

University of South Bohemia in České Budějovice

Faculty of Science

**Expressed sex ratio in populations of the moss
Hamatocaulis vernicosus (Scorpidiaceae) in the
Czech Republic with consideration of its cryptic
species.**

RNDr. Thesis

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České Budějovice

2019

This thesis should be cited as:

Manukjanová, A., 2019: Expressed sex ratio in populations of the moss *Hamatocaulis vernicosus* (Scorpidiaceae) in the Czech Republic with consideration of its cryptic species. RNDr. Thesis, University of South Bohemia, Faculty of Science, České Budějovice, Czech Republic, 24 pp.

Annotation

This thesis aims at sex expression and sex ratio in cryptic species of a rare fen moss *Hamatocaulis vernicosus*. The sex expression and expressed sex ratio were compared at various hierarchical levels. Additionally, micromaps of expressed sex ratio in patches at 21 localities, including 5 localities where both clades co-occur have been created.

Declaration [in Czech]

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České Budějovice, 5.8.2019

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Manukjanová A., Štechová T. & Kučera J. (2019). Expressed sex ratio in populations of the moss *Hamatocaulis vernicosus* (Scorpidiaceae) in the Czech Republic with consideration of its cryptic species. – *Cryptogamie-Bryologie* 40: 41–58. (IF=1.09)

AM performed most of the sampling, lab work, data evaluation and writing the manuscript. TS assisted with the field sampling and choosing localities. JK assisted with the data evaluation and writing the manuscript.

Expressed sex ratio in populations of the moss *Hamatocaulis vernicosus* (Scorpidiaceae) in the Czech Republic with consideration of its cryptic species

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Running title: Sex ratio of *Hamatocaulis vernicosus*

Abstract

We assessed the sex expression and expressed sex ratio in populations of the moss *Hamatocaulis vernicosus* (Mitt.) Hedenäs at 21 localities in the Czech Republic. Despite its extremely rare sporophyte production, the species had a high sex expression (59% of shoots); however, the method of its calculation had a major impact on results. The micromaps of individual localities showed that male and female plants tend to grow in separate clusters, while only 7% of patches contain both sexes, which may affect the frequency of fertilization. The overall F:M sex ratio of stems was 1.03; however, the 62% of localities showed female-biased sex ratio.

As the species is known to consist of two cryptic species that are presumably sexually incompatible, we also assessed the expressed sex ratio of barcoded shoots at the localities with populations of both cryptic species growing together. The cryptic species differed neither in their sex expression nor in the sex ratio. However, the overall seemingly well-balanced sex ratio at localities often obscured situations when severe mate limitation in one of the cryptic species occurred.

Keywords: bryophyte – cryptic species – *Hamatocaulis vernicosus* – reproduction – sex ratio

Résumé

Rapport entre les sexes dans les populations d'une mousse rare *Hamatocaulis vernicosus* (Mitt.) Hedenäs (Scorpidiaceae) en République Tchèque et ses espèces cryptiques.

Les auteurs ont étudié l'expression sexuelle et le rapport entre les sexes des populations de *Hamatocaulis vernicosus* (Mitt.) Hedenäs dans 21 localités de République Tchèque. En dépit de sa production de sporophyte extrêmement rare, l'espèce a une très importante expression sexuelle, cependant, la méthode de calcul a un impact majeur sur les résultats. L'analyse spatiale de distribution montre que les plantes mâles et femelles tendent à croître dans des groupes séparés, tandis que des ensembles mixtes sont très rares, ce qui peut affecter la fréquence de fertilisation. Le rapport global entre les sexes, mais 61% des localités est biaisée par une majorité femelle. Comme l'espèce est connue pour comprendre deux espèces

cryptiques qui sont probablement incompatibles, les auteurs ont également évalué le sexe ratio des tiges avec des codes barres pour les populations mixtes. Les espèces cryptiques diffèrent ni dans l'expression sexuelle, ni dans le ratio sexuel. Cependant, le rapport général, apparemment bien équilibré entre les sexes dans les localités masque souvent des situations où une limitation importante des partenaires dans l'une des espèces cryptiques se produit.

Mots clés: Bryophyta, espèce critique, reproduction, sexe ratio.

Introduction

Sexual reproduction plays a key role in maintaining the genetic diversity and long-range dispersal of bryophytes. Although vegetative reproduction is common in most bryophyte groups and some species are even known to reproduce only vegetatively, sporophyte production occurs in most of the species at least occasionally. Fertilization in bryophytes depends on the presence of liquid water, which is needed for delivery of motile spermatozooids to the egg cell (Glime & Bisang, 2017). This requires the close proximity of male and female gametangia (Longton, 1976). Approximately 50% of bryophytes are unisexual, in contrast to mere 4% of vascular plants (Shaw, 2000; Glime & Bisang, 2017). This may entail spatial segregation of the sexes, particularly in cases of spore establishment from long-distance dispersal. Another complication might be the low sex expression or markedly biased sex ratio (Bisang & Hedenäs, 2005). Absent, generally low or regionally and temporally oscillating sporophyte production seems to be common in dioicous pleurocarpous moss species (Longton & Miles, 1982; Pépin, *et al.*, 2013).

Unlike in vascular plants, prevailing bryophyte sex ratio seems to be female-biased (Longton & Schuster, 1983; Bowker *et al.*, 2000; Bisang & Hedenäs, 2005), although male-biased (Shaw *et al.*, 1992; Bisang & Hedenäs, 2005; Holá *et al.*, 2014), as well as balanced ratios (Bowker *et al.*, 2000; Bisang & Hedenäs, 2005) were reported as well. Skewed sex ratio may result from different factors or a combination thereof (reviewed by Glime & Bisang (2017). Stark *et al.*, (2000) and Haig (2016) suggested that female-biased sex ratio is a consequence of higher investments into antheridia production in prezygotic phase, than into archegonia which developed because of the high importance of female plants as sporophyte bearers. Sex ratios at the level of spore development have only been studied in a few mosses and the results mostly showed the expected balanced ratio which is a result of undisturbed meiosis (Stark *et al.*, 2010; Bisang *et al.*, 2017). However, expressed sex ratios in adults may be female-biased despite the balanced sex ratio of spores, as shown in a study of *Drepanocladus lycopodioides* (Brid.) Warnst. (Bisang & Hedenäs, 2013; Bisang *et al.*, 2017). Higher mortality of male sporelings, slower growth of male plants, as well as sexual differences in ecology and desiccation tolerance may add to the reasons for female-biased sex ratio (Newton, 1972; McLetchie, 1992, 2001). Differential expression of gametangia, biased towards higher proportion of sexually non-expressing shoots among genetically male individuals was called “shy male hypothesis” (Stark *et al.*, 2005). It was observed in *Syntrichia ruralis* (Hedw.) F. Weber & D. Mohr (Mishler & Oliver, 1991) but not in *Syntrichia caninervis* Mitt. (Stark *et al.*, 2005) or *Drepanocladus lycopodioides* (Bisang & Hedenäs, 2013).

The pleurocarpous moss *Hamatocaulis vernicosus* (Mitt.) Hedenäs is considered threatened in most European countries, is listed in Annex 2 of the EU Habitats Directive

(92/43/EEC), and is confined to non-calcareous rich fens, which are classified as an endangered habitat at the European scale. The reproduction biology of *Hamatocaulis vernicosus* has been studied to some extent, however, none of the studies addressed the differences between its cryptic species. Pépin *et al.* (2013) studied the causes of its sporophyte absence in the French Central Massif. They found that these likely resulted from the generally unfavourable site conditions, causing the sporophyte abortion during winter, and limited mate availability or sometimes even absence of the other sex in populations, preventing thus the fertilisation. Bisang *et al.* (2014) revealed that in their dataset originating mainly from Sweden, *H. vernicosus* had higher-than-average sex expression as compared to 10 wetland species of Calliergonaceae and Amblystegiaceae, while its sporophyte production was average. In their study, based mostly on herbarium specimens, the sex expression of *H. vernicosus* was 63% while most of other species expressed gametangia in less than 50% of samples.

H. vernicosus consists of two separate lineages, which were regarded cryptic species by Hedenäs & Eldenäs (2007), based on the pattern of sequence variation at the studied loci. The cryptic species are termed hereafter ‘clade 1’ and ‘clade 2’, respectively, following the convention used by Hedenäs & Eldenäs (2007). The clades were shown to have their own history and distribution pattern, despite the apparently overlapping ecology and morphology. At parts of Central Europe and in southern Scandinavia, the two clades occur sympatrically. Comparative studies of sex ratio among cryptic species are extremely rare in bryophytes. To our knowledge, only Buczkowska *et al.*, (2006) showed that the lineages of the hepatic *Aneura pinguis* (L.) Dumort. *Representing* cryptic species, differed in their expressed sex ratio and sex expression levels.

Here, we investigated the sex expression and expressed sex ratio in both cryptic species of *H. vernicosus* using molecularly barcoded individuals. We compared different approaches to sex expression and expressed sex ratio by assessing the parameters at different levels. The study was carried out at localities which contained populations of only one or both cryptic species. At localities where both clades are present we depicted the spatial distribution of the two clades and their sex. We hypothesized that in mixed populations with uneven proportion of clades or their spatial segregation, availability of mating partners might be severely limited even when the overall sex ratio and expression is balanced, leading to false and/or over-optimistic conclusions with respect to conditions underlying sexual reproduction at the localities.

Material and methods

Sampling

Samples were collected at 21 localities of *Hamatocaulis vernicosus* between 2013 and 2017 (Table 1). Selection of localities for the study, which represent almost one third of recently known localities in the Czech Republic, was based on a preliminary screening of clade distribution in the country to ensure the regional balance. The distance among localities was mostly at least several kilometres, but in cases when local populations were closer, the localities were considered distinct if separated by more than 200 m of unsuitable habitat. This was the case of the macro-localities Zhůří (localities Zhůří 1, Zhůří 2) and Boží Dar (localities Boží Dar 1 and 2). Populations were sampled evenly over the whole locality depending on population

size (Table 1). To decrease the probability of sampling from the same clone, patches were sampled at a distance of at least 20 cm apart.

For the sex ratio assessment, ten neighbouring well-developed shoots were collected from each patch; the average patch size was about 5×5 cm. In very small populations covering less than a few dm² of very loose turfs (in this study the locality Bažiny), only one shoot per patch was sampled to avoid over-collecting. In addition, some shoots needed to be excluded in course of the laboratory examination because they were broken or damaged. In total, we inspected 3767 shoots from 420 patches.

To determine the optimal sampling time with respect to gametangia development and observability, we compared the observed sex expression in spring (21 May 2013) and early autumn (22 September 2013) at one locality (V Lisovech). Repeated sampling at the locality V Lisovech proved the observed difference in sex expression between spring and autumn assessments, being higher during the autumn sampling (96% vs. 78%, Appendix 1). The difference was obviously caused by the better gametangia development in autumn – neither too young and undistinguishable, nor too old, falling from shoot and decomposing.

Table 1 Localities included in this study with the information about GPS position (WGS 84) and sampling pattern. In mixed populations, the total number of barcoded patches/shoots belonging to clade 1 and 2 are specified.

locality	N (°)	E (°)	date of visit	clade	elevation (m a.s.l.)	population size (number of patches)	shoots inspected
Bažiny	50.2964	16.2997	7.10.2013	1	620	7	7
Boží Dar 1	50.407	12.9006	24.9.2017	2	1000	6	59
Boží Dar 2	50.4057	12.8985	24.9.2017	1	1010	10	65
Břehyně	50.581	14.7189	19.9.2015	1	280	29	290
Červený rybník u Pihele	50.7353	14.5529	26.10.2013	1	300	29	290
Hrádecká bahna	49.7132	13.659	2013	1	400	21	210
Kostelní vrch	49.0556	13.4603	30.10.2015	1	970	19	124
Louky v Jeníkově	49.7385	15.9645	18.10.2013	1	630	8	80
Na Oklice	49.4042	15.3945	22.9.2013	1	660	13	104
Novozámecký rybník	50.6125	14.5853	19.9.2015	1	255	8	24
Panská	49.6019	16.1688	17.10.2013	2	720	15	131
Ratajské rybníky	49.7694	15.9339	17.10.2013	1	590	16	152
Ruda	49.1453	14.6908	22.4.2013	1	415	19	190
Řeka	49.6666	15.853	18.10.2013	1+2	555	49	436 (45+363)
Skalské rašeliniště	49.9182	17.2114	8.10.2013	2	680	14	120
Šimanovské rašeliniště	49.4504	15.4467	1.5.2013	1+2	605	14	136 (87+10)
Šmauzy	49.197	13.2622	30.10.2015	1	1030	16	146
V Lisovech (autumn)	49.247	15.2788	22.9.2013	1	650	26	212
V Lisovech (spring)	49.247	15.2788	21.5.2013	1	650	24	223
Vidlák	50.5244	15.2174	7.10.2013	1+2	280	37	370 (80+250)
Zhůří 1	49.1725	13.3317	2.11.2013	1+2	900	24	240 (80+150)
Zhůří 2	49.1707	13.3326	5.10.2015	1+2	960	16	158 (50+99)

Clade determination and mixed-clade localities

It was not possible to barcode molecularly every single shoot to its respective clade with respect to cost of such an approach. However, under the assumption of high clonality of fen mosses with respect to the high proportion of vegetative reproduction (Poschlod & Schrag, 1990), we assumed that one barcoded plant from each patch represents the clade identity of the whole patch in majority of cases. One shoot from each patch was barcoded into its respective cryptic species using one of the methods (ITS sequencing, PCR-RFLP of ITS, amplification of specific SSR loci) described in Manukjanová *et al.* (2018). In mixed-clade populations, we assessed 2-3 shoots from each patch with the same methods as described above. When both male and female plants were present in same patch, we preferred to barcode one shoot of each sex to enhance number of tested genotypes. Only patches with shoots belonging to only one clade were used for analyses which distinguished between clades. This approach enabled us to treat all shoots as barcoded to their respective clades, even though we had to exclude 10 of the 420 patches. Plants of only *Hamatocaulis vernicosus* clade 1 occurred at 13 of the 21 investigated localities, only clade 2 occurred at three others, and five localities supported the occurrence of both cryptic species. The samples from each locality are stored in herbarium CBFS.

Sex determination

Presence of perigonia and perichaetia was assessed under the dissection microscope using 45× magnification and presence of antheridia and archegonia was verified under compound microscope (magnification 400×) in a few cases from each locality.

Although sex markers for *H. vernicosus* have not yet been developed, we tried to estimate sex of non-expressing shoots by the indirect method based on expected clonality. As Teleganova & Ignatov (2007) suppose, non-expressing shoots from male patches were considered as non-expressing males and vice versa. The data from mixed sex patches and from sexually non-expressing patches were not evaluated. The female to non-expressing putative female and male to non-expressing putative male ratios were counted for the whole dataset and for individual localities separately.

Data analyses

The position of each patch was drawn into a field sketch that was later transformed into a GIS layer and supplemented with information about sex expression and number of male/female shoots. The maps of patches for each locality showing the rates of shoots were created using the QGIS v. 2.6 software (QGIS Development Team 2015).

Sex expression was assessed both for all shoots, irrespectively of the clade identity, and for the distinguished clades separately. Moreover, we compared the results based on the assessment on different pooling levels with respect to patch and locality identity. First, we assessed the rate of expressing shoots irrespectively of the patch and locality identity (hereafter termed “*shoots*”). Second, we counted the mean of the shoot expression rate at individual localities (“*mean of shoots at localities*”). Third, we assessed the rate of patches containing sex expressing shoots to all patches in the study irrespectively of the locality identity (“*patches*”), and fourth, we assessed the mean of the preceding pooling level assessed at the individual localities (“*mean of patches at localities*”), analogically to the second level. Finally, we assessed the percentage of localities containing sex expressing shoots (“*localities*”). The same levels of pooling hierarchy were used for the assessment of sex ratio, counting the rate of female

to male shoots/patches/localities (F:M). The rate of patches/localities where both sexes are present (F+M) was counted as well.

The difference in sex expression and expressed sex ratio at individual localities was tested using one-way ANOVA in the Statistica 13.0 software (Statsoft, 2016). The individual values for the analyses were counted those for each locality separately (“shoots at localities”, “patches at localities”). We also tested the difference in sex expression between clades 1 and 2 using the same approach.

Results

Sex expression

Sex expression of *Hamatocaulis vernicosus* regardless of the cryptic species in the study area totalled 58.8% of assessed shoots, while it ranged between 0 and 96% at individual localities (Fig. 1), with the mean value of 52.9%. The differences in sex expression of shoots among localities were statistically significant (“shoots at localities”, $F(21)=4.7338$, $p<0.001$). The expression at the higher levels of evaluation hierarchy was considerably higher: 81.8% of patches and over 95% of localities expressed the gametangia (Table 2).

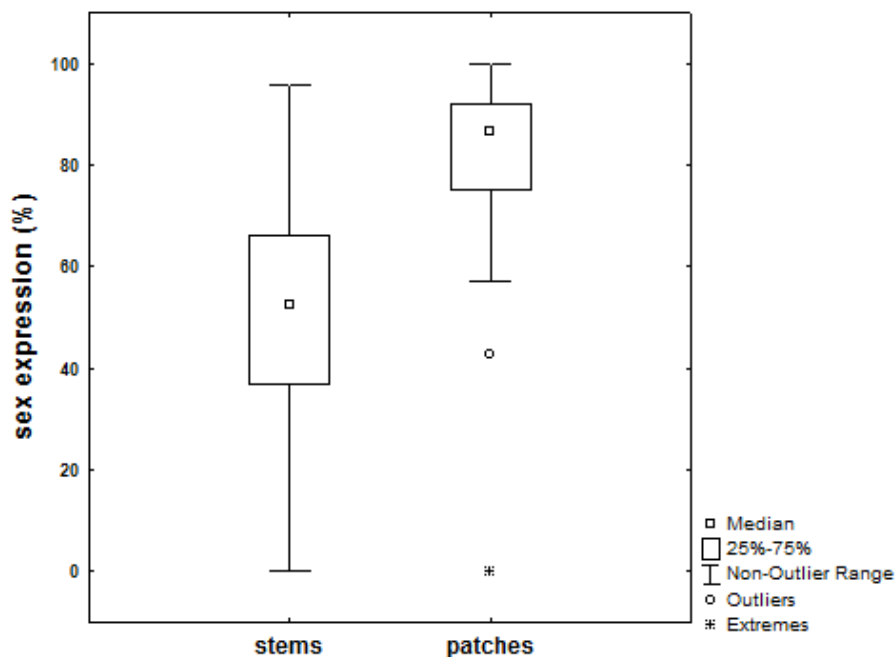


Fig. 1 The sex expression of *Hamatocaulis vernicosus* in the Czech Republic at individual localities assessed at two levels of pooling hierarchy - (“shoots at localities” and “patches at localities”).

Differences in sex expression between the two clades of *Hamatocaulis vernicosus* at individual localities were not statistically significant using either of the assessment approaches (“shoots at localities”; $F(1;24) = 0.1263$; $p = 0.7254$) and “patches at localities” ; $F(1;24) = 2.8633$; $p = 0.1036$).

The female to non-expressing putative female ratio was 1.69 (“mean of shoots at localities“ 1.43) and male to non-expressing putative male 3.23 (“mean of shoots at localities“ 2.06).

Table 2 The sex expression of *Hamatocaulis vernicosus* clades in the Czech Republic assessed at different hierarchy levels

assessment level	undistinguished		clade 1		clade 2	
	N	% of sex expressing	N	% of sex expressing	N	% of sex expressing
shoots	3544	58.8	2204	55.2	1182	65.9
shoots at localities	21	52.9	8	51.4	18	54.9
patches	395	81.8	258	77.9	123	91.1
patches at localities	21	78.6	8	75.6	18	90.5
localities	21	95.3	8	94.2	18	100

Table 3 Sex ratio in *Hamatocaulis vernicosus* in the Czech Republic at different levels of evaluation hierarchy considering the barcoded clades.

assessment level	clade	% male	% female	% only non-expressing	% F+M	F:M
shoots	undistinguished	29.03	29.77	41.20		1.03
	cl 1	26.52	28.71	44.70		1.08
	cl 2	35.79	30.12	34.09		0.84
mean of shoots at localities	undistinguished	25.23	27.71	47.10		1.10
	cl 1	25.48	25.85	48.60		1.01
	cl 2	25.33	29.62	45.10		1.17
patches	undistinguished	36.36	44.44	18.20	7.08	1.22
	cl 1	36.05	49.22	22.10	7.36	1.37
	cl 2	44.72	49.59	8.90	3.25	1.11
mean of patches at localities	undistinguished	37.01	49.47	21.40	7.83	1.34
	cl 1	45.00	60.65	24.40	8.10	1.35
	cl 2	36.95	46.72	9.50	15.12	1.26
localities	undistinguished	71.43	80.95	4.66	57.14	1.13
	cl 1	70.59	76.47	5.78	52.94	1.08
	cl 2	87.50	75.00	0.00	62.50	0.86

Sex ratio

The sex ratio at the level of shoots was female-biased at 62% of investigated localities (56% localities in clade 1 and 63% of clade 2). In contrast to sex expression, the sex ratio was not much different at different levels of evaluation hierarchy (Table 3) but differed slightly between clades, depending on the method used. However, the differences were not statistically significant. The difference in sex expression between shoots (“shoots at localities”; $F(1;40) = 0.1183$; $p = 0.7327$) as well as patches (“patches at localities” $F(1;40) = 1.519$; $p = 0.2250$) at individual localities was not statistically significant.

Although the sex ratio of *H. vernicosus* s.l. and in individual clades in the whole studied region was only slightly biased, the situation at individual localities was much more diverse. At ten of the 21 investigated localities, only male or female shoots of the respective clade were found. At localities with both sexes, various levels of male or female-biased ratios in plants of the respective clades occurred (Fig. 2). Neither the sex expression, nor the ratio of barcoded plants at individual localities followed any apparent geographical pattern in the Czech Republic (Fig. 3).

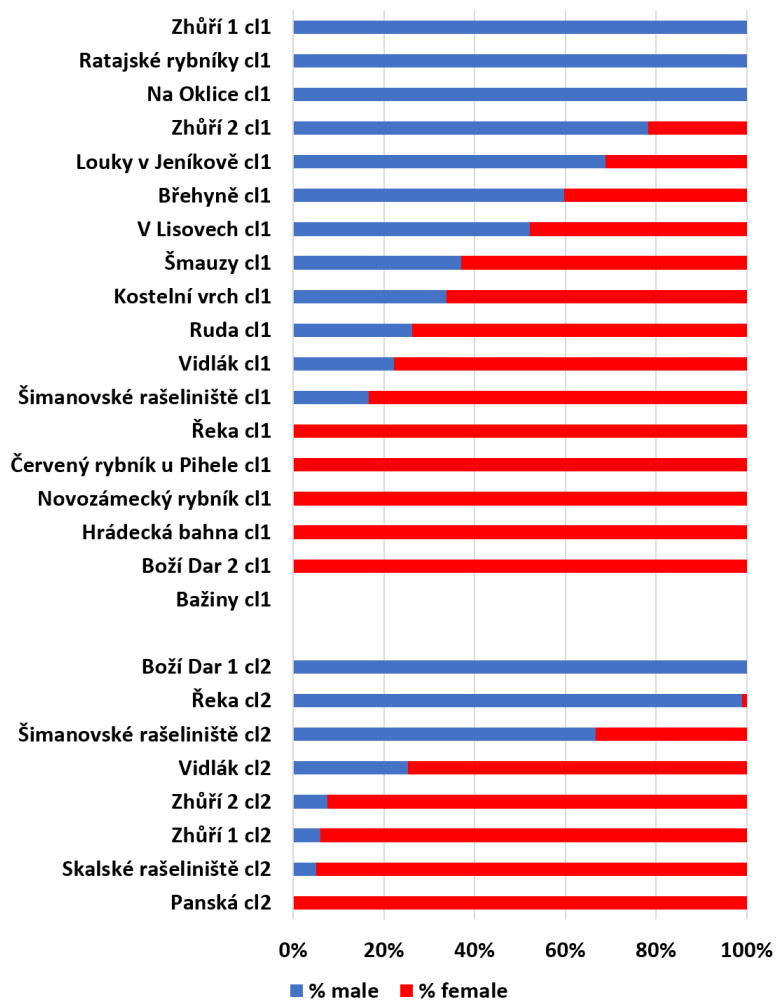


Fig. 2 The expressed sex ratio at studied localities of *H. vernicosus*. In mixed populations, only single-clade patches were used for the assessment.

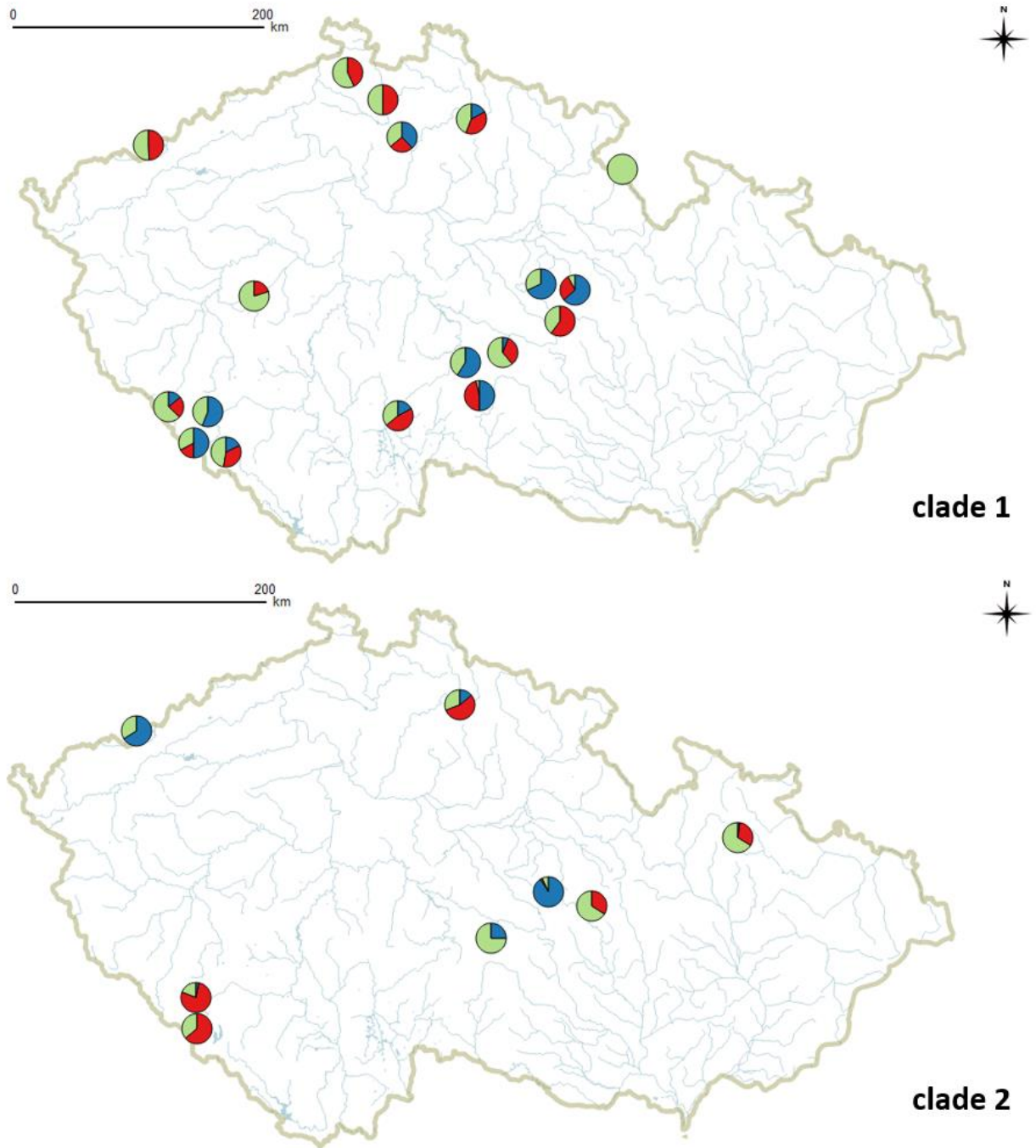


Fig 3 Rates of male (blue), female (red) and non-expressing (green) plants at studied localities of *Hamatocaulis vernicosus* clade 1 and 2.

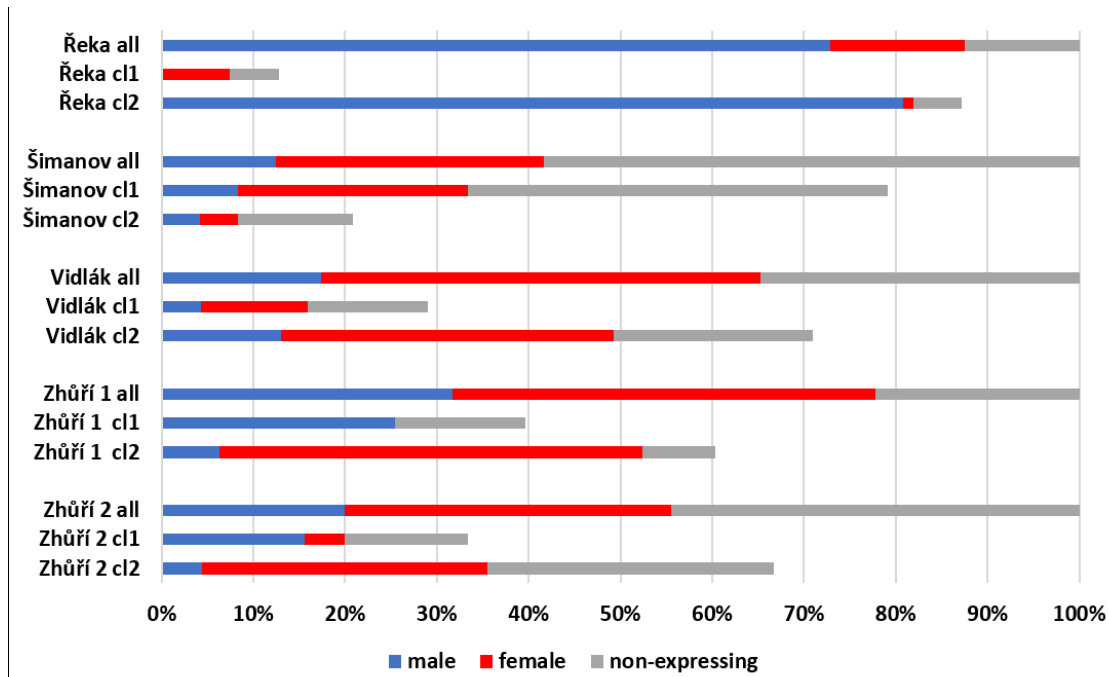


Fig. 4 Sex ratio at localities with co-occurring cryptic species. All – without distinguished clades, cl1 – clade 1, cl2 – clade 2. Only barcoded shoots were used to create this graph.

At localities where both *H. vernicosus* clades are present, both sexes did not always occur in each of them (Fig. 4), although male and female plants, irrespectively of the clade, were always found. For example, at the locality Zhůří 1, the overall sex ratio is close to 1:1, but clade 1 has only male plants, while clade 2 consist mostly of female plants.

Intensive sampling pattern at individual localities enabled us to assess the sex ratio of barcoded plants in individual patches, although the number of patches where both sexes were present was extremely low. The maps of spatial distribution of sexed patches show a high level of clustering of patches with plants of the same sex (Fig. 5, Appendix 2). The map of spatial distribution of patches at the locality Zhůří 1 also shows that from 24 studied patches, only one (grey) had plants of both clades present (Fig. 5). The clades are obviously clustering together and the patches with both clades that indicate transition zone are extremely rare.

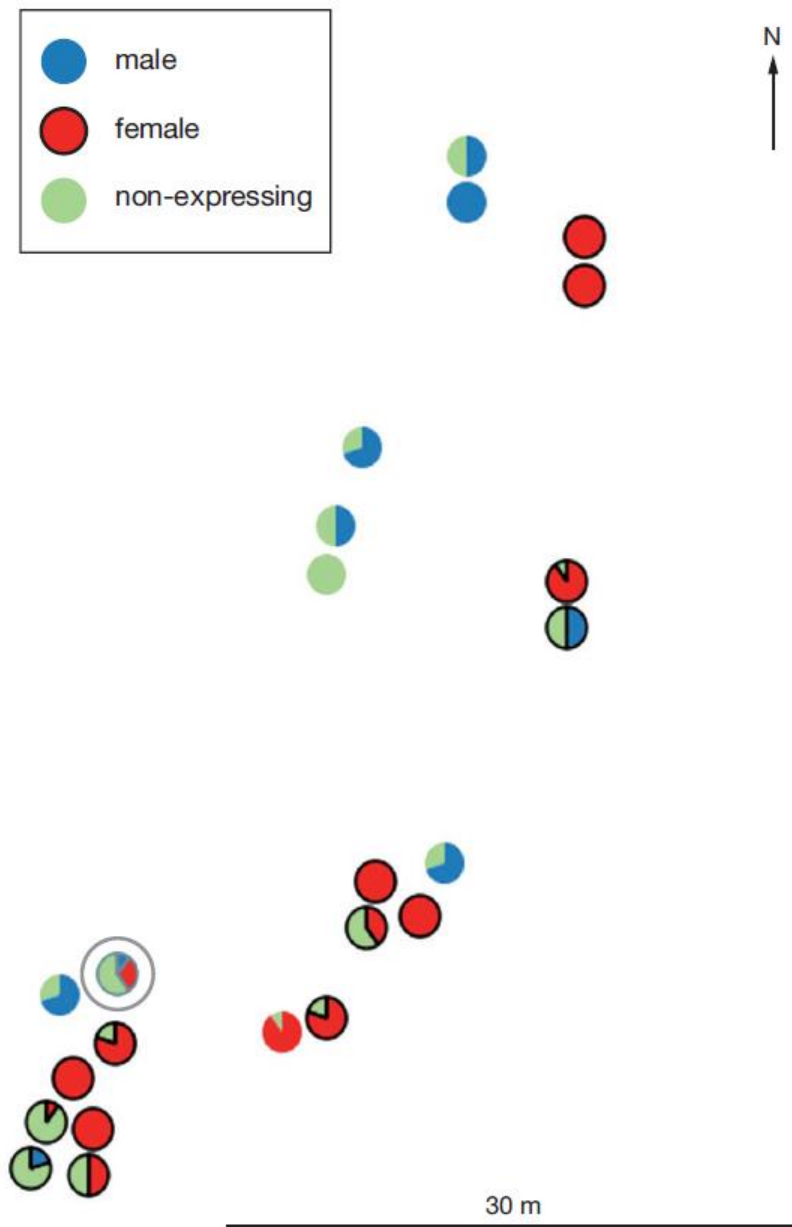


Fig. 5 The sex ratio in mixed population at locality Zhůří 1. Unbordered pie charts refer to clade 1, bordered ones represent clade 2. The patch in the larger circle contained plants of both clades, so this patch must be excluded from evaluating sex ratio in separated clades.

Discussion

Sex expression

The expression of gametangia in *Hamatocaulis vernicosus* at localities in the Czech Republic was higher (59% of shoots, 82% of patches, more than 95% of localities) than reported for the species from both France (30% of shoots, less than 70% of localities; Pépin *et al.*, 2013) and Scandinavia (63% of specimens, Bisang *et al.*, 2014) The latter method, assessing the sex expression in non-randomly collected specimens of unequal size is different from our definition of patches, but it can with some limitations be compared to our approach.

Lower rates of sex expression in the other published papers could be explained either by less favourable environmental conditions (Eppley *et al.* 2011), smaller sampling effort or the

effect of inappropriate sampling time. The suboptimal environmental conditions might indeed have been the case for the lower expression of *H. vernicosus* in Massif Central, as acknowledged by Pépin *et al.* (2013) in their discussion of reasons for unrecorded sporophyte development. The results can nevertheless also be affected by the sampling time, as shown by our repeated sampling at the locality V Lisovech. The latter cause might have affected the results published by Bisang *et al.* (2014), who inspected mostly herbarium specimens, sampled at various localities over the whole growing season, which necessarily increased the probability of encountering shoots where gametangia were absent only due to the inappropriate sampling time. While the best sampling time for discovery of gametangia was autumn, sporophytes were only found during spring sampling in our region.

We were able to demonstrate the difference in sex expression between the cryptic species of *H. vernicosus*, although the number of specimens was rather low for clade 2 to be sufficiently representative. Similar result was found by Buczkowska *et al.* (2006), who found variation in proportion of fertile to non-expressing gametophytes among the cryptic species of *Aneura pinguis*. However, even in that study, the number of specimens of individual cryptic species was rather low.

The sex expression of genetically male and female plants could not be directly compared in our study. The sex primers published for *Drepanocladus trifarius* (Hedenäs *et al.*, 2010), although known to amplify in another related species, *Drepanocladus lycopodioides* (Bisang *et al.*, 2010; Bisang & Hedenäs, 2013), did not work in *H. vernicosus* (Holá & Košnar, unpublished data). However, our estimate using the indirect approach did not indicate the difference in the ratio of non-expressing shoots in male patches from that of non-expressing female shoots in female patches (cf. Appendix 2). On the contrary, female patches contained more non-expressing shoots. Thus, the “shy male hypothesis”, describing the lower sex expression in male shoots (Stark *et al.*, 2005), does not seem to be true for *H. vernicosus* in the study area. In another study, which studied the sex of non-expressing shoots using sex-specific PCR primers (Bisang & Hedenäs, 2013), the authors did not find any difference in the level of expression between male and female plants of *Drepanocladus lycopodioides*.

Sex ratio

The overall sex ratio in *Hamatocaulis vernicosus* at studied localities was seemingly balanced. The overall apparent balance, when analysed both spatially according to localities and patches, and separately in individual clades, nevertheless obscures the real situation at sites. More localities (62%) were slightly female biased (F:M = 1.1 using the approach “*mean of shoots at localities*”), while a few large populations were markedly male-biased. The balanced overall sex ratio of *H. vernicosus* in the Czech Republic contrasts with the situation in French Central Massif, where the F:M ratio of expressing individuals (using the “*shoots*” approach) was 3.2 (Pépin *et al.*, 2013). This difference is likely to be caused by the stochasticity of small populations, as it was the case of the above-described male-biased populations (Fig. 2). Our localities contained plants of both sexes more often than it was the case in France (60 vs. 27%; cf. Pépin *et al.*, 2013), which probably was affected by the assessment of generally larger populations in our study.

Interestingly, the theoretically expected balanced sex ratio has not been commonly reported in bryophytes. In their review of the sex ratio in 103 bryophyte species, Bisang & Hedenäs (2005) found that the female-biased sex ratio was more frequent (88% of studies using “shoots” method and 68% of studies using “patches” method). Some species or one of the sexes were also reported regionally non-expressing (cf. also Haig, 2016). Our data and their comparison with the study of Pépin et al. show that the reported bias might significantly be affected by the inadequate sampling from too few or too small populations. Indeed, many of studies reviewed in Bisang & Hedenäs (2005) were based on data from only a few localities.

Barcoding of sexed shoots to the cryptic species (clades) proved that the sex ratio for the individual cryptic species was at some localities extremely skewed and sometimes only single-sex populations of one of the cryptic species occurred at particular localities, although the overall sex ratio was seemingly balanced (Fig. 4). This confirmed our hypothesis that severe mate limitation might exist at many localities in the region, as the cryptic species are likely sexually incompatible. This deepens the dependence of both *Hamatocaulis vernicosus* clades on asexual reproduction, which does not provide genetically diverse individuals capable of adaptation to changing conditions in spite of effectivity in biomass production. In the landscape affected by both climate change and changes caused by human activities, the mate limitation can pose a severe problem for fen bryophytes.

The difference in the sex ratio between cryptic species, reported in the case of *Aneura pinguis* (Buczowska *et al.*, 2006), was not demonstrated in the cryptic species of *H. vernicosus*. However, the reported differences in *Aneura pinguis* might have been strongly affected by the small number of samples of individual cryptic species, as discussed above in the section on sex expression and shown here at individual localities of *H. vernicosus* (Fig. 2).

The higher abundance of clade 2 at most of the localities where both clades co-occur (Fig. 4), raises the question about their competitive abilities and niche differentiation. Although the two cryptic species have not been reported to differ in their ecological preferences (Hedenäs & Eldenäs, 2007), the real situation might be different at least regionally. As most large patches are unisexual and probably clonal at the studied localities, it is unlikely that the reason for greater abundance of clade 2 at mixed localities is the more successful sexual reproduction. Differences in vegetative growth rate between clades seem to be more likely, caused perhaps by slight shifts in ecological preferences of cryptic species, promoting various levels of success in different microhabitats at localities.

Different hierarchy of data evaluation

Different approaches to the assessment of sex expression and sex ratio assess the parameters at different hierarchy levels and therefore accentuate various aspects with respect to the particular study aim. The “shoots” approach best reflects the situation in the population as a whole, while “mean of shoots at localities” gives every locality the same weight. Hence, a single big population with aspects untypical for the majority of populations in the region (in our case, e.g., the population Řeka with plants of clade 2) may obscure the typical pattern and conservation concerns that should be regionally addressed, if “shoots” approach is applied. Similarly, the “shoots” approach cannot reveal the local mate limitation in individual

populations in case that the overall F/M ratio is balanced. The approaches summarising the sex expression or ratio over patches may, perhaps correctly, accentuate the importance of the biological unit, *patch*, which might have the equally important effect for maintaining and propagating the population. The information on how many patches contain shoots of both sexes is vital. Even if a majority of plants in the patches expresses the gametangia, the fertilisation usually only occurs between shoots that are only several centimetres apart (Longton & Schuster, 1983). Whether the approach “*patches*” or “*mean of patches at localities*” is preferred, depends on the weight we want to give to the individual populations in case that these are of markedly unequal size. Finally, the approach “*locality*” sums the rate of expression at localities, highlighting the localities where no expression is present at all. When sex ratio is assessed, the approach “*locality*” is the most simplified way, showing only, whether male and/or female sex is present at locality. Also, the number of localities where both male and female plants are present simultaneously is a crucial information for assessing reproductive potential of species, because localities where only 1 sex is present do not contribute to sexual reproduction.

The “*shoots*” approach is probably the most widely used in bryophyte research (Bisang & Hedenäs, 2005), because of its simplicity. However, various modifications of the “*patches*” approach are also popular (Bisang *et al.*, 2014), even though the definition of patch may differ being either herbarium sample or a patch collected in the field.

Acknowledgments

We are grateful to our colleague Eliška Vicharová for help with specimen sampling and Eva Holá with Jiří Košnar for examining sex markers for *H. vernicosus*. We are also grateful to anonymous referees for considerable improvements of our manuscript.

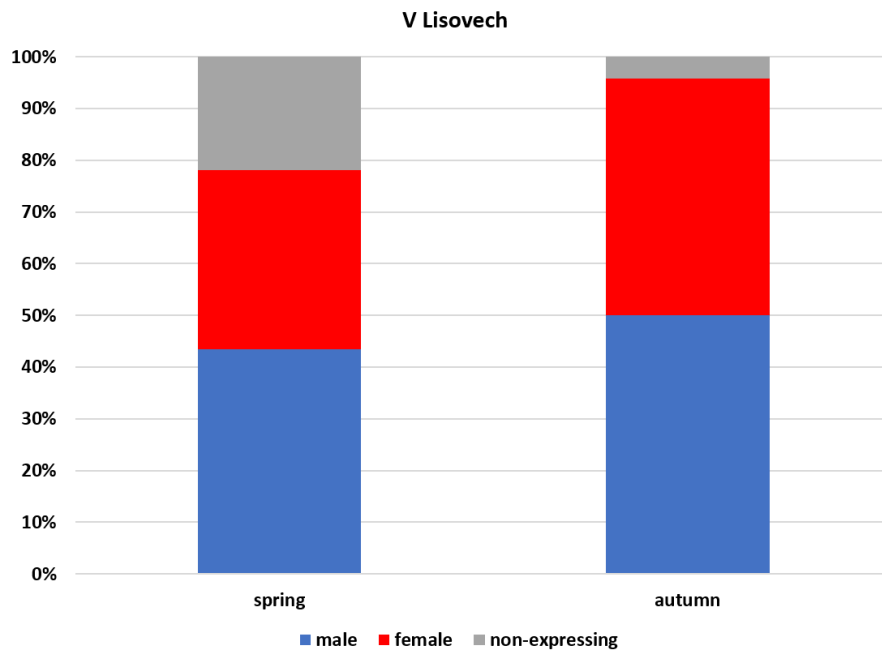
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Appendices

Appendix 1 Seasonal variation of the sex expression and F:M ratio at the locality V Lisovech. 24 patches were inspected on spring and 26 patches in autumn. The difference in sex ratio between the samplings counted by one-way ANOVA was statistically significant ($F(1;48) = 5.0396$; $p = 0.0294$)



Appendix 2a-e Spatial distribution and rates of male (blue), female (red) and non-expressing (green) plants of *Hamatocaulis vernicosus* at individual localities. The small dots show the position of patches, the pie-charts observed sex ratio in the patch. Blue – male, red – female, green – sterile. All maps have same orientation – North-facing upwards. Empty chart shows patches with shoots unfit to study (broken) or confused with similar species (mainly *Scorpidium cossonii* (Schimp.) Hedenäs). Because localities differ in size, each of them has its own scale. The locality Zhůří 1 is located in result section of the article, the spatial data for locality Novozámecký rybník are not available.

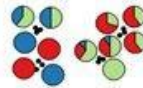
0 10 m



Šmauzy, clade 1



0 30 m



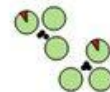
Břehyně, clade 1



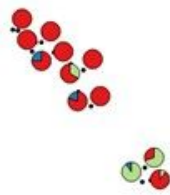
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Hrádecká bahna, clade 1

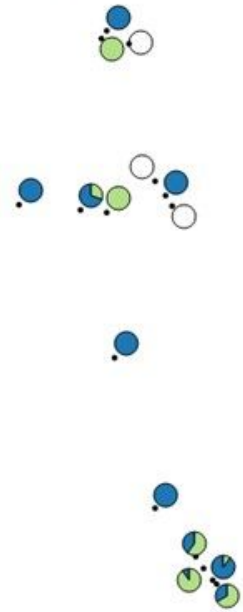


0 30 m



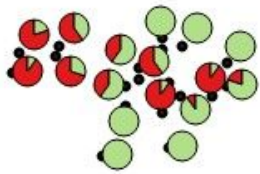
Kostelní vrch, clade 1

0 30 m



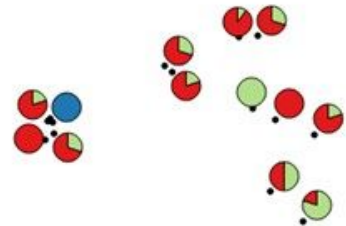
Oklika, clade 1

0 10 m

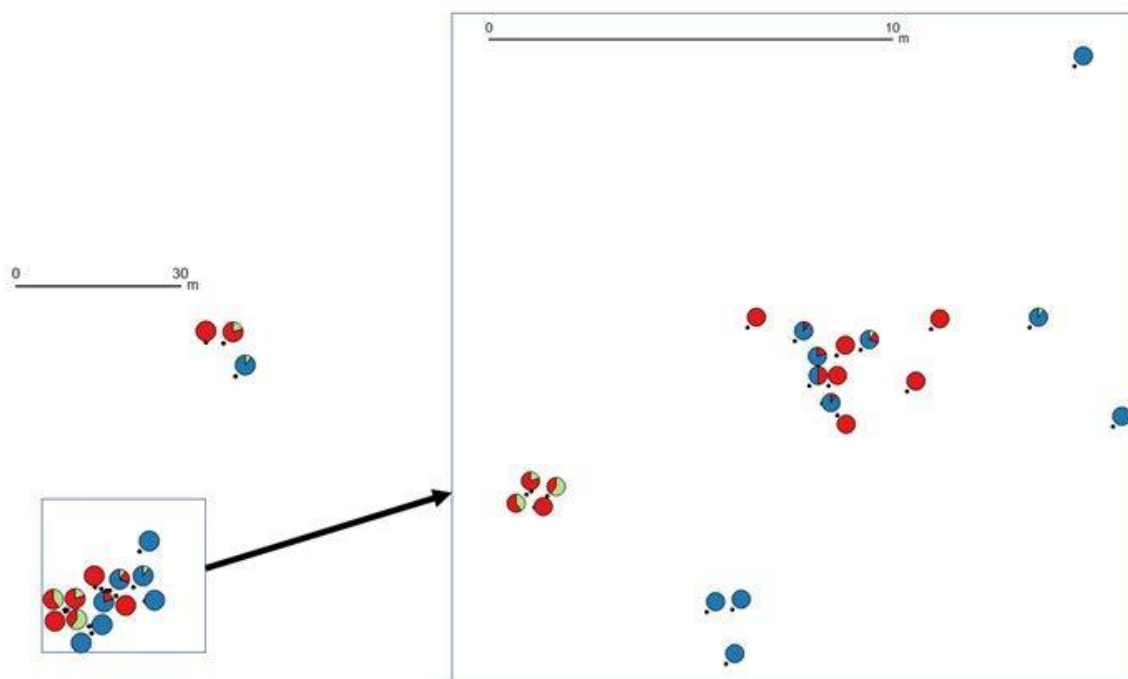


Pihel, clade 1

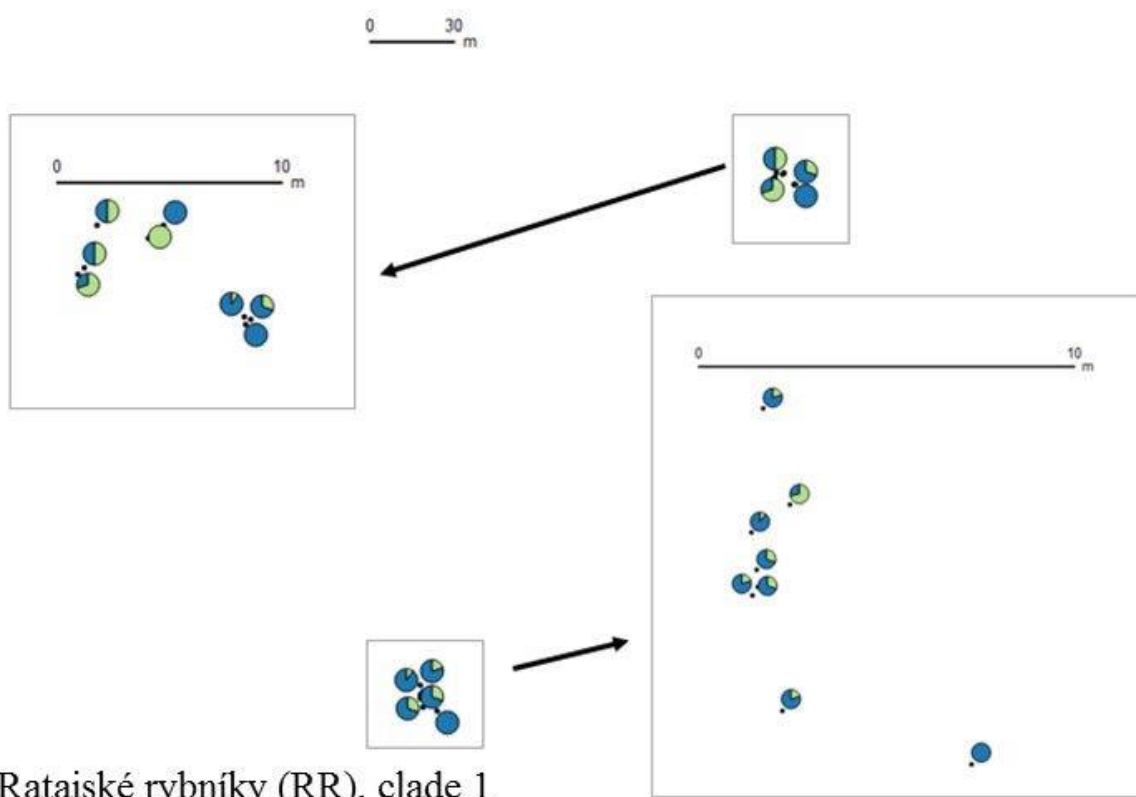
0 30 m



Ruda, clade 1

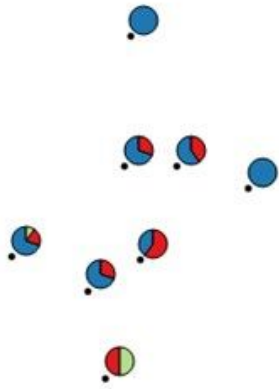


V Lisovech (autumn sampling), clade 1



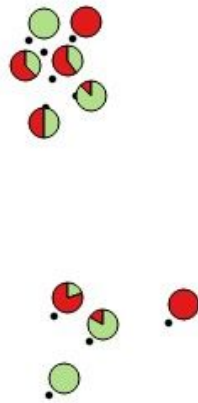
Ratajské rybníky (RR), clade 1

0 1 m



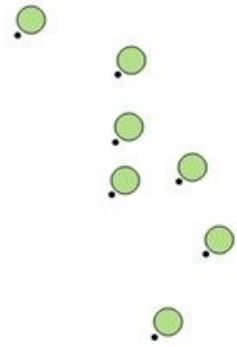
Louky v Jeníkově (LVJ), clade 1

0 3 m



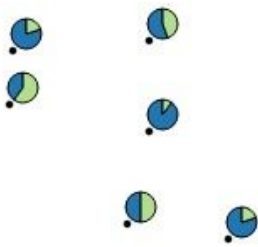
Boží dar 2, clade 1

0 1 m



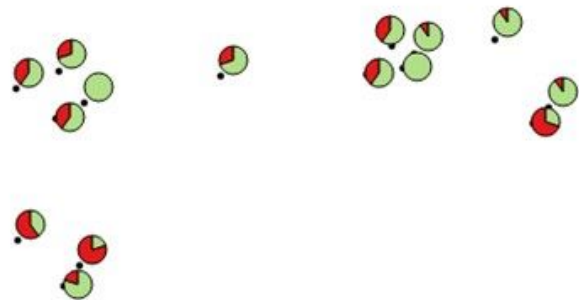
Bažiny, clade 1

0 1 m



Boží dar 1, clade 2

0 10 m



Panská, clade 2

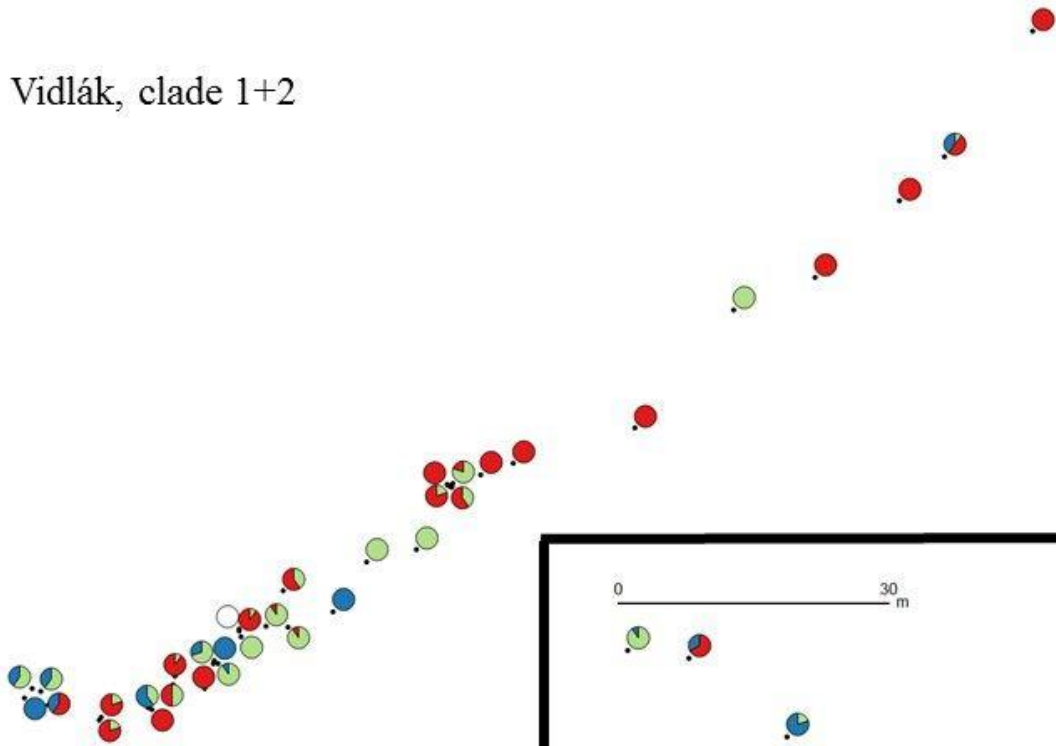
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Skalské rašelinště, clade 2

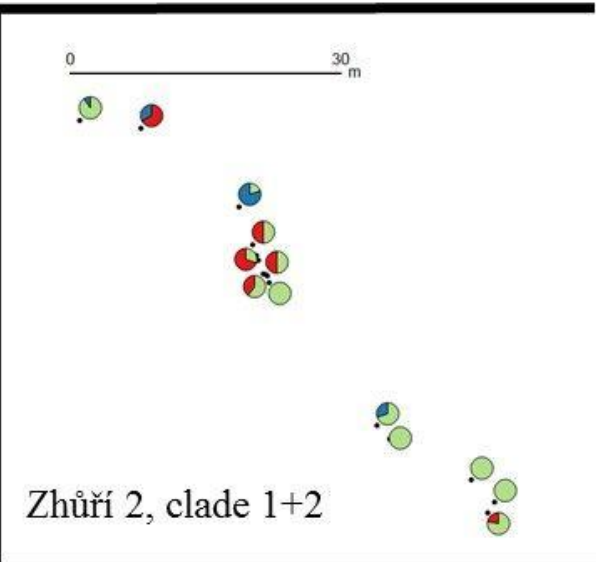
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Vidlák, clade 1+2



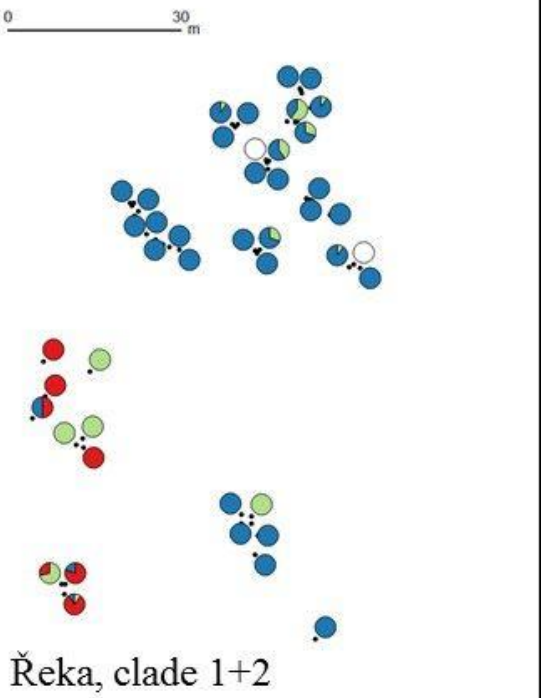
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Zhůří 2, clade 1+2



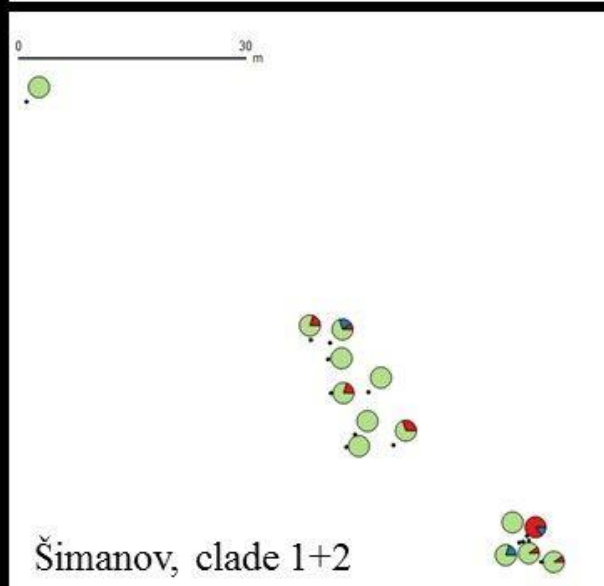
0 30 m

Řeka, clade 1+2



0 30 m

Šimanov, clade 1+2



Appendix 3 Clade barcoding at *Hamatocaulis vernicosus* mixed-clade localities, raw data

patch	stem 1 a		stem 2 b		stem 3 c		clade in patch
	sex	clade	sex	clade	sex	clade	
Řeka							
1	m	2	m	2			2
2	m	2	m	2			2
3	m	2	m	2			2
4	s	2	s	2			2
5	m	2	m	2			2
6	m	2	m	2			2
7	m	2	m	2			2
8	m	2	m	2			2
9	m	2	m	2			2
10	m	2	m	2			2
11	s	2	s	2			2
12	x	x	x	x			x
13	m	2	m	2			2
14	m	2	m	2			2
15	m	2	m	2			2
16	m	2	m	2			2
17	m	2	m	2			2
18	m	2	m	2			2
19	m	2	m	2			2
20	x	x	x	x			x
21	m	2	m	2			2
22	m	2	m	2			2
23	m	2	m	2			2
24	m	2	m	2			2
25	m	2	m	2			2
26	m	2	m	2			2
27	m	2	m	2			2
28	m	2	m	2			2
29	m	2	m	2			2
30	m	2	m	2			2
31	m	2	m	2			2
32	x	x	x	x			x
33	m	2	m	2			2
34	m	2	m	2			2
35	m		m				
36	f	1	f	1	x	x	1
37	f	2	f	1	m		both
x	x	x	x	x			x
41	s	1	s		s	1	1
42	f	1	f	1	f	1	1
43	x	x	x	x	x	x	x
44	s	1	s	1	s	1	1
45	s	1	x	x	x	x	1
46	f	1	x	x	x	x	1
47	m	2	x	x	x	x	2
48	m	2	m	2	m	2	2
49	m	2	s	2	m	2	2
50	s	2	s	2	s	2	2
51	m	2	m	2	m	2	2
52	m	2	m	2	m	2	2
53	m	2	m	2	m	2	2
54	f	1	m	2	f	1	both
55	f	1	s	1	f	1	1
56	f	1	m		f	1	1
Šimanov	sex	clade	sex	clade	sex	clade	
1	f		s		s	1	1
2	f		f	1	f		1
3	s		s		s		
4	m	1	s	1	m	1	1
5	s	1	s	1	s		1
6	f	1	s	1	s	1	1
7	f		s		f	1	1
8	s	2	s	2	s	1	both
9	s		s	2	s	1	both

patch	stem 1 a		stem 2 b		stem 3 c		clade in patch
	sex	clade	sex	clade	sex	clade	
10	f	1	s		f	1	1
11	s		s		s		
12	f	2	s		m	2	2
13	f		s	1	f	1	1
14	s	1	s	1	s	1	1
Vidlák	sex	clade	sex	clade	sex	clade	
1	m	2	m	2			2
2	s	2	m	2			2
3	m	2	s	2			2
4	f	1	m	1			1
5	f	2	s	2			2
6	s	2	f	2			2
7	m	1	s	1			1
8	f	2	f	2			2
9	s	2	f	2			2
10	f	1	s	1			1
11	f	1	f	2			both
12	s	2	m	2			2
13	m	2	m				2
14	s	2	m	2			2
15	s	1	s	1			1
16	f	1	f	1			1
17	x	x	x	x			
18	s	2	f	2			2
19	f	2	s	1			both
20	f	2	s	2			2
21	m	2	m	2			2
22	s	1	s	1			1
23	s	2	s	2			2
24	f	2	s	2			2
25	f	2	s	2			2
26	f	2	f	1			both
27	s	2	f	2			2
28	f	2	f	2			2
29	f	2	f	2			2
30	f	2	f	2			2
31	f	2	f	2			2
32	s	2	s				2
33	f	2	f				2
34	f	2	f				2
35	m	1	f	2			both
36	f	2	f				2
37	s	1	f	1			1
38	f	1	s	1			1
Zhůří 1	sex	clade	sex	clade	sex	clade	
1	m	1	s	1	m	1	1
2	m	1	s	1	m	1	1
3	s	1	s	1	s	1	1
4	m	1	m	1	m	1	1
5	f	2	f	2	f	2	2
6	m	1	f	2	f	2	both
7	f	2	f	2	f	2	2
8	s	2	f	2	f	2	2
9	f	2	f	2	f	2	2
10	f	2	s	2	f	2	2
11	m	1	s	1	m	1	1
12	f	2	f	2	f	2	2
13	f	2	f	2	f	2	2
14	s	2	f	2	f	2	2
15	f	2	f	2	f	2	2
16	f	2	f	2	f	2	2
17	f	2	m	2	m	2	2
18	s	1	m	1	m	1	1
19	m	2	s	2	m	2	2
20	f	2	s	2	f	2	2
21	f	2	f	2	f	2	2

patch	stem 1 a		stem 2 b		stem 3 c		clade in patch
	sex	clade	sex	clade	sex	clade	
22	f	2	f	2	f	2	2
23	m	1	m	1	m	1	1
24	s	1	s	1	m	1	1
Zhǔrǐ 2	sex	clade	sex	clade	sex	clade	
1	f	2	s	2	f	2	2
2	f	2	s	2	f	2	2
3	f	2	s	2	f	2	2
4	s		s		s	1	1
5	f	2	s	2	f	2	2
6	f	2	f	2	f	2	2
7	f	1	s	1	f	1	1
8	m	1	s	1	m	1	1
9	f	2	m	1	m	1	both
10	m	1	s	1	s	1	1
11	m	1	s	1	m	1	1
12	f	2	s	2	f	2	2
13	s	2	s	2	s	2	2
14	s	2	s	2	s	2	2
15	m	2	s	2	m	2	2
16	s		s	2	s	2	2