patterns in the genetic variation of two cryptic species (termed also clades) of the rare moss *Hamatocaulis vernicosus* in the Czech Republic were studied using two sets of polymorphic microsatellite loci, developed for each cryptic species separately with respect to cross-amplification failure. Reproductive isolation of the morphologically indistinguishable and commonly co-occurring species was confirmed not only by the absence of cross-compatibility in all but five of the used primers, but also by the obvious absence of gene flow at localities where both cryptic species co-occurred. The genetic diversity expressed in terms of the number of genotypes and Shannon diversity index was higher in clade 1 (southern cryptic species), which is more common in the region, than in clade 2 (northern cryptic species). Both cryptic species also differed in structure of their genetic diversity. While in clade 2, 84% of the variability was among populations, the level of inter-population variability was only 51% in clade 1. The high genetic isolation of populations indicates the detrimental effect habitat fragmentation has had on central European fens. The level of sexual reproduction at most localities was low, especially in the larger clones of clade 2. The effect of genetic pauperization seems to be counteracted by somatic mutations, which were recorded at several localities.

K e y w o r d s: bryophyte, cryptic species, dispersal limitation, *Hamatocaulis vernicosus*, microsatellites, spatial genetic structure

Introduction

Hamatocaulis vernicosus is a dioicous pleurocarpous moss, classified currently in the family *Scorpidiaceae* (Ignatov & Ignatova 2004). In central Europe, it is considered to be in continuous decline (Štechová & Kučera 2007) due to its confinement to a threatened habitat, non-calcareous rich fens (Davidson 2014, Janssen et al. 2016). The decline in the abundance of *H. vernicosus* in Europe resulted in it being listed among the species in Annex 2 of the Habitats Directive (92/43/EEC), which stimulated surveillance of its existing and potential localities, and an interest in obtaining a better understanding of its habitat requirements and other aspects of its biology (Štechová & Kučera 2007, Štechová et al. 2008, 2012a, b, Pépin et al. 2013, Manukjanová et al. 2014). Biological research on *H. vernicosus* became more challenging after Hedenäs & Eldenäs (2007) discovered that this species consists of two separate lineages, which can be considered as cryptic species as they are not morphologically separable (cf. also Manukjanová et al. 2019a). These cryptic species are called clade 1 and clade 2, or southern/northern cryptic species

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(Hedenäs 2018). They are reported to have partly overlapping distributions, with one of them (southern, clade 1) occurring south of the boreal zone in Europe, Peru and Russia, while the northern clade 2 is widespread in Europe occurring most frequently in the boreal zone and is also reported in the USA (Hedenäs & Eldenäs 2007). The genetic diversity of *H. vernicosus* has not yet been studied in detail. Based on the analysis of DNA sequence data from one nuclear and two chloroplast loci in a limited set of samples (Hedenäs & Eldenäs 2007, Hedenäs 2018), haplotype variability is reported in both cryptic species, but no evidence of gene flow between them. This seems to confirm that the two clades function as two distinct biological species, each with its own evolutionary history. Their current sympatric occurrence results probably from reproductive isolation, which evolved in reaction to slight niche differentiation or allopatric origin.

In previous studies, we assessed the distribution of the cryptic species of *H. vernicosus* at all known localities in the Czech Republic including their detailed spatial distribution at localities where they occur sympatrically (Manukjanová et al. 2019a). We recorded the sex ratio at 21 localities in the Czech Republic (Manukjanová et al. 2019b) and concluded that the clades did not significantly differ in their sex expression or sex ratio. As more than one third of the populations consisted of plants expressing only one sex, it is likely that such populations are entirely clonal. The answer to this question can only be resolved using a molecular approach.

Evolutionary histories are reflected in distinct patterns of genetic variability, which can be assessed using hypervariable molecular markers, such as the microsatellites, AFLP or next-generation approaches. Microsatellites (short sequence repeats – SSRs) are still used as a convenient molecular marker for studying genetic diversity at the population level in bryophytes. Apart from being codominant and selectively neutral markers with high levels of polymorphism, they also allow for the assessment of gene flow levels among populations and rates between sexual and asexual reproduction (Rodríguez-Romero et al. 2016). Their major benefit in studies on bryophytes is that the quantity and quality of needed DNA template is low, enabling the use of single shoots for DNA amplification, as well as the use of herbarium material. Moreover, after the first major investment in development of SSR markers, the analysis itself is cost-effective and the results are highly reproducible, allowing for later additions to the dataset.

A comparative study, assessing the population diversity in two closely related pleurocarpous species of the genus *Scorpidium* using SSRs was published by Kophimai et al. (2014). It shows that the rarer of the species tends to harbour most of the intraspecific diversity among populations, reflecting their genetic isolation and dispersal limitation. This study was enabled by a successful cross-amplification of SSR markers at most of the polymorphic loci. In our case, the surprisingly big genetic distance between the two cryptic species of *H. vernicosus* did not allow the use of the SSR set developed for *H. vernicosus* clade 1, necessitating the development of a SSR primer for each clade separately (Manukjanová et al. 2018).

Having developed the SSR markers for both cryptic species of the moss, *H. vernicosus*, we compared the genetic diversity and patterns in genetic variability of different Czech populations of both clades. We assumed that these patterns reflect the different evolutionary histories of the two cryptic species in central Europe. In addition, we tried to determine whether the cryptic species hybridize. Finally, we hypothesized that the rare production of sporophytes and existence of localities with only plants of a single sex

might result in high levels clonality within populations and in extreme cases localities where there is only a single clone.

Material and methods

Sampling

Hamatocaulis vernicosus was sampled at 22 localities between 2013 and 2017 (Table 1), which is more than one third of recently known localities in the Czech Republic. The distance between localities was mostly several kilometres or more and considered distinct if separated by more than 200 m of unsuitable habitat, which was the case for Zhůří (localities Zhůří A, Zhůří B), Boží Dar (localities Boží Dar A, Boží Dar B) and Břehyně (localities Břehyně A, Břehyně B). All the populations at a locality were evenly sampled in the extent depending on population size and spatial distribution (Table 1). To decrease the probability of sampling from the same clone, the patches sampled were at least 20 cm apart. The position of each patch was drawn on a field sketch that was later transferred into a GIS layer (QGIS v. 2.6 software, QGIS Development Team 2015). The information on the expressed sex ratio at localities and sex of individual shoots was obtained during a previous study utilizing the same data set (Manukjanová et al. 2019b).

From each patch, one shoot was chosen for the microsatellite analysis to determine the within-population genetic diversity, allele frequencies and Shannon index. In addition, a second shoot from each patch was collected for additional analyses that determined clonality and genotype diversity at the scale of possible fertilization distances (specifically micromaps, spatial autocorrelation analysis and PCoA of mixed localities), but this data were not used for other analyses and population characteristics. The second stem in patch was analysed only in populations which consisted of more than one multilocus genotype, as the probability of obtaining additional information from analysis of the second shoot in uniform populations was believed to be negligible. The shoots in a patch were taken from plants of different sex whenever possible, to increase the number of detected genotypes. The shoots were barcoded into their respective clades using one of the methods described in Manukjanová et al. (2018). Because of the rarity of clade 2 at Czech localities it was not possible to have an equal representation of populations of both clades; plants of *H. vernicosus* clade 1 occurred at 20 of the selected localities, plants of clade 2 occurred at nine localities at five of which both cryptic species occurred.

SSR genotyping

A set of 12 SSR loci for clade 1 and 11 SSR loci for clade 2 were used in the analysis (Manukjanová et al. 2018, Electronic Appendix 1). The DNA isolation, PCR protocols and fragment analysis followed the methods described in Manukjanová et al. (2018). Microsatellite alleles were coded in terms of the number of SSR motif repeats and scored using GeneMarker v1.80 (SoftGenetics LLC, State College, USA). Samples with more than three missing loci were discarded from the dataset. As some of the methods cannot proceed with the analysis if any data is missing and various programs treat missing data differently, we substituted missing allele data with existing alleles from the genetically

Table 1. Population characteristics of *Hamatocaulis vernicosus*. No = number of samples, Ng = number of multilocus genotypes, Largest MLG = number of samples of the most abundant MLG, Pol = % of polymorphic loci, Nr = number of genotypes resampled in 7 samples, H = Shannon diversity index, r_d = index for multilocus linkage disequilibrium (significance of r_d values is marked as ** P < 0.001; * P < 0.01). The expressed sex at a locality is coded as n.a. = not available, F = female, M = male.

Clade	Locality	Sex	N (°)	E (°)	Elevation (m a.s.l.)	No	Ng	Largest MLG	NG/N	Pol	Nr	r_d	H
1	Bažiny	n.a.	50.2964	16.2997	620	7	**1**	7	0.14	0	1.00	–	0
1	Boží Dar B	F	50.4057	12.8985	1010	10	2	8	0.20	8.33	1.94	–	0.50
1	Břehyně A	M+F	50.5810	14.7189	280	29	15	11	0.52	100.00	5.07	0.441**	2.27
1	Břehyně B	n.a.	50,5845	14,7069	280	9	5	3	0.56	83.33	4.34	0.603**	1.46
1	Hrádecká bahna	F	49.7132	13.6590	400	20	7	6	0.35	66.67	4.48	0.770**	1.74
1	Kostelní vrch	M+F	49.0556	13.4603	970	19	14	4	0.74	100.00	6.14	0.322**	2.51
1	Louky v Jeníkově	M+F	49.7385	15.9645	630	8	2	5	0.25	66.67	2.00	1.000	0.66
1	Novozámecký rybník	F	50.6125	14.5853	255	7	3	4	0.43	33.33	3.00	0.541*	0.96
1	Oklika	M	49.4042	15.3945	660	13	11	2	0.85	83.33	6.46	0.273**	2.35
1	Pihel	F	50.7353	14.5529	300	24	3	22	0.13	16.67	1.54	–0.043	0.33
1	Ratajské rybníky	M	49.7694	15.9339	590	16	10	4	0.63	25.00	5.61	0.028	2.13
1	Ruda	M+F	49.1453	14.6908	415	19	8	6	0.42	91.67	4.47	0.592**	1.79
1	Řeka	M+F	49.6666	15.8530	555	9	3	7	0.33	16.67	2.52	–0.125	0.68
1	Staré jezero	M+F	48.9792	14.8973	445	40	19	11	0.48	100.00	5.49	0.269**	2.58
1	Šimanov	M+F	49.4504	15.4467	605	9	6	2	0.67	50.00	5.22	0.439**	1.74
1	Šmauzy	M+F	49.1970	13.2622	1030	16	7	5	0.44	91.67	4.80	0.328**	1.77
1	V Lisovech	M+F	49.2470	15.2788	650	26	18	6	0.69	100.00	6.03	0.325**	2.66
1	Vidlák	M+F	50.5244	15.2174	280	13	11	2	0.85	91.67	6.41	0.422**	2.35
1	Zhůří A	M	49.1725	13.3317	900	9	8	2	0.89	83.33	6.41	0.447**	2.04
1	Zhůří B	M+F	49.1707	13.3326	960	5	2	4	0.40	33.33	–	1,000*	0.50
2	Boží Dar A	M	50.407	12.9006	1000	6	**1**	6	0.17	0	1.00	–	0
2	Panská	F	49.6019	16.1688	720	15	**1**	15	0.07	0	1.00	–	0
2	Řeka	M	49.6666	15.8530	555	40	3	35	0.08	45.45	1.74	0.674**	0.44
2	Řeřišný	M	50.5046	16.2915	495	13	4	5	0.31	18.18	3.63	–0.028	1.33
2	Skalské rašeliniště	M+F	49.9182	17.2114	680	14	4	11	0.29	54.55	2.61	0.679**	0.75
2	Šimanov	M+F	49.4504	15.4467	605	3	2	2	0.67	9.09	–	–	0.56
2	Vidlák	M+F	50.5244	15.2174	280	25	6	14	0.24	63.64	3.28	0.381**	1.26
2	Zhůří A	M+F	49.1725	13.3317	900	16	5	9	0.31	72.73	3.45	0.329**	1.24
2	Zhůří B	M+F	49.1707	13.3326	960	12	4	5	0.33	63.64	3.44	0.641**	1.24

and spatially closest plant of the same sex at the same locality, even though this approach may lead to a minor underestimate of the actual diversity.

Data analysis

For multilocus genotypes (MLG), their number (Ng), number of resampled MLGs (Nr) for the defined number of samples (we used Nr for seven samples in this study) and Shannon index (H) were calculated using the GenClone 2.0 software (Arnaud-Haond & Belkhir 2007). The difference in the Shannon index between localities where both sexes were recorded and those, where both sexes were not recorded was tested using analysis of variance (ANOVA). The relationship between population size, represented by the number of samples (No), and the number of genotypes (Ng) was tested using linear regression. Both ANOVA and linear regression were calculated in Statistica v. 8 software (Statsoft 2007).

In order to evaluate relationship and whether clade 1 and clade 2 hybridize, populations at five mixed localities were analyzed. We used a reduced dataset consisting of only five loci that were variable in both clades. To maximize the number of samples, both shoots collected from a patch were used for analyses at mixed localities, resulting in 66 individuals of clade 1 and 126 individuals of clade 2, respectively. In addition, we analysed the full dataset from all available localities using those five loci to test whether they form distinct groups in ordination space. The genetic similarity among samples was visualized using Principal Coordinate Analysis (PCoA, Electronic Appendix 2) implemented in GenAlEx 6.5 (Peakall & Smouse 2012) based on PhiPT genetic distances assuming a stepwise mutation model.

The structure of genetic variability within and among populations of each cryptic species was tested based on an Analysis of Molecular Variance (AMOVA) calculated in GenAlEx 6.5. To illustrate the relationships among samples and populations, we used the PCoA based on PhiPT genetic distances among samples. PhiPT value, which is analogous to Rst for codominant data, was used for both AMOVA and PCoA. Pair-wise genetic distances between the different populations were computed using Nei's standard genetic distance D (Nei 1972, 1973). In addition, pair-wise fixation index (Rst) between the different populations were computed in GenAlEx 6.5. A Mantel test was used to explore correlations between genetic and geographic distances and was computed in GenAlEx 6.5 for each cryptic species. To assess whether marker distributions in individual populations resulted from sexual or asexual reproduction, we performed the linkage disequilibrium analysis using MultiLocus 1.3 software (Agapow & Burt 2001). Multilocus linkage disequilibrium was tested using the index of association (r_d) modified to remove the effect of the number of loci analysed. Statistical significance was tested by comparing the observed dataset with the null hypothesis of infinite amount of sex and recombination by random shuffling of the alleles amongst individuals using 500 randomizations.

To test the correlation between geographic and genetic distances and reveal the fine-scale spatial genetic structure (SGS) within localities, a spatial autocorrelation analysis was conducted in SPAGeDi 1.4 software (Hardy & Vekemans 2002) For each clade, mean multilocus pairwise kinship coefficient values (Fij) based on Nason's kinship coefficient (Loiselle et al. 1995) were plotted against the upper boundaries of geographic distance classes (0.01, 0.5, 1, 2, 5, 10, 50, 100 and 2000 m). Significance of the mean Fij per distance class was tested using 500 random permutations of individuals. This analysis also included the second shoot in a patch, which accounts for all values in the first distance class.

The spatial distribution of individual genotypes at localities was visualized using micromaps plotted in Microsoft Excel. The position of individual samples was defined using their UTM coordinates.

Results

Genetic variability and its structure in the two cryptic species

The dataset with one sample analysed per patch contained 452 successfully genotyped shoots (Table 2). The genetic variability of clade 2 was generally lower, with a mean number of MLG per locality of 3.33 as opposed to 7.75 for clade 1. Even when resampled

Table 2. Population characteristics for both *Hamatocaulis vernicosus* clades (based on 1 shoot per patch). No = number of samples, Ng = total number of multilocus genotypes, Ng max = the highest number of multilocus genotypes per locality, mean Ng = mean number of genotypes per locality, mean Nr = mean number of genotypes resampled in 7 samples per locality. For mean values, standard deviation is shown in brackets).

Characteristic	Clade 1	Clade 2
No	308	144
Ng	152	29
mean Ng	7.75 (5.35)	3.33 (1.63)
mean Ng/No	0.52 (0.22)	0.32 (0.18)
mean Nr	4.37 (1.81)	2.51(1.12)
Ng max	19	6
number of alleles per locus	4–22	2–11
mean Shannon index H'	1.55 (0.83)	0.76 (0.51)

Table 3. Analysis of molecular variance (AMOVA) of microsatellite variation recorded in both clades of *Hamatocaulis vernicosus*.

Clade	Source	df	SS	variance
clade 1	Among populations	19	47817	51%
clade 1	Within populations	288	42838	49%
clade 2	Among populations	8	7378	84%
clade 2	Within populations	135	1517	16%

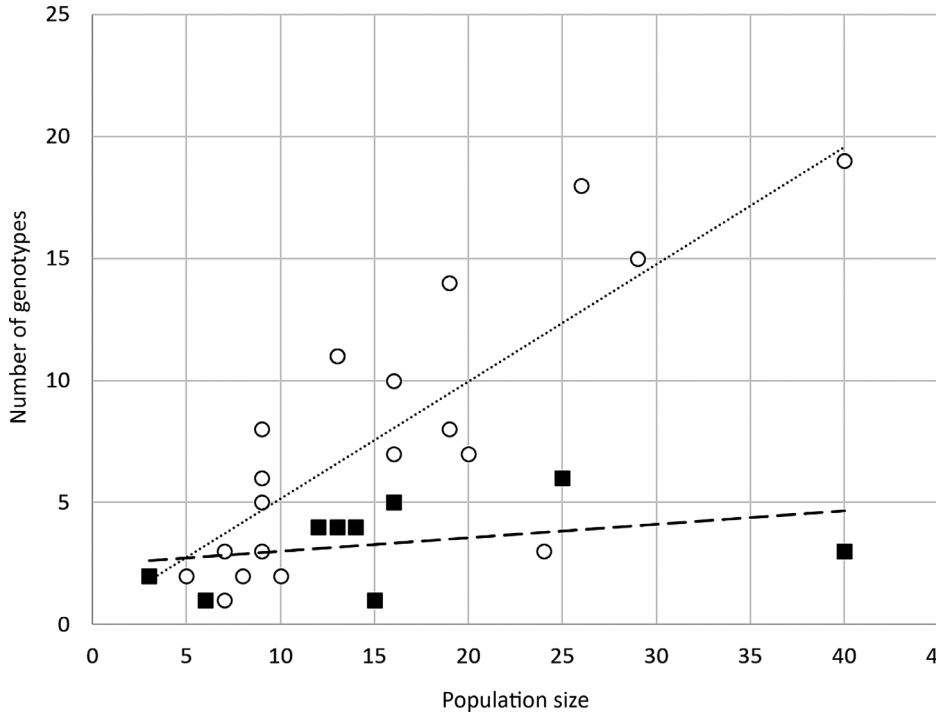

Fig. 1. – Relationship between the number of genotypes (Ng) and population size in *Hamatocaulis vernicosus*. The correlation (r = 0.7848) was significant (P = 0.00004) for clade 1 (○), but not clade 2 (■, r = 0.3499; P = 0.356)

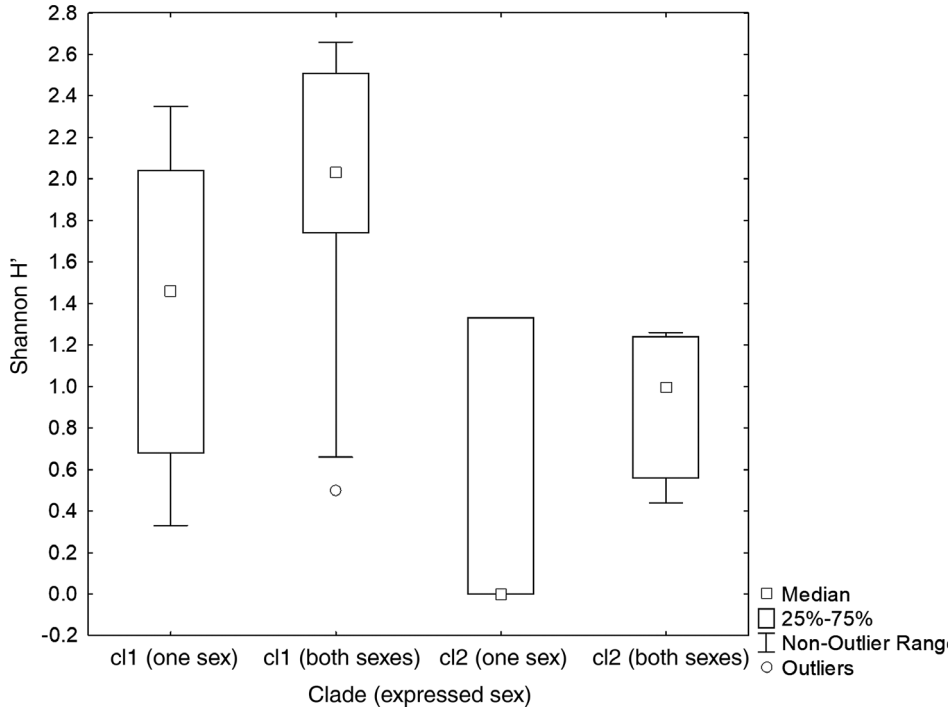

Fig. 2. – The comparison of Shannon indices for populations with both male and female plants expressing gametangia and unisexual populations of clade 1 and 2 of *Hamatocaulis vernicosus*.

for the same number of samples for each locality in order to compensate for different population sizes, clade 1 had almost twice as many MLGs on average (Table 2). The ratio of number of MLG and number of samples at a locality for clade 2 was significantly lower ($F_{1, 27} = 8.659$; $P = 0.0066$), indicating a lower genetic variation than in clade 1. Localities with the highest number of genotypes were usually large and contained plants of both sexes. The population census size (since this term is not frequently used in bryology, we use only population size henceforth in the text) is positively correlated with the number of MLGs, but the test was statistically significant only for clade 1 (Fig. 1). No MLG was shared between localities of clade 1 except for sublocalities Břehyně A and B which shared one MLG, while for clade 2, one MLG was shared between localities Vidlák and Šimanov, which are120 km apart.

The structure of genetic variability within and among populations of clade 1 and clade 2 differed (Table 3). While in clade 1 variability within and among populations was almost equal, populations were less variable in clade 2 and most of the variability was recorded among populations.

In contrast to the situation among localities, we recorded plants with identical multilocus genotypes at each locality, which we considered to be clones (Table 1). Nevertheless, only at one locality of clade 1 (Bažiny) and two of clade 2 (Boží Dar a and Panská) we recorded only a single clone. The clones were variable in size, the largest clone of clade 1 at Pihel contained 22 samples and the largest clone of clade 2 at Řeka contained

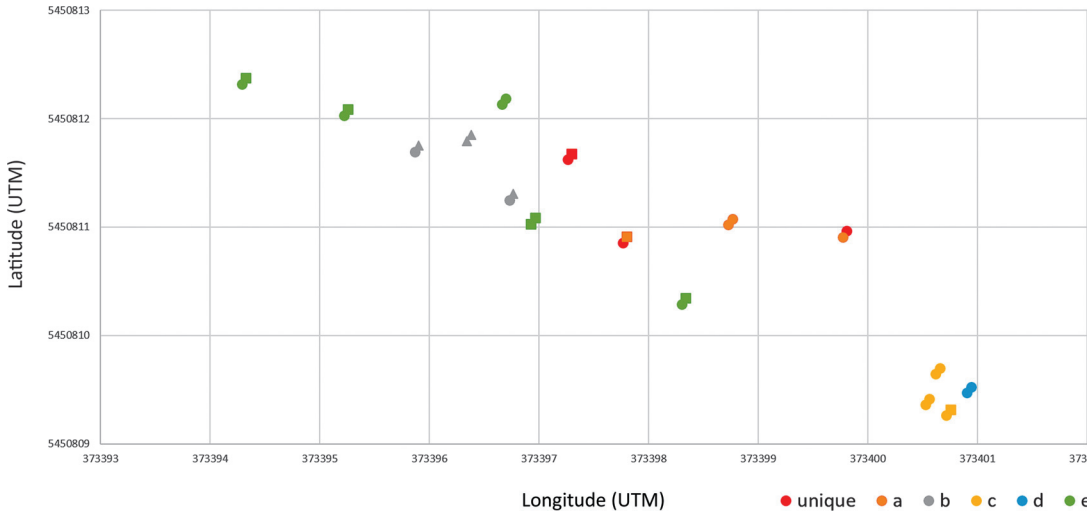

Fig. 3. – Spatial genetic structure of *Hamatocaulis vernicosus* at locality Šmauzy (clade 1). Each clone recorded more than once is depicted using a a different colour, unique genotypes are merged into one category. Symbol shapes represent sex – triangles are males, squares females and circles sterile plants. The position of patches is displayed on a UTM grid.

35 samples. At Boží Dar b, all samples except one and at Pihel, all samples except two, belonged to the same clone, and the differences between samples were for a single locus at which one allele was one repetition longer.

The localities where both male and female plants had gametangia had higher values of Shannon index than localities where only one sex was present (Fig. 2), however, the results were not statistically significant (clade 1: $F_{1, 17} = 3.575$; $P = 0.076$; clade 2: $F_{1, 7} = 2.255$; $P = 0.177$). At Bažiny where there was no sex expression there was only a single MLG. Shoots with the same MLG were usually of the same sex (Fig. 3), although some exceptions were recorded. The ongoing gene-flow between plants of the same clade estimated on low linkage disequilibrium values was recorded only at a small number of localities, with most localities having high values, which indicate the prevalence of asexual reproduction and consequently the documented high level of clonality (Table 1). Interestingly, the linkage disequilibrium values did not differ significantly between clades, despite the large genetic diversity in clade 1 (r_d: $F_{1, 22} = 0.024$; $P = 0.879$).

At localities where the second shoot from a patch was analysed, most of the samples from the same patch (89%) were identical, even though the additional samples analysed (139 for clade 1 and 42 for clade 2) revealed a further 30 MLGs for clade 1 and 10 MLGs for clade 2.

Spatial genetic structure and distribution of genotypes

Genetic distances among plants of both clades were correlated with geographical distance based on Mantel's test (clade 1: $r = 0.133$, $P = 0.01$; clade 2: $r = 0.288$, $P = 0.01$). The gradual decrease in the similarity of the MLGs in the two clades is also indicated by the spatial autocorrelation analysis (Fig. 4). In all distance classes, the values of Fij are positive for

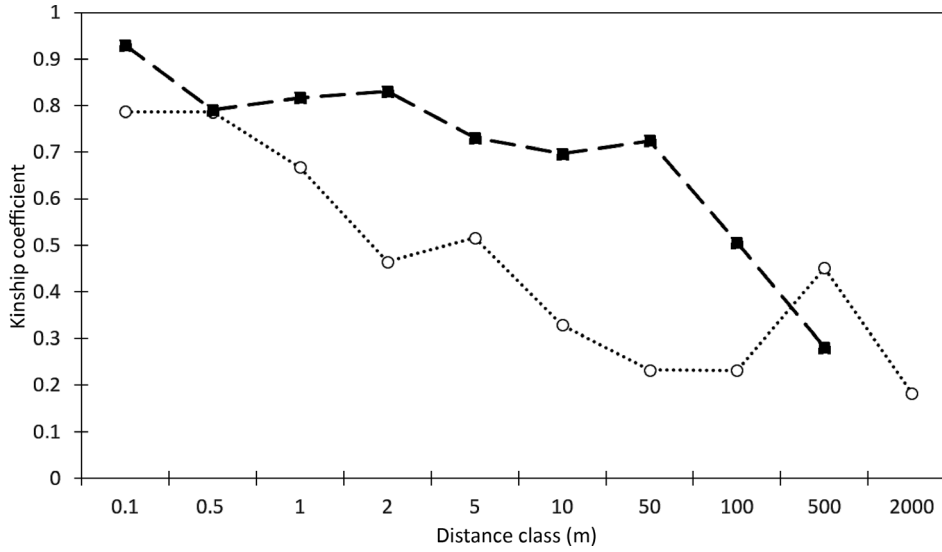

Fig. 4. – Spatial autocorrelation analysis based on microsatellite data for populations of *Hamatocaulis vernicosus* of ○ clade 1 and ■ clade 2. The Nason's kinship coefficients (Fij) are positioned along the x-axis at the mean pairwise distance within each distance class. All the values are statistically significant ($P < 0.001$). The first distance class is based solely on the plants sampled from the same patch.

plants of both clades, although in plants of clade 1, the values gradually decrease with distance while in clade 2, the values remain high up to a distance of 50 metres.

The spatial distribution of genotypes at individual localities revealed high aggregation of plants of the same genotype (Fig. 3, Electronic Appendix 3), particularly at the shortest distances analysed (< 10 cm). At some localities with large clones (e.g., locality Řeka, Electronic Appendix 3), we recorded plants differing in a single repetition within a huge patch of the genotype.

The Nei's genetic distance between localities was higher for clade 1 (0.987) than for clade 2 (0.703), despite the higher average pairwise Rst values for clade 2 (0.54 for clade 1 and 0.78 for clade 2, Electronic Appendix 4). The correlation between Rst and geographic distance was positive for clade 1 ($r = 0.1707$; $P = 0.0186$), but non-significant for clade 2 ($r = 0.0286$; $P = 0.8683$).

Genetic differentiation of the two cryptic species at mixed localities

Relationships among populations of clade 1 and 2 are illustrated using the PCoA analysis of samples based on a limited set of five loci, which were variable in both clades. The first analysis, which utilized all the samples in the dataset, revealed a distinct cluster consisting of samples of clade 2 with samples of clade 1 dispersed around it (Fig. 5). The second analysis used only samples from mixed localities (Fig. 6) and revealed even more distinct clusters but not the closeness of the samples of both clades from the same locality. Even in this small sample set of mixed-localities (66 individuals of clade 1 and 126 individuals of clade 2) the number of MLGs in clade 1 was higher than in clade 2 (27 MLGs in clade 1, 22 MLGs in clade 2).

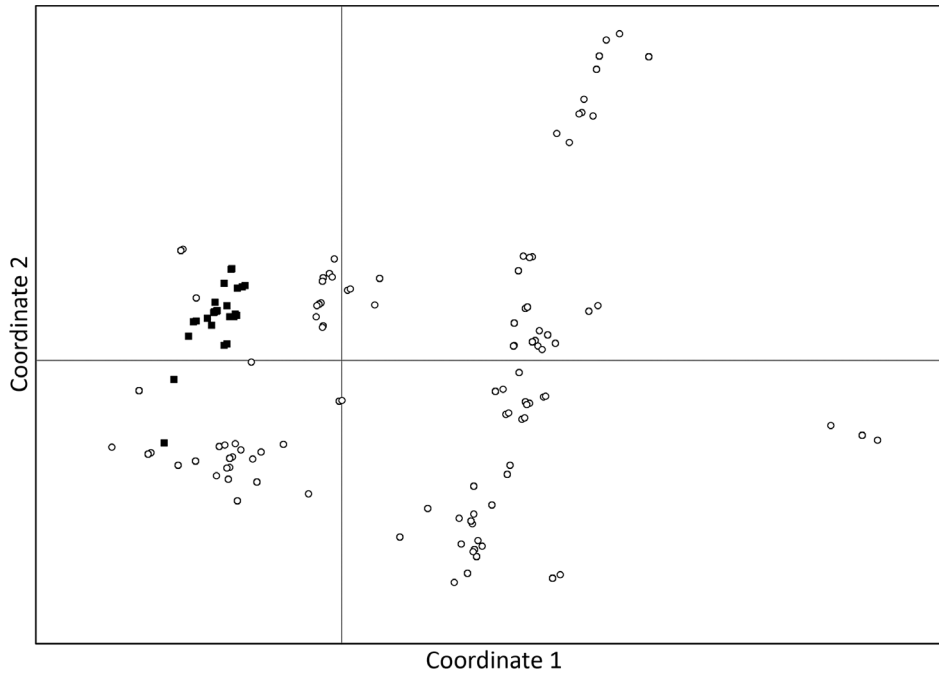

Fig. 5 – The genetic differences of ○ clade 1 and ■ clade 2 at all localities based on five variable loci visualized using principle coordinate analysis.

Discussion

Absence of hybridization among the two cryptic species

Comparison of genetic variability of the two cryptic species of *Hamatocaulis vernicosus* is complicated by the surprisingly strong divergence in their microsatellite loci (Manukjanová et al. 2018). While in other similar studies, cross-amplification of SSR loci in closely related species is generally successful (in pleurocarpous species of *Scorpidium*, Kophimai et al. 2014, and *Sphagnum*, Shaw et al. 2008a, b, Johnson & Shaw 2015, Mikulášková et al. 2017), we only successfully cross-amplified five out of 18 variable SSR designed for both cryptic species of *H. vernicosus*. The absence of ongoing hybridization was also supported by analysis of genetic distances at localities where both clades coexisted (Fig. 6). While the samples from different clades often grew sympatrically at localities, the clades formed distinct clusters in ordination space. The samples from the same localities were not closer to each other than between clades, indicating no gene-flow between the lineages. This confirms that mechanisms maintaining the reproductive isolation between the two cryptic species of *H. vernicosus* are strong and they are distinct biological species, even though they appear to be morphologically and ecologically identical (Hedenäs & Eldenäs 2007, Manukjanová et al. 2019a).

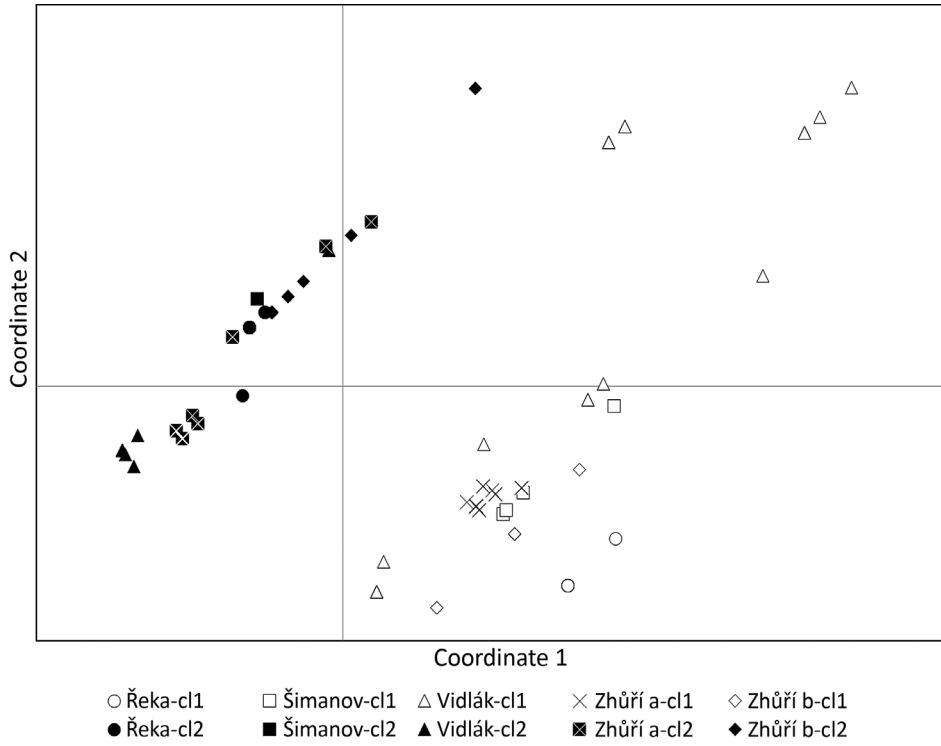

Fig. 6. – The genetic differences of clade 1 and 2 at mixed localities of *Hamatocaulis vernicosus* based on five variable loci visualized using principle coordinate analysis. Plants from same locality are denoted by the same coloured symbol: white for clade 1 and black for clade 2. The first two axes account for 53.6% and 38.1% of the variability, respectively.

Differences in genetic variability and its spatial structure in the two cryptic species

Based on the total number of MLGs, number of resampled MLGs within populations and Shannon index values, clade 1 is more genetically variable than clade 2. This seems to be correlated with the number of localities (almost 10 times more than for clade 2) and population size in the Czech Republic; non-significant relationship between the number of MLGs and population size of clade 2 might result from the low number of populations analysed. The greater genetic variability of clade 1 is consistent with the variability of ITS ribotypes (Manukjanová et al. 2019a), with three ribotypes recorded in Czech populations of clade 1, while clade 2 was uniform. In contrast, clade 2 is more diverse in Scandinavia in terms of the number of ribotypes (Hedenäs & Eldenäs 2007). On the other hand, it is not certain, whether the pattern of genetic diversity between clades holds generally for central Europe, as the number of ribotypes in individual clades reported for localities in Switzerland and western Austria are similar, and hence the small genetic diversity in clade 2 might be specific to the central-eastern European region and not to the more western part of its area of distribution. Interestingly, clade 2 often has bigger populations at localities in the Czech Republic where both clades are present, which would indicate either a competitive advantage in terms of faster growth, or a broader ecological

niche. Different levels of genetic diversity between the clades are congruent with the hypothesis of Hedenäs & Eldenäs (2007), that the clades possibly followed different migration routes after the last glaciation. Clade 2 could have survived the last glaciation in refugia in northern Europe (Hedenäs & Eldenäs 2007, Kyrkjeeide et al. 2014) similar to *Drepanocladus aduncus* (Hedenäs 2008) and *Rhytidium rugosum* (Hedenäs 2015) and probably migrated to the Czech Republic following a single migration route.

Unlike clade 1, most of the variability (84%) of clade 2 was allocated among populations (Table 3), reflecting the genetic isolation among them, and is also correlated with the average geographical distance between populations in the Czech Republic. On the other hand, the mean pairwise Nei's genetic distance between localities was higher in clade 1, despite its lower average pairwise fixation index (Rst values). Pairwise Rst values between localities are higher (for both clades) than those reported in other similar SSR studies on *Polytrichum formosum* (Van der Velde et al. 2001), *Sphagnum* (Szövényi et al. 2008) and *Crossocalyx hellerianus* (Holá et al. 2015), which indicates only a limited gene flow between localities. Particularly surprising was the great genetic differentiation between sublocalities Zhůří A and B (several hundred metres apart) in clade 1, which was higher (Rst = 0.947) than most other pairwise distances (averaging 0.54). The limited gene flow among localities generally results from habitat fragmentation, which has adverse effects on the genetic diversity (Wilson & Provan 2003, Leonardía et al. 2013, Kophimai et al. 2014, Pandey et al. 2016). The serious destruction, degradation and fragmentation of rich fens over the last centuries particularly affected species relying on vegetative reproduction.

Despite the high number of localities studied and samples collected, with the exception of two cases, no shared MLG was recorded at the different localities. One genotype of *H. vernicosus* clade 1 was shared between microlocalities Břehyně A and Břehyně B, which are situated about 1 km apart, which share a similar history and might be a residuum of a single larger population, in addition to the possibility of diaspore dispersal via water or animals. In clade 2, one MLG was shared between two localities more than 100 km apart, which was most probably caused by the accidental homoplasy of the microsatellite loci used (Estoup et al. 2002). The general absence of shared MLGs matches the previously published pattern in the rare epixylic hepatic *Crossocalyx hellerianus* (Holá et al. 2015), for which there has also been a significant decline and fragmentation of its habitat in central Europe.

The genetic diversity of populations of both clades of *H. vernicosus* in the Czech Republic was generally low, with the mean number of resampled MLGs per locality 4.36 for clade 1 and 2.52 for clade 2. Variation within populations based on number of MLGs was similar in both cryptic species to the values reported in a microsatellite study of two taxa of the related genus *Scorpidium* (Kophimai et al. 2014). The dioicous *Scorpidium cossonii* was more diverse (mean Ng = 13.6, Kophimai et al. 2014) than our dioicous clade 1 species of *H. vernicosus* (mean Ng = 7.75) while the diversity in the monoicous *Scorpidium*, *S. revolvens* (mean Ng = 3.5), was close to that of *H. vernicosus* clade 2 (mean Ng = 3.33). However, the studies differed in their sampling pattern (seven samples per each of the five circular plots of 2 m radius within an area 1 ha in Kophimai et al. 2014), as did the number and variability of utilized SSR loci (14 loci for S. *cossonii* and 13 for *S. revolvens*). The smaller number of localities of both *Scorpidium* species (five and four) resulted in only the larger populations being sampled, which tend to have a greater diversity. In both *Scorpidium* and *Hamatocaulis*, a lower genetic diversity is reported for the locally rarer species. Generally,

rare alleles may be lost accidentally while common alleles may become fixed, resulting in low genetic diversity and high differentiation between populations (Frankham et al. 2004). Low genetic diversity of rare species based on other genetic markers (isozymes) is also reported for the moss genus *Plagiomnium* (Wyatt 1992).

Low level of sexual reproduction in both cryptic species

Sporophyte production is extremely rare in *H. vernicosus* (Štechová & Kučera 2007). Over the last two decades it was reported at less than 10 localities in the Czech Republic (Štechová, personal communication). However, since many of the occurrences were at mixed localities, it was not possible to quantify the frequency of sporophytes for the two cryptic species separately. Availability of sexual mates did not contribute statistically significantly to higher genetic diversity, although the localities where both sexes were present had slightly higher Shannon index values. The linkage disequilibrium values at most localities, including the genetically more variable ones, however, were high (Table 1), suggesting that most of the observed genetic variability does not result from successful establishment of new recombinant genotypes formed in course of the ongoing sexual reproduction (Shaw et al. 2008b, Ramaiya et al. 2010). More likely, current genotype diversity may result from population establishment from multiple sources, or from diversification by somatic mutations (see below). This explanation is plausible even in cases of genetically variable populations consisting of only one sex or the possibility of accidently not sampling plants of the other sex.

The micromaps of MLGs at each locality reveal that for both clades, the shoots sampled were usually grouped in clusters with identical MLGs for the same sex. The extreme case was recorded in a small population of clade 1 (Louky v Jeníkově), where all male plants had one MLG and all female plants another, which indicates the absence of sexual reproduction. Generally, the expansion in identical MLGs implies that vegetative spreading is the most important means of reproduction in *H. vernicosus* (reported earlier by During & van Tooren 1987). The expansion of clones is reported in several other molecular studies on bryophytes (Pfeiffer et al. 2006 for *Rhytidium*, Brzyski et al. 2018 for *Marchantia*). In clade 2, a more gradual decrease in values of the kinship coefficient was recorded (Fig. 4), which indicates a greater spatial extent of vegetative expansion than in clade 1.

Somatic mutations

The pairs of genetically nearly identical MLGs differing by only one repetition at a single locus recorded at Pihel and Boží Dar B indicate the occurrence of somatic mutations. This variation in microsatellite data often results from strand slippage during DNA replication (Levinson & Gutman 1987) in which excision or addition of repeats enables a new match (Schlötterer & Tautz 1992). The somatic mutations can reduce the extent of genetic pauperisation, particularly in asexually reproducing bryophytes, as is proposed in many studies (Newton & Mishler 1994, Skotnicki et al. 2005, Pohjamo et al. 2008, Karlin et al. 2011, Bączkiewicz 2012, Kophimai et al. 2014). Even at localities with a greater genetic diversity a significant part of it might originate from somatic mutations, given the high linkage disequilibrium values (Table 1).

See www.preslia.cz for Electronic Appendices 1–4

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Souhrn

Rozložení genetické variability obou kryptických druhů vzácného mechu *Hamatocaulis vernicosus* v České republice bylo studováno za použití dvou sad mikrosatelitních lokusů, vyvinutých pro každý druh zvlášť (kvůli neúspěšným cross-amplifikacím). Reprodukční izolovanost těchto morfologicky nerozlišitelných, nicméně společně se vyskytujících druhů byla potvrzena nejen absencí kompatibility všech amplifikujících lokusů kromě pěti, ale i absencí genového toku na směsných lokalitách. Genetická variabilita (vyjádřená počtem genotypů a Shannonovým indexem) linie zvané clade 1 (jižní kryptický druh), která je v regionu běžnější, byla vyšší než u linie clade 2 (severní kryptický druh). Zároveň se výrazně lišila struktura jejich variability, jelikož u clade 2 připadalo 84 % na variabilitu mezi populacemi, zatímco u clade 1 pouze 51 %. Vysoká genetická izolovanost v populacích obou linií poukazuje na vážné důsledky fragmentace biotopu, která ovlivňuje středoevropská slatiniště. U obou kryptických druhů byl na většině lokalit zaznamenám malý podíl pohlavního rozmnožování, rozsáhlé klony byly zaznamenány zejména na lokalitách cladu 2. Genetické ochuzení je nicméně vyváženo přítomností somatických mutací, které byly pozorovány na některých lokalitách.

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